Ubiquitin-proteasome-system and enzymes of energy metabolism in skeletal muscle of patients with HFpEF and HFrEF

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Abstract

Background  Skeletal muscle (SM) alterations contribute to exercise intolerance in heart failure patients with preserved (HFpEF) or reduced (HFrEF) left ventricular ejection fraction (LVEF). Protein degradation via the ubiquitin-proteasome system (UPS), nuclear apoptosis, and reduced mitochondrial energy supply is associated with SM weakness in HFrEF. These mechanisms are incompletely studied in HFpEF, and a direct comparison between these groups is missing.

Methods and results  Patients with HFpEF (LVEF ≥ 50%, septal E/e’ > 15 or >8 and NT-proBNP > 220 pg/mL, n = 20), HFrEF (LVEF ≤ 35%, n = 20) and sedentary control subjects (Con, n = 12) were studied. Inflammatory markers were measured in serum, and markers of the UPS, nuclear apoptosis, and energy metabolism were determined in percutaneous SM biopsies. Both HFpEF and HFrEF showed increased proteolysis (MuRF-1 protein expression, ubiquitination, and proteasome activity) with proteasome activity significantly related to interleukin-6. Proteolysis was more pronounced in patients with lower exercise capacity as indicated by peak oxygen uptake in per cent predicted below the median. Markers of apoptosis did not differ between groups. Mitochondrial energy supply was reduced in HFrEF and HFpEF (complex-I activity: −31% and −53%; malate dehydrogenase activity: −20% and −29%; both P < 0.05 vs. Con). In contrast, short-term energy supply via creatine kinase was increased in HFpEF but decreased in HFrEF (47% and −45%; P < 0.05 vs. Con).

Conclusions  Similarly to HFrEF, skeletal muscle in HFpEF is characterized by increased proteolysis linked to systemic inflammation and reduced exercise capacity. Energy metabolism is disturbed in both groups; however, its regulation seems to be severity-dependent.

Keywords  Heart failure; Diastolic heart failure; Skeletal muscle exercise; Ubiquitin-proteasome system genetics; Atrophy; Mitochondria

Introduction

Chronic heart failure (CHF) is one of the leading causes of premature death worldwide. Heart failure with preserved left ventricular ejection fraction (HFpEF) accounts for more than 50% of heart failure cases with an increasing prevalence.¹ As in heart failure with reduced ejection fraction (HFrEF), the primary symptom is exercise intolerance, characterized...
by exertional fatigue and dyspnoea. Decreased peak oxygen consumption (peak VO$_2$) measured by cardiopulmonary exercise testing (CPET) is associated with reduced quality of life and increased mortality. Formerly, a reduced cardiac output, either due to impaired left ventricular contractile function or impaired left ventricular filling, was considered to be the sole driver for the reduction in peak VO$_2$. However, the observation that exercise intolerance in HFrEF patients persists despite rapid normalization of cardiac output by pharmaceutical treatment or cardiac transplantation indicates that peripheral alterations significantly contribute to exercise intolerance. Besides endothelial dysfunction, impaired iron metabolism, and diaphragm weakness, morphological and functional alterations of the skeletal muscles (SM) were identified as key players in HFrEF and recently also HFpEF. Local and systemic chronic inflammation mediates organ dysfunction in HFrEF and seems to be a causal factor of HFpEF.

Analysis of SM biopsies in HFrEF has demonstrated a shift in fibre type composition and a reduction in capillary-to-fibre ratio compared with healthy subjects. This has also been observed in older HFpEF patients, indirectly related to peak VO$_2$. A reduction in cross-sectional area (CSA) of muscle fibres and loss of total muscle bulk, termed muscle atrophy, and its relation to exercise intolerance has been identified in HFrEF. Activation of the ubiquitin-proteasome-system (UPS) and subsequent protein degradation is a main determinant of muscle atrophy in HFrEF. In HFpEF patients, the UPS activity has not been evaluated so far, and direct comparisons of SM alterations in HFpEF vs. HFrEF vs. healthy controls are limited in both animal models and humans.

Therefore, we analysed SM specimens from patients with either HFpEF or HFrEF and apparently healthy control subjects to investigate markers of systemic inflammation, SM proteolysis, and apoptosis as well as markers of mitochondrial energy supply.

Methods

Subjects of three randomized controlled trials with either symptomatic HFpEF (OptimEx-CLIN), or HFrEF (SMARTEX-HF), or control subjects without any signs and symptoms of heart failure (LEICA), in whom SM biopsies at baseline evaluation were available, were included in this study. V. A., S. W., and E. B. W. had full access to all data and take responsibility for its integrity and data analysis.

Patient selection

Detailed information on inclusion criteria of each of the trials have been reported elsewhere. Briefly, all heart failure patients had to be in a chronic disease state according to NYHA class II–III, clinically stable, and on optimal medical treatment for at least 6 weeks, without signs of myocardial ischaemia on stress test or high grade heart valve disease. Left ventricular ejection fraction (LVEF) in HFrEF patients had to be below 35%, whereas LVEF in HFpEF patients had to be $\geq 50\%$ in combination with diastolic dysfunction. Diastolic dysfunction was defined as septal E/e’ $> 15$ or E/e’ $8–15$ in combination with an elevated NT-proBNP level $>220$ ng/L. Healthy control subjects had to be free of heart failure signs and symptoms with NT-proBNP levels within normal range. Significant coronary artery and heart valve disease was excluded in those patients. The study research protocols of all trials were approved by the University of Leipzig Ethics Committee. All subjects provided written informed consent before entry into the study.

Echocardiography

Every subject underwent a standardized two-dimensional echocardiography examination according to current echocardiography guidelines using a commercial ultrasound system (Vivid 7, GE Health Medical, Milwaukee, Wisconsin). All images were recorded using harmonic imaging and stored digitally for analysis. Two-dimensional images were recorded using a temporal resolution of at least 60 frames per second; TDI frames were recorded at a rate of $>100$ frames per second. All data were read and analysed by investigators who were blinded to conditions.

Cardiopulmonary exercise testing

Twenty-four hours after sampling of biomaterials CPET was performed with standard equipment for indirect calorimetry (ZAN600, nSpire Health GmbH, Oberthulba, Germany) in an incremental protocol until exhaustion on a bicycle ergometer. In CHF patients, the protocol comprised a 10 Watt increase in workload every minute, starting at 20 Watt. In control subjects, workload was increased progressively every 3 min in steps of 25 Watt. The mean of the three highest 10 s consecutive measurements was identified as peak VO$_2$. Respiratory quotient and other related values are reported from this time point. Per cent predicted peak VO$_2$ was calculated using the Wasserman and Hansen equation. CPET personnel were not blinded to group assignment, but analysis was performed separately by an independent investigator.

Skeletal muscle biopsy

Percutaneous needle biopsies were obtained from the middle part of the vastus lateralis muscle under local anaesthesia. The biopsies were snap-frozen in liquid nitrogen and stored at $-80^\circ$C.
Quantification of circulating inflammatory markers

Serum was obtained from blood samples of all patients by centrifugation and frozen in liquid nitrogen and stored at −80°C until further analysed. Serum concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), and high-sensitive C-reactive protein (hs-CRP) were quantified using a commercially available specific enzyme-linked immunoabsorbent assay (ELISA) [R&D Systems, Heidelberg, Germany (IL-6, IL-1β), Cusabio, Houston, TX, USA (hs-CRP)] according to the manufacturer protocol. Samples were assayed in duplicate.

Protein expression

For western blot analysis, SM tissue was homogenized in Relax 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, 10 mmol/L creatine phosphate, and pH 7.4) containing a protease inhibitor mix (Inhibitor mix M, Serva, Heidelberg, Germany) and sonicated. Protein concentration was determined (BCA assay, Pierce, Bonn, Germany) and aliquots (5–20 μg) were separated by SDS-polyacrylamide gel electrophoresis (PVDF) and incubated overnight at 4°C using the following primary antibodies: muscle ring finger-1 (MuRF-1) (1:1000; Abcam, Cambridge, UK), muscle atrophy F-box (MAFbx) (1:1000; Abcam, Cambridge, UK), ubiquitin linkage-specific proteasome activity (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

Data were analysed using SPSS version 25 (IBM Corporation, Armonk, USA). Continuous variables are depicted as mean values ± standard error of the mean (SEM). Normal distribution was tested applying the Shapiro–Wilk test. One-way ANOVA or Kruskal–Wallis, as appropriate, followed by two-sided post hoc test was used to analyse differences between groups. Categorical variables are given as numbers and percentages and were tested applying the Fisher’s exact test. Bivariate correlation coefficients between protein expression or enzyme activities and clinically meaningful parameters within the whole dataset were calculated using two-sided Pearson or Spearman, as appropriate. A P-value of <0.05 was considered statistically significant.

Results

Clinical data and SM biopsy samples were available in 12 control subjects, 20 HFrEF, and 20 HFrEF patients. Diagnosis of CHF was made more often within 1 year of study inclusion in the HFrEF group compared with HFrEF (8 vs. 4 points, P < 0.05). The diagnostic score of the Heart Failure Association of the European Society of Cardiology, HFA-PEFF score, was ≥5 points in 19 patients and 4 points in one patient in the HFrEF group.23 HFrEF patients comprised the youngest group, were predominantly male, had a high prevalence of coronary artery disease, and had lower systolic blood pressure compared with HFrEF patients (Table 1). Both medical and cardiac implanted device therapy were more prevalent in HFrEF compared with HFrEF (Tables 1 and 2). HFrEF patients were predominantly female, had higher body mass index compared with controls, all had a history of arterial
hypertension (not significantly different between groups), and more often atrial fibrillation with concomitant oral anticoagulation (Tables 1 and 2). In both CHF groups, patients reported symptoms of dyspnoea predominantly New York Heart Association class II, NT-proBNP levels were significantly elevated, and kidney function chronically impaired compared with control. The incidence of diabetes mellitus, dyslipidaemia, chronic obstructive pulmonary disease, or peripheral arterial occlusive disease was not significantly different between groups (Table 1).
Echocardiographic findings

Echocardiographic analysis is summarized in Table 3. It revealed significantly enlarged left ventricular (LV) diameters and volumes in HFrEF patients compared with control and HFpEF. This was associated with a severely impaired LVEF and a higher rate of minor mitral regurgitation. In HFpEF patients, LVEF was nearly identical compared with control, but septal wall was significantly thicker compared with control and HFrEF. Consequently, LV mass index (LVMI) was significantly higher in HFrEF and also tendentiously in HFpEF vs. control (P = 0.07). In both CHF groups, diastolic function was impaired indicated by reduced tissue Doppler velocities of the septal and lateral mitral annulus (e0) and increased E/e0 ratio. In both CHF groups, left atrial volume normalized to body surface area (LAVI) was found to be increased compared with control suggesting impaired LV filling. Doppler velocity of tricuspid regurgitation was not significantly increased in HFrEF or HFpEF compared with control, and therefore, relevant pulmonary hypertension is unlikely.

Cardiopulmonary exercise testing

Data from CPET were available from all patients and are shown in Table 4. Respiratory exchange ratio (RER) was close

Table 3 Echocardiographic findings

|               | Control n = 12 | HFrEF n = 20 | HFpEF n = 20 | Statistics               |
|---------------|----------------|--------------|--------------|--------------------------|
| LVEF, %       | 64 ± 2         | 64 ± 1       | 29 ± 1***    | vs. Control and HFpEF    |
| LVEDD, mm     | 48 ± 2         | 45 ± 1       | 68 ± 2***    | vs. Control and HFpEF    |
| LVESD, mm     | 32 ± 2         | 30 ± 1       | 58 ± 2***    | vs. Control and HFpEF    |
| LVEDV, mL     | 79 ± 2         | 87 ± 5       | 232 ± 17***  | vs. Control and HFpEF    |
| LVESV, mL     | 27 ± 2         | 31 ± 2       | 165 ± 13***  | vs. Control and HFpEF    |
| Septum, mm    | 10.3 ± 0.6     | 13.9 ± 0.6***| 10.8 ± 0.6   | vs. Control and HFpEF    |
| LVMI, g/m²    | 87 ± 6         | 138 ± 17     | 151 ± 8***   | vs. Control              |
| LA diameter, mm| 36 ± 2         | 45 ± 2***    | 48 ± 2***    | vs. Control and HFpEF    |
| LAVI, mL/m²   | 17 ± 2         | 43 ± 3***    | 42 ± 4***    | vs. Control and HFpEF    |
| E wave, cm/s  | 73 ± 9         | 101 ± 6*     | 87 ± 8       | vs. Control and HFpEF    |
| A wave, cm/s  | 67 ± 7         | 94 ± 7*      | 69 ± 7       | vs. Control and HFpEF    |
| E/A ratio     | 1.1 ± 0.1      | 1.1 ± 0.1    | 1.6 ± 0.3    | n.s.                     |
| DT, ms        | 229 ± 10       | 208 ± 18     | 204 ± 30     | n.s.                     |
| IVRT, ms      | 119 ± 9        | 91 ± 6*      | 122 ± 13     | vs. HFrEF               |
| e0 septal, cm/s| 8.3 ± 1.4      | 5.1 ± 0.3**  | 4.6 ± 0.5**  | vs. Control and HFpEF    |
| e0 lateral, cm/s| 9.9 ± 1.5      | 8.2 ± 0.7    | 6.1 ± 0.6*   | vs. Control and HFpEF    |
| E/e0 septal   | 9.6 ± 0.9      | 20.1 ± 1.3***| 21.2 ± 2.7***| vs. Control and HFpEF    |
| E/e0 lateral  | 8.1 ± 1.0      | 12.7 ± 1.0***| 16.2 ± 1.9***| vs. Control and HFpEF    |
| MR grade 0/I/II, n (%) | 9/3/0 (75/25/0) | 9/11/0 (45/55/0) | 2/17/1 (10/85/5)* | vs. Control and HFpEF |
| TR velocity, m/s | 2.3 ± 0.1 | 2.6 ± 0.1 | 2.8 ± 0.1 | n.s.                     |
| PAPs, mmHg    | 22 ± 1         | 29 ± 2       | 33 ± 3       | n.s.                     |

A, peak velocity flow in late diastole caused by atrial contraction; DT, E-wave deceleration time; E, peak velocity blood flow from ventricular relaxation in early diastole; e0, early diastolic velocity of the septal mitral annulus; IVRT, isovolumetric relaxation time; LA, left atrium; LAVI, left atrial volume index; LVEDD, left ventricular end diastolic diameter; LVEDV, left ventricular end diastolic volume; LVEF, left ventricular ejection fraction; LVE, left ventricular end systolic diameter; LVESV, left ventricular end systolic volume; LVMI, left ventricular mass index; MR, mitral regurgitation; PAPs, systolic pulmonary artery pressure; TR, tricuspid regurgitation.

*P < 0.05.
**P < 0.01.
***P < 0.001.

Table 4 Cardiopulmonary exercise testing

|               | Control n = 12 | HFrEF n = 20 | HFpEF n = 20 | Statistics               |
|---------------|----------------|--------------|--------------|--------------------------|
| RER           | 1.11 ± 0.02    | 1.11 ± 0.02  | 1.09 ± 0.02  | n.s.                     |
| RER > 1.1, n (%)| 6 (50)        | 11 (55)      | 12 (60)      | n.s.                     |
| Maximum heart rate, bpm | 136 ± 7 | 114 ± 7 | 121 ± 5 | n.s.                     |
| Load, Watt    | 100 ± 9        | 93 ± 6       | 100 ± 6      | n.s.                     |
| VO2 at VT1, mL | 1007 ± 68     | 940 ± 47     | 1073 ± 63    | n.s.                     |
| Peak VO2, mL/min | 1495 ± 128   | 1541 ± 91    | 1541 ± 114   | n.s.                     |
| Peak VO2/BW, mL/min/kg | 19.1 ± 1.1 | 17.4 ± 1.3 | 17.4 ± 1.2 | n.s.                     |
| Peak VO2% predicted, % | 91 ± 6 | 92 ± 6 | 73 ± 5* | vs. Control and HFpEF |
| Ventilation, L/min | 50 ± 4        | 61 ± 5       | 60 ± 5       | n.s.                     |

BW, body weight; RER, respiratory exchange ratio; VO2, oxygen uptake; VT1, ventilatory threshold 1.

*P < 0.05.
**P < 0.01.
***P < 0.001.
to 1.1 in all groups indicating exhaustion at CPET termination. Between groups, there was no significant difference in maximum load, ventilation, peak VO2, and peak VO2/kg body weight. Because predicted oxygen uptake was higher in the HFrEF group due to age and body height, peak VO2 in percent of predicted value was significantly reduced (73 ± 5%) in comparison with control and HfPEF. In patients with HfPEF, beta-blocker treatment was associated with lower heart rate at rest and at maximum effort, whereas RER, maximum load, and peak VO2 were not significantly different from patients without beta-blocker therapy (data not shown).

### Correlation analysis

Systemic inflammation, indicated by elevated IL-6, directly correlated to NT-proBNP ($r = 0.336$; $P < 0.05$). Correlation analysis for proteasome activity is summarized in Table 5. Proteasome activity was significantly correlated to both parameters, IL-6 and NT-proBNP. Furthermore, proteasome activity was related to the expression of E3 ligases and ubiquitinated proteins as well as common echocardiographic alterations of both CHF entities, for example, LAVI, LVMi, and E/e'. A weak negative association with peak VO2 per cent predicted did not reach statistical significance. However, in subjects with peak VO2 per cent predicted below median (83.5%), proteasome activity was significantly higher compared with those with oxygen uptake above median ($5.15 ± 0.73$ mU/mg vs. $3.42 ± 0.46$ mU/mg, $P < 0.05$).

There were no significant associations between mitochondrial complex-I activity and IL-6, NT-proBNP, other parameters of energy metabolism, proteasome activity, echocardiographic parameters, or peak VO2 (Table 5).

### Sex and age matched subgroup

Due to significant differences in essential patient characteristics between groups a subgroup of eight patients per group were matched for sex ($P = 1.00$) and age ($P = 0.63$). Differences between groups disappeared for height but remained, at least numerically, for higher BMI in HfPEF and higher IL-6 serum concentration in HfPEF and HFrEF compared with control (Supporting Information, Table S1). Analysis of proteolysis (Supporting Information, Figure S1), apoptosis (Supporting Information, Figure S2), and energy metabolism (Supporting Information, Figure S3) showed similar results on trend in comparison with the full dataset.

### Discussion

Skeletal muscle atrophy and metabolic alterations—potential mechanisms of impaired exercise tolerance in heart failure—are incompletely understood, and especially a comparison between HfPEF and HFrEF is missing. This study for the first time directly compares the activity of the UPS, markers of apoptosis, and energy metabolism in SM specimens of CHF patients either with reduced or preserved LVEF and apparently healthy control subjects.

### Patient characteristics

Patients with HfPEF were about 10 years older and more often female compared with HFrEF patients, who suffered more often from ischaemic heart disease.
reflects findings from other trials and is related to pathophysiology.\textsuperscript{15,17,24,25} Definite diagnosis of HFpEF was given in 19 out of 20 patients of the HFpEF group according to the HFA-PEFF score. Clinical and echocardiographic assessment of both groups revealed classical findings of stable CHF in NYHA class II–III with markedly elevated NT-proBNP, increased LVMI, dilated left atrium, and impaired diastolic LV function compared with control subjects. In contrast, the cardiovascular risk factor profile, for example, overweight, arterial hypertension, and dyslipidaemia, was similar between groups. Because inhibitors of the renin-angiotensin-system were frequently used in both CHF groups (95%), its impact on study results was probably minor.\textsuperscript{26} Peak VO\textsubscript{2} per cent predicted was significantly impaired in HFrEF. We would have expected higher peak VO\textsubscript{2} levels in control subjects.\textsuperscript{7,9,15,27} This might be related to differences in age and gender, the overall sample size, and minor discrepancies in CPET protocol between groups and otherwise indicates

**Figure 1** The protein expression of MuRF-1 (A), MAFbx (B), and ubiquitinated proteins (UB-K48) (C), and the activity of the proteasome (D) was measured in skeletal muscle specimens of control subjects (Control) and patients with heart failure with preserved (HFpEF) or reduced left ventricular ejection fraction (HFrEF). For protein expression, representative western blots and quantitative analysis are shown. Values are shown as means ± SEM.
that control subjects were more or less sedentary and untrained. Within a subgroup of patients matched for sex and age, similar results for inflammation, proteolysis, apoptosis, and energy metabolism were seen. These findings suggest that the hereinafter discussed SM pathologies are intrinsic alterations of CHF and neither a result of chronic deconditioning nor related to age-dependent sarcopenia, gender mismatch, or risk factor profile.11,28,29

Inflammation

In line with previous data, we found increased inflammation, indicated by IL-6, in CHF patients compared with control.6,11 The significant correlation of IL-6 and NT-proBNP levels is consistent with a relation of the inflammatory response to the severity of the CHF syndrome. This is supported by a positive correlation of IL-6 and proteasome activity. In HFrEF, the SICA-HF trial demonstrated an association of increased IL-6 levels with SM wasting and worse prognosis.30 Experimental models suggest a causal relationship between IL-6 and the activation of the UPS and subsequent SM wasting.31

Regulation of the ubiquitin-proteasome-system within the skeletal muscle

Skeletal muscle mass and muscle fibre size are mainly determined by protein turnover. An imbalance between protein synthesis and degradation in favour of the latter is associated with muscle atrophy, a reduction in absolute force production, and exercise intolerance. The muscle-specific ubiquitin E3 ligases MuRF-1 and MAFbx catalyse the rate-limiting step of the ubiquitination process and subsequent proteasome-dependent degradation of proteins in experimental studies (for a review, see Adams et al.13). Even though Forman et al. did not detect differences in mRNA expression of MuRF-1 and MAFbx (Atrogen-1) in HFrEF patients compared with control,32 our data are in line with findings from Gielen et al. showing an up-regulation of MuRF-1 on mRNA and protein level.11 Consistently, we also found an increase in ubiquitinated proteins, both correlating to a higher activity of the proteasome itself and, therefore, clearly confirming the activation of the UPS in HFrEF. The higher proteasome activity in patients within the lower range of peak VO₂ per cent predicted (below the median) points out the clinical relevance of the UPS in CHF. In fact, exercise training as an interventional approach was shown to reduce MuRF-1 and ubiquitinated proteins accompanied by an increase in CSA of the quadriceps muscle, SM force, and peak VO₂.11,12

Our data for the first time extend these findings of UPS activation to the group of HFrEF patients. MRI scans in HfPEF visualized a fatty degeneration of the quadriceps muscle compared with control subjects and HFrEF and indicate a relevant contribution of SM atrophy to impaired exercise tolerance in HFrEF.15,33 Even though peak VO₂ was found to be essentially preserved in our HFrEF cohort and comparable with a sedentary control group, our data indicate an induction of SM atrophy early in the course of HFrEF and underscores the prominent impact of inflammation on HFrEF
The impact of UPS activation as a pre-clinical marker of CHF-associated SM impairment needs to be further established.

**Intramuscular apoptosis**

Apoptosis of nuclei in SM fibres has been shown in animal experiments of hindlimb suspension\(^2^9\) and HFrEF patients\(^1^0,3^4\) and might contribute to SM atrophy. Among others, reactive oxygen species increase mitochondrial membrane permeability with subsequent cytochrome c release and activation of caspase-3, which is a major executioner of apoptotic nuclear and cytoskeletal fragmentation. BCL2 blocks the release of cytochrome c and therefore serves an anti-apoptotic role.\(^3^5\)

In a small group of HFrEF patients, Vescovo et al. found reduced BCL2 and increased caspase-3 levels in SM associated with an elevated number of apoptotic nuclei. The latter
negatively correlated with CSA and peak VO₂.¹⁰ This is in contrast to our data showing no difference in BCL2 and activated caspase-3 neither in HfPEF nor in HFrEF. Interestingly, Adams et al. detected apoptotic nuclei only in patients with severely reduced peak VO₂ (mean 12.0 mL/min/kg) and comparable with the patient group studied by Vescovo et al., but not in patients with more preserved exercise tolerance (mean peak VO₂ 18.2 mL/min/kg), which was comparable with our cohort.³⁴ Therefore, nuclear apoptosis seems to be of minor relevance for SM atrophy in both CHF entities and might be confined to more advanced disease states.

**Energy metabolism of the skeletal muscle**

Muscle function not only depends on the availability of contractile proteins but also on sufficient energy supply by mitochondria. The significant reduction of complex-I activity not only in HFrEF but also in HfPEF indicates a reduction in oxidative capacity, because complex-I is suggested the rate-limiting step in overall respiration and therefore central in energy metabolism.³⁶ This is supported by reduced activity of MDH representing the citrate cycle and, therefore, glycolytic and oxidative capacity. Nevertheless, it seems that in HfPEF, the SM compensates this complex-I reduction by increasing complex-II activity and protein expression. This compensation mechanism is absent in HFrEF. Recently, indirect measurement of reduced mitochondrial content and function by porin expression and citrate synthase activity revealed similar results in a group of older HfPEF patients, correlated with peak VO₂, and suspected to contribute to abnormal SM oxygen utilization.³⁷ Conflicting results come from a study of respiration in skinned muscle fibres with markedly impaired oxidative capacity in HFrEF compared with physically active controls but not different from healthy sedentary controls.²⁸ However, the gold standard for direct measurement of mitochondrial energy production *in vivo* is phosphorous resonance spectroscopy under exercising conditions. Using this technique, alteration in metabolic response to exercise in HFrEF where described already 30 years ago.⁷ Recently, Weiss et al. determined an increased fatigability of exercising muscles associated with early, rapid declines in high-energy phosphates, significantly delayed rate of creatine phosphate recovery, and reduced oxidative capacity in patients with HFrEF and HfPEF compared with healthy controls.¹⁵

Taking into account that the severity of metabolic and mitochondrial alterations was related to the degree of physical limitation in either HfPEF or HFrEF in different studies,¹⁵,²⁸ we postulate that the significantly higher reduction in complex-I activity in HFrEF compared with HfPEF in our study is independent of CHF ethology and related to disease severity as expressed by lower peak VO₂ per cent predicted and higher NT-proBNP on trend.

The rapid depletion of creatine phosphates with exercise in CHF might be partially explained by a reduction in creatine kinase, a key enzyme for shuttle of high-energy phosphates from the mitochondrial oxidative phosphorylation to the cytosolic myosin filaments. We found an impaired CK activity in HFrEF patients but an up-regulation in HfPEF. If this phenomenon is based on compensatory mechanisms in failing energy supply or rather a disease specific alteration is unclear. Previous studies on CK activity in HFrEF are inconclusive.¹⁵,²⁷,²⁸ Further research is necessary to elucidate the complex regulation of energy metabolism in the course of different CHF entities.

**Differences between heart failure with preserved left ventricular ejection fraction and heart failure with reduced left ventricular ejection fraction**

Summarizing the molecular changes in SM (*Table 6*), it became evident that alterations in HFrEF are more pronounced compared with HfPEF. In both CHF entities, the catabolic system is activated, evident by the up-regulation of MuRF-1 and proteasome activity, whereas the impairment of the mitochondrial energy generation (complex-I and MDH activity) is more prominent in HFrEF. These
differences, possibly explained by the higher level of inflammation in HFrEF, are in accordance with data from animal models and may be one reason for the impaired exercise capacity in HFrEF. Furthermore, this supports the single syndrome notion, which suggests that CHF—indeed of its ethology—is a continuum with multiple phenotypes between both extremes.

**Limitations**

This study is limited by a small sample size of the control group, hindering the detection of small differences between groups, the comparison of subgroups, for example, ischaemic vs. non-ischaemic ethology, and propensity score matching to control for confounding factors, for example, BMI, NYHA class, peak VO₂, NT-proBNP, or medical treatment. Therefore, the differentiation of disease-specific vs. disease duration or severity related alterations is limited. However, access to SM biopsies in humans, especially in healthy controls, is limited. The combination of molecular data with functional parameters of SM strength and endurance would have strengthened the paper. We did not analyse markers of protein synthesis such as insulin-like growth factor 1 or myostatin for a more complete description of protein turnover due to a shortness of biopsy material. For a more comprehensive evaluation of energy production, mitochondrial respiration (total ATP production) should ideally have been measured in isolated organelles or saponin skinned muscle fibres taking into account the complex interaction and modulation of respiratory enzymes including supercomplex formation. Unfortunately, this is impossible in frozen specimens. Tissue staining for apoptotic cells would have been necessary to rule out programmed cell death independent from caspase-3.

**Conclusions**

This study for the first time directly compares the activity of the UPS, markers of apoptosis and energy metabolism in SM specimens in CHF patients either with reduced or preserved LVEF and healthy control subjects. In both CHF entities, the UPS is clearly activated on the level of E3 ligases, amount of ubiquitinated proteins and proteasome activity, and significantly related to IL-6 levels. The UPS, therefore, might serve as therapeutic target in HFrEF and HFpEF. In contrast, intramuscular apoptosis seems to be of minor impact for SM atrophy and exercise intolerance in our cohort. The regulation of SM energy metabolism is more complex with a down-regulation of complex-I activity and MDH in both CHF groups, indicating disrupted mitochondrial respiration and citrate cycle, whereas CK activity is differentially regulated with reduction in HFrEF and elevation in HFpEF. If this references a compensatory mechanism in different disease severity or differentiates HFpEF from HFrEF needs to be further elucidated.

**Conflict of interest**

Ephraim Winzer reports personal fees from Boehringer-Ingelheim, Novartis, and CVRx outside of this study. Norman Mangner reports personal fees from Edwards LifeScience, Medtronic, Biotronik, Novartis, Sanofi Genzyme, and AstraZeneca outside the submitted work. Stephan Gielen reports personal fees from Amgen, AstraZeneca, Novartis, Daiichi-Sankyo, and Sanofi-Aventis outside and without relevance to the submitted work. Volker Adams, Sebastian Wunderlich, Jennifer Hommel, Katrin Esefeld, Martin Halle, Øyvind Ellingsen, Emeline M. Van Craenenbroeck, Ulrik Wisløff, Burkert Pieskem and Axel Linke declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The protein expression of MuRF-1 (A), MAFBx (B) and ubiquitinated proteins (UB-K48) (C) and the activity of the proteasome (D) was measured in skeletal muscle specimens of control subjects (Control) and patients with heart failure with preserved (HFrEF) or reduced left ventricular ejection fraction (HFrEF) matched for sex and age. Values are shown as means±SEM.

Figure S2. The protein expression of BCL2 (A) and caspase-3 (B) was measured in skeletal muscle specimens of control subjects (Control) and patients with heart failure with preserved (HFpEF) or reduced left ventricular ejection fraction (HFrEF) matched for sex and age. Values are shown as means±SEM.

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