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Supplemental Material

Molecular mechanisms of diaphragm myopathy in humans with severe heart failure

1. **Methods**

All patients gave written informed consent. The trial was approved by the local ethics committee, complied with the Declaration of Helsinki, and is registered at www.clinicaltrials.gov (Unique Identifier: NCT02663115). Patients with severe chronic heart failure (CHF) and reduced left ventricular ejection fraction (LV-EF) scheduled for left ventricular assist device implantation and patients with coronary artery disease (control) undergoing elective CABG with no signs or echocardiographic features of CHF were eligible for this study. Patients were included at the Heart Center Leipzig between January 2016 and December 2017. Age had to be 40-70 years. Inclusion and exclusion criteria are shown below.

Inclusion criteria CHF

- Age 40-70 years
- Reduced LV-EF ≤ 35% due to ischemic or dilated cardiomyopathy for at least 1 year and
- Reduced cardiac output (cardiac index ≤2,4 l/min/m²) or
- Reduced maximum oxygen consumption (VO_{2peak} ≤17 ml/min/m²).
- Individual optimal medical therapy
- Heart Team decision for left ventricular assist device implantation
- Written informed consent for study participation

Inclusion criteria control

- Age 40-70 years
- Coronary artery disease requiring elective CABG,
- Preserved LV-EF ≥50%
- Stable clinical situation without cardiac decompensation within the last 6 months
- Written informed consent for study participation

Exclusion criteria for both groups

- Mechanical ventilation within the preceding 3 months
- Forced expiratory volume within one second (FEV1) <70% and/or medication aimed for lung disease including α-antagonists, β-mimetics and/or inhaled corticosteroids
- Lung fibrosis
- Elevated diaphragm diagnosed by X-ray or ultrasound or known paresis of N. phrenicus
- Chronic kidney disease state 4 and 5, e.g. glomerular filtration rate (GFR) <30 ml/min/1.73 m² and/or dialysis-dependency
- Acute renal failure
- Immunosuppressive therapy
- Liver failure Child-Pugh B and C
- Relevant ventricular dysrhythmia (Lown >IV b)
- Acute myocardial infarction in the preceding 3 months
- Decompensated vitium cordis
- Age ≤40 years and ≥70 years
- Pregnancy

Clinical assessment

Echocardiography was performed according to current standards [57] using a Vivid 9 system (General Electric Healthcare, Chalfont St. Giles, Great Britain). Diaphragm thickness was measured using a linear probe (12 MHz) and three measurements were averaged per side. Coronary angiography was performed according to local standards and right heart catheterization was performed in CHF only assessing right atrial and ventricular pressure, pulmonary pressure, pulmonary capillary wedge pressure and cardiac output. All patients underwent cardiopulmonary exercise testing (CPX) on a bicycle ergospirometer

(ZAN600, nSpire Health GmbH, Oberthulba, Germany) to determine the individual exercise capacity (i.e. VO_{2peak}) applying an incremental protocol until exhaustion. Respiratory muscle strength was measured using Flowhandy ZAN100USB (nSpire Health GmbH, Oberthulba, Germany). $P_{0.1}$ (the decrease in airway pressure at 100 ms after commencement of a tidal inspiratory effort against an occluded airway), maximum inspiratory (Pi_{max}) and expiratory force (Pe_{max}) were measured and the highest value out of five attempts with <10% variability was recorded. The $P_{0.1}/Pi_{max}$ ratio was calculated to prevent underestimation of neural respiratory drive due to weakness of the inspiratory musculature. [28] Static and dynamic lung parameters, in particular vital capacity (VC), FEV1, the ratio FEV1/VC, residual volume (RV), resistance and diffusion capacity were assessed applying bodyplethysmography. Blood analysis was performed for clinically indicated parameters including nt-proBNP.

Diaphragm biopsy

A diaphragm biopsy was taken under direct vision from the left anterior quadrant of the costal diaphragm during left ventricular assist device insertion or coronary bypass operation immediately after sternotomy/left-anterior thoracotomy. The probe was immediately transferred to ice-cold saline and cleaned from connective tissue and blood and separated for subsequent histological, molecular, electron microscopic, contractile force measurements of diaphragm fibers, and isolated mitochondrial functional analyses.

Histology

For quantification of fiber-type distribution and cross sectional area (CSA), paraffin-embedded diaphragm sections (3 µm) were stained and incubated as previously described. [51] Briefly, paraffin-embedded diaphragm sections were incubated for 1 h after antigen retrieval with a primary antibody to slow myosin heavy chain (1/75 dilution, Abcam, Cambridge, UK). After extensive washing with PBS, the sections were incubated with an anti-mouse peroxidase secondary antibody (1 h, Sigma, Taufkirchen, Germany, 1:250), followed by AEC (3-amino-9-ethylcarbazole) staining. Positive-stained (type I fibers) and negative-stained fibers (type II fibers) were counted and circumscribed by image analysis software (Zen imaging software, Zeiss, Jena, Germany).

Analysis of isolated mitochondria

Isolation and analysis of mitochondria was carried out as described previously. [55] For isolation of mitochondria, 500 µl of the homogenate was centrifuged at 100xg for 10 minutes, and the supernatant containing subsarcolemmal (SSM) mitochondria was stored. The pellet was treated with Nagarse (5mg /g wet weight) for 30 seconds and centrifuged at 5900xg for 5 minutes. The resulting pellet was resuspended with KEA (180 mmol/L KCL, 10 mmol/L ethylenediamine tetraacetic acid, and 0.5% albumin) and centrifuged at 100xg for 10 minutes. The supernatant containing interfibrillar mitochondria was transferred to the tube containing SSM and spun down 3 times at 5900xg for 10 minutes. The last pellet was diluted with one third KME (100 mmol/L KCl, 50 mmol/L 3-(N-morpholino)propanesulfonic acid, and 0.5 mmol/L ethyleneglycol-bis-[ß-aminoethylether]-N,N,N´,N´-tetraacetic acid) of used homogenate. All steps were carried out at 4°C.

Oxygen consumption of isolated mitochondria was measured using a Clark-type oxygen electrode (Strathkelvin) at 25°C [60] as linearity of respiratory measurements has been described between 22 and 41 °C. [59] Mitochondria were incubated in a solution consisting of 100 mmol/L KCl, 50 mmol/L MOPS, 1 mmol/L EGTA, 5 mmol/L KH2PO4 and 1 mg/ml fatty acid free bovine serum albumin at pH 7.4. The rate of oxidative phosphorylation was measured using 10 mmol/L glutamate/2.5 mM malate, 20 µmol/L palmitoylcarnitine/2.5 mmol/L malate, 10 mmol/L palmitoyl-CoA (PCoA)/2.5 mmol/L carnitine/2.5 mmol/L malate, 5 mmol/L pyruvate/2.5 mmol/L malate, or 10 mmol/L succinate/3.75 µmol/L rotenone, 0.5 mM tetramethylhydroquinone (DHQ)/3.75 µmol/L rotenone, 0.5 mmol/L tetramethylphenylendiamine (TMPD)/3.75 µmol/L rotenone as substrates and ADP (100 µmol/l and 1 mmol/L) as stimulus. The ADP-stimulated oxygen consumption (state 3 respiration) and the ADP-limited oxygen consumption (state 4) in the respiratory chamber, , the respiratory control index (RCI; state 3 divided by state 4 respiration), and the ADP/O ratio (ADP added per oxygen consumed) were determined as previously described. [52]

To determine mitochondrial subpopulation size and complexity, we performed flow cytometric analyses using a fluorescence-activated cell sorting (FACS) Calibur (Becton Dickinson, Heidelberg, Germany) as previously described. [53] Individual parameters (size and complexity) were assessed using specific

light sources (laser and photomultiplier tube) and specific detectors. Freshly isolated mitochondria (isolation procedure provided healthy and intact mitochondria) were diluted in buffer and analyzed 2 h after isolation. Gated events (100,000 events/sample) were subsequently examined using the forward scatter detector (FSC; 488-nm argon laser and diode detector) and sideward scatter detector (SSC; photomultiplier tube and 90° collection lens) and represented in FSC versus SSC density plots. The geometric mean (in arbitrary units), representing FSC (linear scale), was used as an indicator of size, whereas values from SSC (logarithmic scale) were used to indicate complexity in the subpopulations. Although FSC is proportional to the individual mitochondria particle size, the absolute value remains an arbitrary unit.

Electron microscopy

Electron Microscopy (EM) studies were performed as previously described. [56] Briefly, small muscle specimens were fixed with glutaraldehyde (2.5%, pH 7.4), post fixed with osmium tetroxide (2%), dehydrated and embedded in resin. Longitudinally oriented ultra-thin sections were obtained at different level of deepness from 1 to 3 small blocks and stained with uranyl acetate and lead citrate. Ultra-thin sections of transversally oriented blocks were obtained only for the most significant findings. The grids were observed using a Philips CM120 electron microscope (80 kV; Philips Electronics NV, Eindhoven, The Netherlands) and were photo-documented using a Morada camera (Olympus Soft Imaging System, Hamburg, Germany).

Contractile force measurements

Contractile force measurements of diaphragm fibers were performed as described previously. [45, 46] The part of the biopsy assigned for contractility measurements was stored in relax/glycerol (Rx/Gly, see below for all solution compositions) with a high concentration of protease inhibitors (Rx/Gly high) and initially placed at a roller band for 24 hours at 4°C. Finally, Rx/Gly high was substituted with RxGly containing lower concentrations of protease inhibitors (Rx/Gly low) and stored at - 20°C until further use. An average of 12 fibers per subject were evaluated.

Single diaphragm fibers were isolated in a relaxing solution (pCa 9.0) at 5ºC and clipped with 2 aluminium T-clips at a length of approximately 1-1.5 mm. The clipped diaphragm fibers were incubated for 10 minutes in cold (5°C) skinning solution containing relaxing solution and 1% Triton X-100 to permeabilize the plasma membrane enabling activation of myofilaments with exogenous calcium. Subsequently, the fibers were mounted horizontally on two stainless-steel hooks in a relaxing solution filled chamber (200 µL) with a glass coverslip-bottom on the stage of an inverted microscope (Zeiss, The Netherlands). One of the hooks was attached to a force transducer (model 403A Aurora Scientific Inc, Ontario, Canada), which has a resonance frequency of 10 kHz, whereas the other end was attached to a servomotor (model 315C, Aurora Scientific Inc.; Aurora, Ontario, Canada) which has a rise time of 250 µs. The fiber was microscopically examined for injury; in case of damage, loss of cross-striation or other irregularities fibers were excluded. Diaphragm fibers were stretched to optimal length by adjusting the sarcomere length to 2.5 µm using dedicated Aurora software. To ensure stable attachment of the diaphragm fiber in the clips throughout the mechanical protocol, the fiber was maximally activated prior to the experiment in activation solution (pCa 4.5), and when necessary restretched to sarcomere length 2.5µm. Diaphragm fiber dimensions were measured with a camera device coupled to the objective. Diaphragm fiber length was determined with 100x magnification, depth and width were measured with 400x magnification (an elliptical cross section of the diaphragm muscle fiber was assumed). Fibers were sequentially transferred from relaxing solution (pCa 9.0), pre-activation solution, submaximal activation solutions (pCa 7.0, 6.2, 6.0, 5.8, 5.4) to maximal activation solution (pCa 4.5). All measurements were performed at 20°C and data data were automatically collected using a data acquisition board (sampling rate 10 kHz). For each fiber the cross sectional area (CSA), maximal absolute force (mN), maximal normalized force or tension (mN/mm2) and calcium sensitivy (pCa 50 and hill slope) were assessed.

At the end of the force measurements, single diaphragm fibers were detached from the force transducer and servo-motor and placed in 25 µl of SDS sample buffer for identifying the Myosin Heavy Chain isoform composition of each fiber by sodium decocylsulfate-polyacrylamide gel electrophoresis. The detailed protocol is described below.

Relax-glycerol solution (Rx/Gly) consisted of 50% (v/v) glycerol and relaxing solution. Rx/Gly low contained the following protease inhibitors (in mmol/L): 1.0 DTT, 0.24 PMSF, 0.04 leupeptin, 0.01 E64. Rx/Gly high contained higher concentrations of leupeptin and E64 (in mM): 1.0 DTT, 0.24 PMSF, 0.4

leupeptin, 0.1 E64. All solutions for the contractile measurements had an ionic strength of 180mM and pH 7.1. The relaxing solution had a negative logarithm of free calcium concentration (pCa) of 9.0 and comprised of 5.89 mmol/L Na2ATP, 6.48 mmol/L MgCl2, 40.76 mmol/L Kpropionate (Kprop), 100 mmol/L BES, 6.97 mmol/L EGTA, 14.50 mmol/L CrP and low concentration of freshly added protease inhibitors. Skinning solution consisted of relaxing solution with 1% Triton X-100 and protease inhibitors (in mmol/L) 1.0 DTT, 0.24 PMSF, 0.04 leupeptin, 0.01 E64. The pre-activation solution consisted (in mmol/L) of 5.87 Na2ATP, 0.1 EGTA, 6.42 MgCl, 41.14 Kprop, 100 BES, 14.50 CrP and 6.9 HDTA. Activating solutions (pCa 4.5) consisted of 5.97 mmol/L Na2ATP, 7.0 mmol/L CaEGTA, 6.28 mmol/L MgCl, 40.64 mmol/L Kprop, 100 mmol/L BES and 14.50 mmol/L CrP. By accurate mixing of the relaxing solution and maximally activation solution, sub-maximal activation solutions with pCa 7.0, 6.2, 6.0, 5.8, and 5.4 were made.
Prior to sodium decocylsulfate-polyacrylamide gel electrophoresis, the samples were dissolved in SDS sample buffer and denaturated by boiling for 2 minutes. The SDS sample buffer contained 62.5 mmol/L Tris.HCL, 2% (wt/vol) SDS, 10% (v/v) glycerol, and 0.001% (wt/vol) bromophenol blue at a pH of 6.8 and stored at 20°C until used. The stacking gel contained 4% acrylamide (pH 6.8) and the separating gel 7% (pH 8.8) with 30% glycerol (v/v). Loaded sample volumes were 8 µL per lane. We used human reference samples of diaphragm bundles in a 1:200 dilution of SDS sample buffer (~9.0 ng/µL) to asses migration patterns and verify isoforms of the myosin heavy chain. The gels were run for 24 hours at 15°C and a constant voltage of 275 V. E8 Gels were fixated overnight in a solution of 50%methanol and 10% acetic acid. Finally, the gels were stained with SYPRO® Ruby protein gel stain (Life Technologies, Thermo Fisher Scientific) and imaged by Amarsham Imager 600 (GE Healthcare Life Sciences, The Netherlands). Fibers expressing the slow isoform were classified as slow-twitch fibers and fibers expressing the 2a and 2x isoform were classified as fast-twitch fibers.

Western Blot analysis
For western blot analyses, frozen diaphragm was homogenized in relaxing buffer (90 mmol/L HEPES, 126 mmol/L potassium chloride, 36 mmol/L sodium chloride, 1 mmol/L magnesium chloride, 50 mmol/L EGTA, 8 mmol/L ATP, and 10 mmol/L creatine phosphate, pH 7.4) containing a protease inhibitor mix (Inhibitor Mix M, Serva, Heidelberg, Germany) and sonicated. Protein concentration of the supernatant was determined (bicinchoninic acid assay, Pierce, Bonn, Germany), and aliquots (5–20 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and incubated overnight at 4°C using the following primary antibodies: myosin heavy chain (mixture of 1:1000 MHC-slow (M6421), 1:1000 MHC-fast (M4276), Sigma, Darmstadt, Germany), LC3 (1:1000, L7543, Sigma, Darmstadt, Germany), porin (Ab154856), Ubiquitin (linkage-specific K48) (Ab140601), MuRF1 (Ab183094), SERCA2A (Ab2861), Mfn2 (Ab124773), Nox2 (Ab129068), MAFbx (Ab168372) (all 1/1000, Abcam, Cambridge, UK), Nox4 (1:500, NB110-58849, Novus Biologicals, Wiesbaden, Germany), UCP3 (1:1000, PA5-29560, Thermo Fischer Scientific, Rockland, USA), and SERCA1 (Sc-515162), p62 (Sc-28359) (all 1:200, Santa Cruz Inc., Heidelberg, Germany), Drp1 (12957-I-AP), PGC1alpha (66369-1-lG) (all 1:1000, Proteintech, Manchester, UK). Membranes were subsequently incubated with a horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit POD (1:10000, AP187P) or rabbit anti-mouse POD (1:5000, A9044), both Sigma-Merck, Darmstadt, Germany), specific bands were visualized by enzymatic chemiluminescence (Super Signal West Pico, Thermo Fisher Scientific Inc., Bonn, Germany), and protein expression was quantified using a one-dimensional scan software package (Bio-1D software, Vilber Lourmat, Eberhardzell, Germany). Measurements were normalized to the loading control GAPDH (1/30 000, 5G4, HyTest Ltd, Turku, Finland). Only protein bands within the linear range for the specific target protein and its loading control GAPDH were used for quantification.

Immunoprecipitation of ryanodine receptor 1
Tissue samples were homogenized in relaxing buffer, sonicated, and protein concentration was determined (bicinchoninic acid assay, Pierce, Bonn, Germany). An aliquot (100µg) of the tissue homogenate was incubated in IP buffer (25 mmol/L Tris, 150 mmol/L NaCl, pH 7.2) with 4 µg of anti-RyR1 antibody (Abcam, Cambridge, UK) at 4°C overnight. Afterwards 100µl of Protein G agarose slurry (Thermo Scientific, Rockford, USA) were added and incubated for two hours at room temperature with gentle agitation. Thereafter, the agarose was washed four times with IP buffer and the final pellet was resuspended in SDS lysis medium. Aliquots were size fractionated on SDS gradient gels (4-15%,

Bio-Rad Laboratories, Feldkirchen, Germany) and transferred (2h, 100V at 4°C) to a PVDF membrane. The membrane was incubated with specific antibodies against RyR1 (1:1000, Abcam, Cambridge, UK) and FKBP12 (1:200, Santa Cruz Inc., Heidelberg, Germany), followed by a horseradish peroxidase conjugated secondary antibody. Specific bands were visualized by enzymatic chemiluminescence (Super Signal West Pico, Thermo Fisher Scientific Inc., Bonn, Germany), and protein expression was quantified by densitometry using a one-dimensional scan software package (Bio-1D software, Vilber Lourmat, Eberhardzell, Germany).

Enzyme activity measurements

Diaphragmatic tissue was homogenized in relaxing buffer and aliquots were used for enzyme activity measurements. Enzyme activities for succinate dehydrogenase (SDH, EC 1.3.5.1), citrate synthase (CS, EC 2.3.3.1), and mitochondrial complex I were measured spectrophotometrically as previously described in detail. [54, 58, 61]

Proteasome activity

Proteasome activity in the cytosolic fraction was measured as previously described. [48] Briefly, chymotrypsin-like activity was assayed using the fluorogenic peptide Suc-LLVY-7-amino-4-methylcoumarine (Biomol, Hamburg, Germany). Proteins (20 µg) were incubated with reaction buffer (0.05 mol/L Tris–HCl, pH 8.0, 0.5 mmol/L EDTA) and the labelled peptide (40 µmol/L), with the kinetics of the reaction recorded using a spectrofluorometer (Tecan Safir 2, Tecan, Crailsheim, Germany) at an excitation of 380 nm and emission at 440 nm. Only the proportion of the reaction that could be inhibited by MG132 (20 µmol/L, Sigma, Taufkirchen, Germany) was regarded as chymotrypsin-like activity. For the calculation of enzymatic activity, a calibration curve of free amino-4-methylcoumarine (Sigma, Taufkirchen, Germany) was recorded and values then determined as milliunits per milligram protein.

Statistical analysis

The statistical analysis was performed using SPSS Statistics version 25.0 (IBM Corporation, Armonk, USA). Categorical variables are expressed as numbers and percentage and were compared with the use of the chi-squared test or Fisher exact test as appropriate. To be uniform throughout the figures and tables, continuous variables are expressed as mean and 95%-confidence interval irrespective of its distribution, which was assessed by the Shapiro-Wilk-Test. However, groups were compared using the Students t-test or Mann-Whitney-U-Test according to normal vs. non-normal distribution. Multiple test correction across the manuscript was not performed Due to limited biopsy size, not all parameters were determined in all biopsies, exact numbers and all values depicted in the *Figures* are given in *Table I in the data supplement*. Correlation analyses were performed using bivariate Pearson correlation to evaluate the association of both potential biological pathways predominantly known from animal studies and of molecular measures with three defined clinical parameters (diaphragm thickness, VO_{2peak}, VE/VCO_2). A two-sided p-value <0.05 was considered significant.

Molecular data acquisition and results analysis throughout the experiments were performed in a blinding fashion by pseudonymization of the probes for the technical staff. An experienced and blinded cardiologist (NM) analyzed echocardiography and diaphragm ultrasound acquired by non-blinded examiners (EW, JJN). Acquisition and interpretation of the exercise tests and respiratory muscle strength measurement was not blinded due to the obvious phenotype and concomitant treatment of the patients.

For all the representative images in the manuscript, demonstrative examples reflecting the group mean trend were selected.

Supplemental Results

Online Table I: Values and n depicted in the Figures

	Control	CHF	Mean difference	p-value
Figure 1				
Pi_{max} [kPa]	7.68 (95%-CI 6.34; 9.03) (n=21)	4.78 (95%-CI 3.59; 5.96) (n=18)	-2.91 (95%-CI -4.67; -1.14)	1.9E-3*
Pe_{max} [kPa]	9.46 (95%-CI 8.02; 10.89) (n=21)	7.11 (95%-CI 5.15; 9.07) (n=18)	-2.35 (95%-CI -4.65; -0.04)	0.046*
$P_{0.1}$ [kPa]	0.27 (95%-CI 0.20; 0.33) (n=21)	0.26 (95%-CI 0.20; 0.31) (n=18)	-0.006 (95%-CI -0.09; 0.08)	0.882*
$P_{0.1}$/ Pi_{max}	0.04 (95%-CI 0.03; 0.05) (n=21)	0.08 (95%-CI 0.04; 0.11) (n=18)	0.04 (95%-CI 0.007; 0.07)	0.024†
Right hemidiaphragm [mm]	2.43 (95%-CI 2.25; 2.61) (n=21)	1.92 [95%-CI 1.56; 2.27) (n=18)	-0.51 (95%-CI -0.88; -0.14)	1.4E-4†
Left hemidiaphragm [mm]	2.37 (95%-CI 2.15; 2.59) (n=21)	1.82 (95%-CI 1.49; 2.15) (n=18)	-0.55 (95%-CI -0.92; -0.17)	5.3E-3*
Mean diaphragm [mm]	2.40 (95%-CI 2.23; 2.57) (n=21)	1.87 (95%-CI 1.54; 2.20) (n=18)	-0.53 (95%-CI -0.87; -0.19)	1.4E-4†
Figure 2				
Slow fiber [%]	54.15 (95%-CI 43.83; 64.47) (n=7)	66.91 (95%-CI 57.42; 76.40) (n=6)	12.76 (95%-CI 0.21; 25.31)	0.047*
Fast fiber [%]	45.85 (95%-CI 35.53; 56.17) (n=7)	33.09 (95%-CI 23.60; 42.58) (n=6)	12.76 (95%-CI 0.21; 25.31)	0.047*
CSA slow fiber [µm²]	2788 (95%-CI 2137; 3439) (n=7)	1819 (95%-CI 1166; 2473) (n=6)	-969 (95%-CI -1787; -151)	0.024*
CSA fast fiber [µm²]	3253 (95%-CI 2125; 4381) (n=7)	1588 (95%-CI 1106; 2067) (n=6)	-1665 (95%-CI -2831; -499)	9.4E-3*
MuRF1/GAPDH [arb. units]	1.00 (95%-CI 0.85; 1.15) (n=16)	1.46 (95%-CI 1.30; 1.62) (n=11)	0.46 (95%-CI 0.25; 0.67)	3.9E-4†
MAFbx/GAPDH [arb. units]	1.97 (95%-CI 1.67; 2.27) (n=19)	1.29 (95%-CI 0.89; 1.69) (n=12)	-0.68 (95%-CI -1.16; -0.21)	2.0E-3†
Ubi-K48/GAPDH [arb. units]	2.07 (95%-CI 1.77; 2.36) (n=18)	3.17 (95%-CI 2.30; 4.04) (n=12)	1.10 (95%-CI 0.36; 1.85)	0.020*
Proteasome activity [mU/mg)	3.47 (95%-CI 2.72; 4.22) (n=16)	5.02 (95%-CI 3.62; 6.41) (n=9)	1.55 (95%-CI 0.20; 2.89)	0.026*
Nox2/GAPDH [arb. units]	1.71 (95%-CI 1.27; 2.15) (n=18)	4.75 (95%-CI 2.90; 6.60) (n=12)	3.03 (95%-CI 1.55; 4.52)	4.2E-3*
Nox4/GAPDH [arb. units]	0.23 (95%-CI 0.10; 0.36) (n=15)	2.34 (95%-CI 0.68; 4.01) (n=12)	2.11 (95%-CI 0.72; 3.51)	1.4E-3†
Carb. MHC/MHC [arb. units]	0.55 (95%-CI 0.50; 0.60) (n=6)	0.73 (95%-CI 0.50; 0.95) (n=6)	0.18 (95%-CI 0.02; 0.38)	0.015†
Figure 3				
Citrate synthase activity [U/g IFMSSM]	1.15 (95%-CI 0.84; 1.47) (n=12)	0.53 (95%-CI 0.25; 0.81) (n=9)	-0.62 (95%-CI -1.04; -0.21)	4.3E-3†
Mitochondrial size [arb. units]	176 (95%-CI 142; 211) (n=10)	121 (95%-CI 83; 156) (n=5)	-55 (95%-CI -107; -4)	0.037*

State 3 respiration [natomsO/min/mg protein]	185 (95%-CI 152; 219) (n=11)	85 (95%-CI 50; 120) (n=6)	-100 (95%-CI -149; -51)	5.4E-4*
State 4 respiration [natomsO/min/mg protein]	132 (95%-CI 94; 170) (n=11)	57 (95%-CI 43; 70) (n=6)	-75 (95%-CI -126; -25)	1.1E-3†
Respiratory Control Index	1.52 (95%-CI 1.21; 1.83) (n=11)	1.47 (95%-CI 1.14; 1.80) (n=6)	-0.05 (95%-CI -0.50; 0.40)	0.819*
ADP/O ratio [nmol ADP/natomsO]	0.68 (95%-CI 0.52; 0.84) (n=10)	0.81 (95%-CI 0.60; 1.03) (n=6)	0.14 (95%-CI -0.11; 0.38)	0.246*
Figure 4				
Porin/GAPDH [arb. units]	1.41 (95%-CI 0.94; 1.87) (n=16)	3.07 (95%-CI 1.94; 4.21) (n=12)	1.66 (95%-CI 0.61; 2.72)	1.5E-3†
Citrate synthase activity [mU/mg]	18.40 (95%-CI 16.20; 20.60) (n=17)	23.18 (95%-CI 18.53; 27.83) (n=9)	4.78 (95%-CI 0.57; 8.98)	0.028*
Citrate synthase activity per porin/GAPDH	15.61 (95%-CI 11.09; 20.13) (n=15)	8.33 (95%-CI 5.47; 11.19) (n=9)	-7.28 (95%-CI -13.31; -1.26)	7.2E-3*
Succinatdehydrogenase activity [mU/mg]	1.23 (95%-CI 1.04; 1.44) (n=16)	1.27 (95%-CI 1.11; 1.44) (n=12)	0.04 (95%-CI -0.22; 0.29)	0.785*
Succinatdehydrogenase activity per porin/GAPDH	1.04 (95%-CI 0.68; 1.41) (n=16)	0.53 (95%-CI 0.25; 0.82) (n=12)	-0.51 (95%-CI -0.98; -0.04)	9.7E-3†
Mitochondrial complex-I activity [mU/mg]	4.38 (95%-CI 3.19; 5.57) (n=14)	2.58 (95%-CI 1.49; 3.66), (n=9)	-1.80 (95%-CI -3.45; -0.17)	0.033*
UCP3/GAPDH [arb. units]	0.49 (95%-CI 0.34; 0.62) (n=17)	1.11 (95%-CI 0.49; 1.73) (n=12)	0.62 (95%-CI 0.11; 1.14)	0.052*
MFN2/GAPDH [arb. units]	0.21 (95%-CI 0.13; 0.29) (n=17)	0.49 (95%-CI 0.33; 0.64) (n=11)	0.28 (95%-CI 0.12; 0.42)	1.4E-3†
DRP1/GAPDH [arb. units]	0.33 (95%-CI 0.29; 0.37) (n=15)	0.54 (95%-CI 0.34; 0.73) (n=12)	0.21 (95%-CI 0.04; 0.38)	0.041†
PGC1alpha/GAPDH [arb. units]	0.30 (95%-CI 0.26; 0.34) (n=19)	0.45 (95%-CI 0.28; 0.61) (n=12)	0.14 (95%-CI 0.01; 0.27)	0.032*
Ratio LC3-I/LC3-II [arb. units]	4.06 (95%-CI 3.33; 4.79) (n=16)	5.71 (95%CI 4.61; 6.81) (n=12)	1.66 (95%-CI 0.45; 2.86)	9.0E-3*
p62/GAPDH [arb. units]	0.08 (95%-CI 0.07; 0.09) (n=17)	0.16 (95%-CI 0.12; 0.20) (n=11)	0.08 (95%-CI 0.04; 0.11)	1.4E-3*
Figure 5				
rel. FKBP12 to RyR1	2.69 (95%-CI 0.59; 4.79) (n=4)	1.01 (0.21; 1.81) (n=4)	-1.68 (95%-CI -3.40; -0.01)	0.050*
SERCA1/GAPDH [arb. units]	0.18 (95%-CI 0.15; 0.21) (n=17)	0.25 (95%-CI 0.18; 0.33) (n=13)	0.07 (95%-CI 0.003; 0.14)	0.044*
SERCA2A/GAPDH [arb. units]	1.64 (95%-CI 1.19; 2.08) (n=18)	2.82 (95%-CI 1.78; 3.86) (n=11)	1.18 (95%-CI 0.26; 2.11)	0.036*
Calpain activity [mU/mg]	3.30 (95%-CI 2.70; 3.89) (n=14)	4.40 (95%-CI 2.60; 6.21) (n=9)	1.11 (95%-CI -0.36; 2.58)	0.643†

pCa50 [mM]	5.90 (95%-CI 5.85; 5.90) (n=12)	5.81 (95%-CI 5.75; 5.87) (n=9)	-0.09 (95%-CI -0.17; -0.02)	0.014*

Values are expressed as mean with 95%-confidence interval. All p-values are two-sided and were not corrected for multiple testing. Continuous variables were tested for normal distribution applying the Shapiro-Wilk-test.

* unpaired Students t-test.

† Mann-Whitney-U-Test.

Online Table II: Cardiopulmonary exercise test and clinical diaphragm assessment

	control n=21	CHF n=18*	P-value
Cardiopulmonary exercise test			
Exercise duration [s]	650 (590; 709)	364 (297; 430)	7.1E-8†
Absolute work load [watt]	118 (108; 128)	73 (61; 85)	7.1E-7†
Relative work load [%]	82 (77; 88)	46 (37; 55)	5.7E-9†
Absolute VO_{2peak} [ml*min^{-1}*kg^{-1}]	23.1 (20.9; 25.3)	15.1 (12.7; 17.5)	1.0E-5†
Relative $VO2_{peak}$ [%]	104 (96; 112)	57 (48; 67)	2.1E-9†
VO2 at VT [ml*min^{-1}*kg^{-1}]	18.1 (16.6; 19.6)	12.2 (9.9; 14.4)	2.8E-5†
Ve/VCO_2 slope	27.8 (26.3; 29.2)	39.0 (33.9; 44.1)	2.5E-4†
Respiratory exchange ratio	1.1 (1.07; 1.11)	1.1 (1.06; 1.13)	0.831†
HR at rest [bpm]	80 (72; 88)	84 (77; 91)	0.463†
HR at peak exercise [bpm]	133 (121; 146)	110 (99; 122)	0.014‡
Sys. BP at rest [mmHg]	127 (119; 134)	98 (93; 103)	1.5E-7†
Dia. BP at rest [mmHg]	77 (72; 82)	65 (61; 69)	8.9E-4‡
Sys. BP at peak exercise [mmHg]	176 (163; 188)	116 (107; 125)	5.7E-9†
Dia. BP at peak exercise [mmHg]	87 (82; 92)	72 (65; 78)	5.2E-4†
Clinical diaphragm assessment			
$P_{0.1}$ [kPa]	0.27 (0.20; 0.33)	0.26 (0.20; 0.31)	0.882†
Pi_{max} [kPa]	7.7 (6.3; 9.0)	4.8 (3.6; 6.0)	1.9E-3†
Pe_{max} [kPa]	9.5 (8.0; 10.9)	7.1 (5.1; 9.1)	0.046†
$P_{0.1}/Pi_{max}$	0.04 (0.03; 0.05)	0.08 (0.04; 0.11)	0.024‡
MVV [l/min]	110 (98; 122)	92 (81; 102)	0.022†
Right Hemidiaphragm [mm]	2.4 (2.2; 2.6)	1.9 (1.6; 2.3)	1.4E-4‡
Left Hemidiaphragm [mm]	2.4 (2.1; 2.6)	1.8 (1.5; 2.2)	5.3E-3†
Mean Diaphragm [mm]	2.4 (2.2; 2.6)	1.9 (1.5; 2.2)	1.4E-4‡

Values are expressed as mean with 95%-confidence interval. VT indicates ventilatory threshold; BP, blood pressure; MVV, maximal voluntary ventilation. 1 kPa = 10.19 cmH_2O

All p-values are two-sided and were not corrected for multiple testing. Continuous variables were tested for normal distribution applying the Shapiro-Wilk-test.

* CPX was performed in all patients except one CHF patient.

† unpaired Students t-test

‡ Mann-Whitney-U-Test.

Online Table III: Invasive data on left and right heart catheterization of CHF patients.

Parameter	mean (95%-confidence interval) (n)
Systolic blood pressure [mmHg]	98 (91; 104) (n=17)
Diastolic blood pressure [mmHg]	67 (61; 72) (n=17)
Left ventricular endddiastolic pressure [mmHg]	22 (16; 28) (n=11)
Right atrial pressure [mmHg]	17 (12; 22) (n=10)
Systolic pulmonary artery pressure [mmHg]	54 (48; 60) (n=17)
Mean pulmonary artery pressure [mmHg]	38 (34; 42) (n=17)
Mean pulmonary capillary wedge pressure [mmHg]	29 (25; 32) (n=17)
Transpulmonary gradient [mmHg]	10 (7; 13) (n=17)
Pulmonary vascular resistance [dynes*s/cm^5]	257 (169; 345) (n=17)
Systemic vascular resistance [dynes*s/cm^5]	1545 (1211; 1879) (n=10)
Cardiac output (according to Fick) [l/min]	3.3 (2.6; 3.9) (n=17)
Cardiac index (according to Fick) [l/min/m²]	1.6 (1.3; 1.8) (n=17)
Cardiac output (according to Thermodilution) [l/min]	3.7 (3.1; 4.2) (n=15)
Cardiac index (according to Thermodilution) [l/min/m²]	1.9 (1.6; 2.2) (n=15)

Catheterization was performed in 17 of 18 CHF patients. Values are expressed as mean with 95%-confidence interval. 80dynes*s/cm^5 = 1 Wood Unit

Online Table IV: Correlation analysis of Nox2 and Nox4 with other molecular and *in-vivo* findings

	Nox2			Nox4		
	Correlation coefficient	**p-value**	**n**	**Correlation coefficient**	**p-value**	**n**
Catabolic markers						
MuRF1	0.40	0.049	26	0.47	0.018	25
Ubi-K48	0.67	<0.001	28	0.39	0.050	26
Proteasome activity	0.44	0.037	23	0.07	0.778	20
Mitochondrial/calcium markers						
Citrate synthase	0.52	7.5E-3	25	0.57	4.9E-3	23
UCP3	0.327	0.103	26	0.69	2.1E-4	24
SERCA1	0.01	0.969	28	0.57	2.8E-3	25
CPX/*in-vivo* diaphragm markers						
Relative VO_{2peak}	-0.72	1.1E-5	29	-0.53	4.7E-3	27
VE/VCO_2 slope	0.56	1.5E-3	29	0.45	0.020	26
Diaphragm thickness	-0.52	3.3E-3	30	-0.41	0.034	27

Correlation analysis were performed by bivariate Pearson correlation. Values are correlation coefficient, two-sided p-value, and number of patients included in the analysis.

Online Table V: Mitochondrial respiratory function with different substrates of diaphragm muscle in patients with heart failure compared to healthy subjects

	Control	CHF	p-Value
PC/Malate			
Max. resp.	185 (152; 219) (n=11)	85 (50; 120) (n=6)	5.4E-4*
ADP-limited resp.	132 (94; 170) (n=11)	57 43; 70) (n=6)	1.1E-3†
RCI	1.52 (1.21; 1.83) (n=11)	1.47 (1.14; 1.80) (n=6)	0.819*
ADP/O ratio	0.68 (0.52; 0.84) (n=10)	0.81 (0.60; 1.03) (n=6)	0.246*
PCoA/Car/Malate			
Max. resp.	180 (136; 225) (n=10)	74 (45; 103) (n=5)	2.9E-3*
ADP-limited resp.	159 (119; 198) (n=10)	57 (36; 77) (n=5)	1.6E-3*
RCI	1.14 (1.09; 1.20) (n=10=	1.30 (1.14; 1.45) (n=5)	0.012*
ADP/O ratio	0.60 (0.49; 0.71) (n=9)	0.73 (0.51; 0.96) (n=5)	0.169*
Glutamate/Malate			
Max. resp.	181 (150; 213) (n=12)	83 (68; 99) (n=7)	4.0E-5†
ADP-limited resp.	123 (95; 151) (n=12)	49 (39; 60) (n=7)	1.0E-4*
RCI	1.57 (1.28; 1.86) (n=12)	1.74 (1.36; 2.13) (n=7)	0.419*
ADP/O ratio	0.68 (0.55; 0.80) (n=12)	0.70 (0.54; 0.87) (n=7)	0.778*
Succinate/Rotenone			
Max. resp.	195 (151; 238) (n=12)	111 (69; 153) (n=7)	0.011*
ADP-limited resp.	137 (103; 170) (n=12)	79 (56; 102) (n=7)	5.1E-3*
RCI	1.44 (1.29; 1.60) (n=12)	1.41 (1.11; 1.71) (n=7)	0.804*
ADP/O ratio	0.42 (0.32; 0.52) (n=11)	0.47 (0.22; 0.73) (n=7)	0.791†
DHQ/Rotenone			
Max. resp.	359 (234; 482) (n=10)	169 (79; 260) (n=6)	0.027*
ADP-limited resp.	243 (161; 326) (n=10)	111 (69; 154) (n=6)	6.3E-3*
RCI	1.47 (1.33; 1.62) (n=10)	1.49 (1.09; 1.89) (n=6)	0.899*
ADP/O ratio	0.39 (0.30; 0.49) (n=10)	0.50 (0.19; 0.81) (n=5)	0.400*
TMPD/Rotenone			
Max. resp. (100mM)	385 (267; 505) (n=10)	150 (45; 257) (n=6)	7.6E-3*

Values are expressed as mean with 95%-confidence interval and number of patients included. Max. resp. indicates maximal respiration (state 3) and is given as natomsO/min/mg protein, ADP-limited resp., ADP-limited respiration (state 4) in natomsO/min/mg protein, RCI, respiratory exchange index, ADP/O, ADP added per oxygen consumed in nmol ADP/natomsO, PC, palmitoylcarnitine, PCoA, Palmitoyl-CoA, Car, carnitine, DHQ, tetramethylhydroquinone, TMPD – tetramethylphenylendiamin
All p-values are two-sided and were not corrected for multiple testing. Continuous variables were tested for normal distribution applying the Shapiro-Wilk-test.
* unpaired Students t-test.
† Mann-Whitney-U-Test.