Exercise Training Attenuates MuRF-1 Expression in the Skeletal Muscle of Patients with Chronic Heart Failure Independent of Age: The Randomized Leipzig Exercise Intervention in Chronic Heart Failure and Aging (LEICA) Catabolism Study

Running title: Gielen et al.; Muscle wasting in aging and heart failure

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Abstract:

Background - Muscle wasting occurs both in chronic heart failure (CHF) and in normal aging and contributes to exercise intolerance and increased morbidity/mortality. However, the molecular mechanisms of muscle atrophy in CHF and their interaction with aging are still largely unknown. We therefore measured the activation of the ubiquitin-proteasome system (UPS) Min muscle biopsies of CHF patients and healthy controls (HC) in two age strata and assessed the age-dependent effects of a 4-week endurance training program on the catabolic-anabolic balance.

Methods and Results - 60 CHF patients (30 patients≤55 years, mean age 46±5 years; 30 patients≥65 years, 72±5 years) and 60 healthy controls (HC, 30≤55 years, 50±5 years; 30≥65 years, 72±4 years) were randomized to 4 weeks of supervised endurance training or to a control group. Before and after the intervention vastus lateralis muscle biopsies were obtained. The expression of cathepsin-L, the muscle-specific E3 ligases MuRF-1 and MAFbx were measured by real-time PCR and confirmed by Western blot. At baseline MuRF-1 expression was significantly higher in CHF patients versus HC (mRNA: 624±59 versus 401±25 rel. units; p=0.007). After four weeks of exercise training MuRF-1 mRNA expression was reduced by -32.8% (p=0.02) in CHF patients ≤55 years and by -37.0% (p<0.05) in CHF patients ≥65 years.

Conclusions - MuRF-1, a component of the ubiquitin-proteasome system involved in muscle proteolysis, is increased in the skeletal muscle of patients with heart failure. Exercise training results in reduced MuRF-1 levels, suggesting that it blocks UPS activation, and does so in both younger and older CHF patients.

Clinical Trial Registration Information - ClinicalTrials.gov; Identifier: NCT00176319.

Key words: aging; chronic heart failure; exercise training; ubiquitin proteasome system
Introduction

Chronic heart failure (CHF) is a disease of the elderly: The prevalence of CHF increases from less than 2% for below the age of 60 to 9% in men and 5% in women between 60 and 79 years of age. In octogenarians 15% of all men and 13% of all women are affected.

The symptomatic effects of CHF are often aggravated in elderly patients because of an overlap between the physiologic age-related decline in exercise capacity and the disease-related functional reduction. The combination of both may potentiate permanent disability and/or need for care. Additionally, it has been found that more than 35% of individuals over the age of 65 years have evidence of sarcopenia, which denotes an excessive loss of muscle mass with advancing age and frailty. Despite the relevance of exercise intolerance resulting in increased need for day/home care for patients, insurance companies, and welfare systems alike it is unknown to which extent the loss of muscle mass and muscle force among elderly CHF patients is age- or disease-related. We hypothesized that a sarcopenia-cachexia overlap could potentially be found in elderly CHF patients.

The Leipzig Exercise Intervention in Chronic heart failure and Aging (LEICA) study was therefore designed to investigate the catabolic-anabolic imbalance in the skeletal muscle associated with CHF in two different age strata, ≤55 years and ≥65 years in comparison to healthy controls of the same age group. Additionally, patients and controls in each age stratum were prospectively randomized to four weeks of supervised endurance training or usual care to study if age would attenuate the gains in exercise capacity and the anabolic activation in the skeletal muscle associated with cardiac rehabilitation.

In the last decade weight loss and muscle wasting were identified as novel predictors of adverse prognosis in chronic systolic heart failure (CHF). Basically, maintenance of lean muscle mass depends on an intricate balance between anabolic (growth hormone (GH), insulin-
like growth factor-I (IGF-I), myostatin) and catabolic factors (TNF-α, IL-1β, IFN-γ). We have previously shown in CHF that the basal expression of IGF-1 is reduced⁵ while catabolic factors like TNF-α, IL-1β, and IL-6 are significantly increased⁶. Based on animal experiments the most important mechanism for intracellular degradation of striated muscle proteins is the ubiquitin-proteasome system (UPS). Damaged cytosolic proteins (mutant or misfolded) and long-lived proteins such as components of the contractile apparatus of striated muscles⁷ are linked to four or more ubiquitin molecules (ubiquitination) to be targeted towards the proteasome, where they are unfolded and cleaved into short peptides and amino acids. Three types of enzymes are involved in ubiquitination: Ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3)⁸. Two E3 ligases – muscle ring finger 1 (MuRF-1) and muscle atrophy F-box (MAFbx) – were identified as being specifically expressed in striated muscle tissue and are potentially involved in the CHF-related muscle wasting⁹. Proinflammatory cytokines, such as TNF-alpha or IL-1 stimulate the expression of MuRF-1 and MAFbx, increase protein ubiquitinylation, and thereby accelerate UPS-mediated protein degradation⁹-¹¹.

Aim of the LEICA Catabolism Study was specifically to analyze the role of the two muscle-specific atrogenes (i.e. MuRF-1 and MAFbx) for the catabolic-anabolic balance in the skeletal muscle of stable CHF patients versus healthy controls in the same age stratum and their response to the anabolic stimulus of a 4-week supervised endurance training intervention in muscle biopsies of the vastus lateralis muscle before and after the intervention.

Methods

Patients

Patients with CHF and healthy controls (HCHC) with normal LV ejection fraction in two age strata (≤55 years and ≥65 years) were recruited. Patients/subjects of each of the four groups
(HC≤55 years, HC≥65 years, CHF≤55 years, CHF≥65 years) were then prospectively randomized to either four weeks of supervised aerobic exercise training or to usual care.

**Inclusion Criteria**

CS with normal systolic LV function (>55%), without signs or symptoms of CHF and without evidence of coronary artery disease during coronary angiography were included. CHF patients referred to the Heart Center Leipzig between June 2005 and April 2008 were enrolled based on the following clinical inclusion criteria: (1) documented heart failure by signs, symptoms, and ventriculographic evidence of reduced LV function (LV ejection fraction [LVEF] <40%) as a result of dilated cardiomyopathy or ischemic heart disease; (2) exercise capacity at baseline >25 W; and (3) clinical stability for at least 3 months before entry into the study. To achieve a difference in mean age of approximately 20 years between the young and old cohort CS and patients ≤55 years and ≥65 years were included.

**Exclusion criteria** were significant valvular heart disease (> grade II), uncontrolled hypertension, insulin-dependent diabetes mellitus, history of LV tachycardia/ fibrillation without implanted cardioverter defibrillator (ICD), peripheral vascular disease, pulmonary disease, or orthopaedic comorbidities precluding exercise training.

The LEICA study research protocol was approved by the University of Leipzig Ethics Committee and all patients provided written informed consent before entry into the study. The study was registered at ClinicalTrials.gov (NCT00176319).

**Training Intervention**

Patients were randomly assigned to either a training group or an inactive control group by drawing a sealed envelope with intervention assignment enclosed. Four baskets of envelopes (n=30 each) were prepared for each of the four cohorts: HC ≤55 years, HC ≥65 years, CHF patients ≤55 years, and CHF patients ≥65 years. Cardiopulmonary exercise testing,
echocardiography, skeletal muscle biopsy, and venous blood samples were obtained at baseline and after the training intervention. The exercise intervention consisted of 4 supervised training sessions per weekday each for 20 minutes (excluding 5 min of warming-up and cooling-down) using a bicycle ergometer (Ergofit Inc., Pirmasens, Germany). Workloads were adjusted so that 70% of the symptom-limited maximum oxygen uptake was reached. In addition, patients and HC were expected to participate in one group training session of 60 minutes per week. Group sessions consisted of walking, calisthenics, and ball games.

Patients assigned to the control group received usual clinical care by their physicians. All examinations including exercise testing were repeated at 4-weeks follow-up. Patients and HC continued on their individually tailored medication. Medication included up-titrated angiotensin converting enzyme inhibitors and beta blockers in all CHF patients. Patients with LV-EF<30% or in NYHA functional class III were maintained on stable spironolactone/eplerenone medication for a minimum of 3 months prior to study enrolment and throughout the study period.

Assessments

Cardiopulmonary Exercise Testing

Exercise testing was performed on a calibrated, electronically braked bicycle (ZAN600, ZAN, Bad Hall, Austria) in an upright position. Workload was increased progressively every 3 minutes in steps of 25 Watts beginning at 25 Watts. Respiratory gas exchange data were determined continuously. Exercise was terminated when patients were physically exhausted (as indicated by a RER >1.1) or developed severe dyspnea/dizziness or peripheral muscle fatigue.

Echocardiography

The echocardiographic studies were performed according to current echocardiography guidelines at baseline and the end of the study by an experienced echocardiographer blinded to patient status and group assignment. Intraobserver variabilities of key parameters were below 5%,
interobserver variabilities were below 10%. For determination of left ventricular ejection fraction (LVEF) and ventricular volumes three consecutive cardiac cycles were analyzed on a GE Vivid 7 echocardiography system (Software: EchoPAC™ Dimension ’06, GE Healthcare, Chalfont St Giles, Great Britain; Simpson method-of-disk) and averaged for each patient.

**Muscle-CT**

As an established marker of skeletal muscle mass the cross sectional area of the quadriceps muscle was measured by CT at baseline and after 4 weeks (Siemens Somatom Plus 4, Erlangen, Germany)\(^1\).

**Force Measurement**

The maximal isometric Force of the M. quadriceps was measured using a dynamometer (Schnell M3 Diagnos, Peutenhausen, Germany) with a minimal resolution <1 Newton. The maximal voluntary isometric force was measured as an average out of 5 contractions which were performed after at least 60 seconds of recovery. Force endurance was measured as the achieved time holding 50% of the maximal isometric force.

**RNA Isolation and Quantification of mRNA Expression**

Total RNA was isolated from frozen biopsies (RNeasy, Qiagen, Hilden, Germany) and reverse transcribed into cDNA using random hexamers and Sensiscript reverse transcriptase (Qiagen, Hilden, Germany). An aliquot of the cDNA was used for quantitative RT-PCR (IQ5 cycler, BioRad, Munich, Germany). Gene expression levels were normalized to the expression of 18S-rRNA. The primers/probes and conditions are detailed in Attachment 1 (Supplemental Material).

**Quantification of protein expression**

Frozen tissue samples were homogenized in lysis buffer and protein expression was quantified by Western blot using specific antibodies to MuRF-1, MAFbx (generated in rabbits by our group...
as recently described). After incubation with a horseradish peroxidase-conjugated secondary antibody, specific bands were visualized by enzymatic chemiluminescence (Super Signal West Pico, Pierce, Bonn, Germany). Loading differences were controlled by re-probing the blot with an antibody against GAPDH (Hytest, Turku, Finland). Ubiquitinylation was quantified using a specific antibody only detecting ubiquitinylated proteins, whereas free ubiquitin is not recognized (MBL, Woburn, CA). Quantification of the protein expression was analyzed using a 1-D densitometric analysis software package (Scanalytics, Rockville, MD, USA).

**Measurement of Parameters of the GH/IGF-I Axis and TNF-α**

Blood samples were collected from both patients and healthy controls in the morning after a fasting period of 12 h. Growth hormone was measured by immunofluorometric assay using the AutoDELFIA System (Wallac, Turku, Finland). Serum levels of IGF-I were measured after acid ethanol extraction by a competitive solid phase immunoassay according to the method of Kratzsch et al\(^5\). Serum levels of IGF binding protein 3 (IGFBP-3) were determined using a commercially available ELISA (DSL, Sinsheim, Germany). All samples were run in duplicates and the average value of the two measurements is reported. The sensitivity of the assays as well as the intraassay and interassay coefficients of variation have been previously published\(^5\). All technicians were blinded for patient status (i.e. study entry or finish) and assignment (i.e. training or control group).

Serum concentrations of tumor necrosis factor-α (TNF-α) were measured by a specific high sensitive enzyme-linked immunoabsorbent assay kit (Quantakine, R&D Systems, Minneapolis, USA) with a sensitivity <0.18 pg/ml. All samples were run in duplicates and the average value of the two measurements is reported.

**Statistical Analysis**
Based on data from animal experiments the change in MuRF-1-mRNA-expression was used as the primary endpoint for the sample size calculation. A difference in treatment effect between training and control groups of 0.3 relative units was assumed with an estimate of the standard deviation of 0.2 relative units. With an $\alpha$ error of 0.05 and a $\beta$ error of 0.10 (power 90%), minimal group size was estimated to be 15 patients in each group.

Longitudinal data were reanalyzed by a multifactorial ANOVA with repeated measurements: CHF, exercise and age group, respectively as between-subject factors; time (baseline, 4 weeks) as within-subject factor. Starting in each case with full factorial models non-significant factors and interactions were omitted. Only significant main effects and interactions with acceptable effect size ($\eta^2>0.1$) were reported. Additionally, comparisons within the eight subgroups over time were analysed by paired t test. $\chi^2$ tests were performed to compare groups on categorical variables. For uncommon variables, Fisher’s exact test was applied. All tests were performed as two-sided at a significance level of 0.05. Pearson’s linear correlation coefficient was applied to detect associations between changes in exercise capacity, force duration and changes in MuRF-1-mRNA expression. SPSS versions 14.0 and 19.0 (IBM Germany GmbH, Munich, Germany) were used for statistical analysis.

All data are presented as mean ± standard error of mean (SEM).

Results

Baseline Characteristics and Clinical Data

From September 2005 to August 2008 a total of 60 healthy controls (HC) and 60 CHF patients were enrolled into the study. In both cohorts, 30 patients/subjects were ≤55 and ≥65 years, respectively (Figure 1).

As expected, hypertension, non insulin-dependent diabetes mellitus, and dyslipidemia
were more prevalent at baseline among elderly HC compared to younger HC (Table 1).

At baseline, younger and older CHF patients in the training and the control groups did not differ with respect to etiology or duration of CHF, ejection fraction or the rate of implanted cardioverters/defibrillators (ICDs). Medical therapy was similar in both groups and remained unchanged during the study period. All CHF patients were on guideline-oriented optimal medical therapy including ACE-inhibitors, β-blockers, aldosterone-antagonists, and diuretics as indicated (Table 1). As a result of the age stratification, an age difference of more than 20 years was observed between two age strata (Table 1).

Within the four weeks of the study period, no serious adverse events, including cardiac decompensations, hospitalizations due to worsening of heart failure, revascularization procedures, acute myocardial infarction or life-threatening ventricular arrhythmias occurred. There were no significant changes in body weight in all study subjects.

The functional changes (on maximal aerobic exercise capacity, left ventricular (LV) ejection fraction and LV diameters) induced by the training intervention are described in detail in Attachment 2 of the Supplemental Material.

Regulation of Skeletal Muscle Protein Catabolism

Age-Related Baseline Differences

In CHF patients both mRNA and protein expression of MuRF-1 were significantly higher as compared to HC (Table 2, Figure 2). However, mRNA and protein levels of MAFbx, the second muscle-specific E3-ligase, and mRNA-levels of catepsin-L were not elevated in muscle biopsies of patients with CHF versus HC.

Training Effects

Catabolic activation of the UPS as measured by MURF-1 mRNA and protein expression was significantly reduced in younger and older CHF patients of the training groups (younger CHF:
mRNA from 629±122 to 423±55 arb. units (p<0.05), protein from 0.70±0.09 to 0.57±0.08 arb.
units (p<0.05), older CHF: mRNA from 621±93 to 391±83 arb. units (p<0.05), protein from
0.92±0.26 to 0.76±0.17 arb. units (p<0.05), Figure 2). MAFbx was unchanged by the training.
The functional impact of reduced MURF-1 levels in trained CHF patients was further
corroborated by a significant reduction of poly-ubiquitinylated protein after 4 weeks (younger by
-38±2% (p<0.05) older by -40±3% (p<0.05), Figure 3). Among CHF patients and HC mRNA
expression of catepsin-L remained unaffected by the exercise intervention.

**Regulation of Skeletal Muscle Protein Anabolism**

**Age-Dependent Baseline Differences**

Compared to HC CHF patients showed significantly reduced IGF-1 mRNA expression in the
skeletal muscle biopsies with no significant difference between younger and older patients
(Table 2, Figure 4). Due to the larger standard error no significant differences between
myostatin levels in CHF patients and HC were detected for each subgroup (n=15). However,
when the baseline data for myostatin expression of all 60 CHF patients and all 60 HC were
pooled a significantly higher baseline myostatin mRNA expression in muscle biopsies of CHF
patients was confirmed (+54.3%, CHF 44.9±6.3 arb. units versus 29.1±3.4 arb. units in HC,
p=0.03). CHF patients had significantly lower GH serum levels versus HC. However, an age-
related decline in GH concentrations was detected neither among HC nor among CHF patients
(Table 2).

**Training Effects**

After training CHF patients had increased IGF-1 mRNA expression (younger pts. from 14.1±1.1
to 26.5±2.1 arb. units (p<0.05) older patients from 13.9±1.3 to 25.7±2.5 arb. units (p<0.05),
Table 2, Figure 4). In the untrained control groups and in all HC no significant change of IGF-1
content could be detected. After 4 weeks of exercise training myostatin mRNA expression remained unchanged in both patients with CHF and healthy controls (Table 2).

**Muscle Cross Sectional Area and Muscle Function**

**Age-Related Baseline Differences**
As compared to age-matched control subjects, the cross-sectional area (CSA) of the quadriceps muscle was significantly lower among patients with CHF. Maximal force and force endurance were also significantly reduced as compared to HC. No age-related differences in muscle function were observed between the younger and the older CHF cohort (Table 2). Older HC had a reduced cross-sectional area as compared to HC ≤55 years. This was accompanied by a significant reduction of maximal force and force endurance among HC ≥65 years (Table 2).

**Training Effects**
After four weeks of training the quadriceps muscle CSA remained unchanged in younger and older patients of the training cohort compared to the untrained control patients. Dynamometric measurements revealed no training related changes of maximal force, however, both younger and older patients with CHF responded to the exercise training intervention with a similar increase of force endurance (younger pts. from 22.8±2.5 to 33.6±2.9s (p<0.001), older pts. from 23.5±4.2 to 33.9±4.s (p<0.05), Table 2). Untrained control patients did not show any significant change regarding the above mentioned parameters.

No changes in muscle CSA and maximal force were observed in younger and older HC. In contrast, force endurance increased in both younger and older HC after training (younger HC from 26.3±2.1 to 39.1±2.1 s (p< 0.05) older HC from 20.2±4.4 to 33.2±2.9s (p< 0.05), Table 2). VO\textsubscript{2 max} and force endurance were significantly correlated in the training cohorts (CHF: r=0.51; p<0.05; HC: r=0.49; p<0.05).
Discussion

Several key messages emerge from this first prospective randomized and age-stratified clinical study to assess the effects of a supervised 4 week endurance training program on the catabolic-anabolic balance in the skeletal muscle of stable non-cachectic CHF patients and control subjects:

Catabolic Activation

The mRNA and protein expression levels of the muscle specific E3-ligase MuRF-1 were selectively up-regulated in vastus lateralis biopsies of CHF patients as compared to control subjects of the same age stratum. MAFbx expression remained unaffected by both the CHF disease process and age. Four weeks of endurance training significantly reduced local MuRF-1 mRNA and protein expression in the skeletal muscle of CHF patients of all ages resulting in decreased protein ubiquitinylation.

Anabolic Activation

Local expression of IGF-I mRNA was significantly reduced among CHF patients as compared to HS in the same age stratum. The present study extends previous studies in the sense that only 4 weeks of endurance training are effective in increasing local IGF-I expression in the quadriceps muscle of patients with CHF. At baseline CHF patients showed elevated myostatin mRNA expression versus HC, however, no significant changes in local skeletal muscle expression levels were observed after the training intervention.

Inflammatory Activation

The LEICA catabolism study confirms and extends the results of previous studies showing an anti-inflammatory effect of endurance training in the skeletal muscle in the sense that 4 weeks of training are sufficient to reduce local TNF-α expression.

Clinical Results
Age did not attenuate the relative gains in maximal exercise capacity and skeletal muscle force endurance after four weeks in patients with CHF.

1. Muscle Wasting and Catabolic Activation in Aging and Heart Failure

One of the strengths of the LEICA catabolism study is that muscle wasting and decline in muscle function with ageing and heart failure were assessed morphometrically (by CT-measurement of muscle CSA), functionally (by determination of maximal isometric force and force endurance of the quadriceps muscle), and on the molecular level. Our findings of decreased muscle CSA, force endurance, and catabolic activation in heart failure patients are consistent on all three levels assessed.

Basically, protein degradation in eucariotic cells can be mediated by [1] cysteine-dependent aspartate specific proteases (caspases)\textsuperscript{15}, [2] lysosomal cathepsins\textsuperscript{16}, [3] calcium-dependent calpains\textsuperscript{17}, and [4] the ubiquitin-proteasome system (UPS)\textsuperscript{18-20}. In recent years the UPS has been identified as the principal regulator of muscle atrophy\textsuperscript{19} (for in-depth review refer to \textsuperscript{21,22}). In the LEICA study we measured L-cathepsin to quantify the role of lysosomal protein degradation. The finding that L-cathepsin was unaffected by either CHF or the training intervention argues against a significant role of lysosomal proteolysis in CHF associated muscle wasting.

The involvement of the UPS in human muscle wasting, was documented in a number of non-inflammatory (spinal cord injury, immobilization) and inflammatory conditions (cancer, sepsis, COPD, ALS, compare table 2 in \textsuperscript{21}). However, no data were yet available on muscle wasting in CHF. We report for the first time that the expression of the muscle-specific E3-ligase MuRF-1 is significantly increased in vastus lateralis muscle biopsies from non-cachectic patients with CHF while MAFbx remains unaltered. This increase is not further enhanced in elderly CHF patients as may have been inferred from animal model of sarcopenia, in which elevated MuRF-1
expression was observed in hindlimb muscles of aged rats\textsuperscript{23}. Similarly, we did not measure any increased expression of MuRF-1 and MAFbx in elderly HC without clinical evidence of sarcopenia. These observations may either be explained by the absence of any increased UPS activity without clinically manifest sarcopenia or by current concepts that the age-related loss of muscle mass is predominantly due to a \textit{decreased anabolic activity} caused by a resistance of skeletal muscle protein synthesis to circulating amino acids\textsuperscript{24}.

The functional relevance of the elevated MuRF-1 expression in the skeletal muscle of young and old CHF patients was confirmed by the increased level of ubiquitinylated proteins. MuRF-1 has several target proteins in skeletal myocytes, including myosin heavy chain and troponin.

The effects of long-term exercise training on MuRF-1 expression have so far only been systematically evaluated in a 60-day bed-rest study with and without a combined endurance-resistance training program\textsuperscript{25}. In this immobilization model for muscle atrophy training prevented the increased protein expression of MuRF-1 in the soleus muscle and maintained fiber cross-sectional area\textsuperscript{25}. On the other hand, bicycle ergometer training exacerbated the expression of three proteasome 26S subunits in a small exercise training study in 6 COPD patients. No mRNA expression measurements of muscle specific E3-ligases were performed in the vastus lateralis biopsies\textsuperscript{23}.

In the LEICA study a significant and consistent decrease in local skeletal muscle MuRF-1 mRNA and protein expression was observed after 4 weeks of endurance training in CHF patients. The relative decline did not differ between younger and elderly patients with CHF. (-32.8\% in CHF patients ≤55 years versus -37.0\% in CHF patients ≥65 years). Again, MAFbx remained unchanged by the training intervention. The reduction in MuRF-1 expression was accompanied by a significant decrease in ubiquitinylated proteins. Taken together these data
confirm a significant attenuation of the elevated baseline UPS activity by endurance training in patients with stable non-cachectic CHF. The findings are consistent with the concept that of the two skeletal muscle-specific E3-ligases MuRF-1 is the primary regulatory factor for UPS activity and muscle atrophy in CHF.

It is well described in experimental studies that MuRF-1 and MAFbx have different substrates/binding partners in the skeletal muscle: While MuRF-1 was found to bind to myosin heavy chain (MHC), titin, and troponin\textsuperscript{27,28} substrates of MAFbx include MyoD and the translation initiation factor eIF3f\textsuperscript{29,30}. The activation pattern of muscle-specific E3 ligases observed in CHF seems to be distinct from experimental models of dexamethasone-induced muscle atrophy, where both MuRF-1 and MAFbx were upregulated\textsuperscript{31}.

The differential regulation of MuRF-1 and MAFbx may be related to the activation of different forkhead transcription factors as indicated by cell-line experiments: While activation of MAFbx gene expression requires DNA-binding of FoxO1 or FoxO3a, upregulation of MURF-1 expression was observed independently of DNA-binding of forkhead transcription factors\textsuperscript{32,33}.

Among the HC aging was associated with a significant decrease in skeletal muscle cross-sectional area (CSA) from 83±4 cm\textsuperscript{2}/82±2 cm\textsuperscript{2} (training and control group, respectively) to 72±1 cm\textsuperscript{2}/73±2 cm\textsuperscript{2}. Such an age-dependent decline in skeletal muscle CSA was not confirmed among CHF patients, who had reduced CSA regardless of age between 69 and 74 cm\textsuperscript{2}. The observation period of just 4 weeks is presumably too short to expect any significant increase in muscle CSA, which would be seen at a minimum of 8 weeks after the initiation of the training program\textsuperscript{34}.

Due to the methodology regarding patient selection (non-cachectic advanced CHF patients and non-sarcopenic healthy controls), biopsy sampling from one single muscle, and the short follow-up period of 4 weeks a number of questions remain to be answered by future studies: (1) Is there a clinical threshold (e.g. related to NYHA functional class or disease
duration) for catabolic activation? (2) Is MuRF-1-related activation of the UPS a continuous process in advanced CHF or do catabolic phases occur intermittently? (3) Are different types of muscle (regarding fiber type composition) affected differently by the wasting process?

2. Anabolic Activation:

The lack of any training-induced changes in serum GH levels and the increase of local skeletal muscle IGF-1 expression have been previously reported and are largely confirmatory. For a detailed discussion please refer to Attachment 3 in the Supplemental Material.

Myostatin is a growth factor belonging to the transforming growth factor family which attenuates skeletal muscle growth. Increased myostatin mRNA expression has been reported e.g. in muscular wasting associated with glucocorticoid administration. The effects of myostatin on skeletal muscle include inhibition of protein synthesis and MyoD expression with an increase of atrogin-1, MuRF-1 and an inhibition of Akt phosphorylation.

In the present study myostatin expression in the skeletal muscle was 54.3% higher in CHF patients as compared to HC. These data are well in line with another recently published biopsy study from our institution in a small group of advanced CHF patients (NYHA III, n=24), which showed a significant increase of myostatin mRNA in skeletal muscle compared to healthy controls. In these advanced stage CHF patients prolonged exercise training over 3 months led to a 36% reduction of myostatin mRNA compared to baseline. The lack of any significant reduction of myostatin expression in the LEICA study is likely to be related to shorter training duration of just 4 weeks.

In other animal models of muscle wasting (hindlimb suspension) myostatin gene expression in the gastrocnemius was not modified. Absence of myostatin expression in myostatin knockout mice did not prevent the loss of muscle mass after hindlimb suspension. On the contrary, these mice are more susceptible to unloading-induced skeletal muscle atrophy.
In recent animal experiments Heineke showed that a heart-specific deletion of myostatin with an Nkx2.5-cre allele prevented skeletal muscle atrophy in heart failure\textsuperscript{39}. In consequence, he postulated that myocardial myostatin expression controls muscle atrophy in heart failure via elevated serum myostatin concentrations\textsuperscript{39}. However, he used a pressure-overload model to induce heart failure, which may not be comparable to the etiology of CHF in humans. The findings of unchanged serum myostatin levels\textsuperscript{37} and increased local myostatin mRNA expression in CHF observed in two independent studies may be more consistent with the relevance of local factors for the regulation of the myostatin system or indicate that serum myostatin levels may be only transiently elevated in earlier stages of CHF and therefore escape detection in stable CHF.

3. Clinical Results

The four week endurance training intervention in the LEICA study was highly effective in improving exercise capacity in both younger and older CHF patients and HC: In relative terms CHF patients increased their peak oxygen uptake by +26\% \leq 55 \text{ years} and by +27\% \geq 65 \text{ years of age}. HC improved by 24\% \leq 55 \text{ years} and by +19\% \geq 65 \text{ years of age}. The complete lack of any attenuation of the relative training effectiveness with increasing age is a strong argument in favor of expanding rehabilitation programs into the elderly patient population. Since the molecular changes in the skeletal muscle biopsies were the primary focus of the present study an in-depth discussion of the clinical training effects is provided in Attachment 3 (Supplemental Material).

Limitations

Healthy controls >65 years were not entirely free from cardiovascular risk factors which was not unexpected in a Western society. As a consequence a number of HC>65 years were on anti-hypertensive medication. However, a stable medication was required for at least three months and medical therapy was kept unchanged during the entire study period. It is therefore unlikely that the study results in HC were influenced by medication. Since the prevalence of diabetes was
higher among CHF patients than among HC and since diabetes was reported to increase UPS activation in animal models of hypoinsulinemic diabetes there is a theoretical possibility that diabetes prevalence could increase the differences in UPS activation. However, all diabetic subjects and patients in the current study suffered from hyperinsulinemic type II diabetes and no human data are available regarding the interaction between this condition and UPS activation in the skeletal muscle.

Since the inclusion criteria as defined by echocardiography and clinical status were identical for both age groups we can only compare exercise capacity and other echocardiographic or molecular parameters between different age groups in similar baseline clinical status. This is, however, fundamental for the study design, which was chosen to compare training-induced changes between different age-groups of CHF patients and HC. We did not aim to recruit a representative sample of all CHF patients. As a result, we did not observe an age-related decline in exercise capacity among CHF patients as evident in the HF-ACTION study. The current study was also not planned with the primary end-point of significant gain in skeletal muscle mass. Had this be an end-point a longer training duration of at least 3 months or the addition of resistance exercise interventions would have been necessary.

Research in sarcopenia has long been difficult due to the lack of an internationally accepted universal definition of the disease conditions. In recent years efforts have been made to establish a consensus definition of sarcopenia which combines anthropometric and functional criteria. However, clear cut-off values for muscle mass are still debated and mainly related to whole-body DEXA scan or body impedance assessment of muscle mass. The lack of these measurements in the current study, which was planned years before any consensus definition was published, makes it difficult to use a current standard definition to exclude sarcopenia in the control cohort. However, using the 2 standard deviation criterium for muscle atrophy advocated
by the Special Interest Group by the Special Interest Group no older control subject had overt sarcopenia. This is corroborated by the mean VO₂ max of >20 mL/kg min in all elderly cohorts.

The findings of the current study do not support the initial hypothesis that age-related sarcopenia overlaps with muscle wasting in chronic heart failure patients. However, CHF patients and older control subjects included in this study did not show sarcopenia/cachexia by clinical definition at baseline despite clear evidence of muscle wasting as shown by CT scan of quadriceps cross-sectional area. It may well be that CHF patients and control subjects in more advanced age (above the age of 80 years) or with more advanced heart failure need to be studied in future clinical trials to definitively prove or refute the sarcopenia-cachexia overlap hypothesis.

**Areas for Future Research:**

It remains unknown if the expression of MuRF-1 is permanently or temporarily elevated in chronic systolic heart failure. The finding that MuRF-1 expression remained unchanged in sedentary control CHF patients and healthy controls over the time of 4 weeks between the two muscle biopsies does not exclude variations in the course of CHF disease progression. To answer this question long-term follow-up studies in CHF patients are needed, in which serial muscle biopsies are obtained at larger time intervals of several months and are correlated to changes in body mass, lean muscle mass, and exercise capacity.

**Conclusions**

The LEICA catabolism study is the first clinical study to specifically measure the interaction between age and disease in modulating the catabolic-anabolic balance in the skeletal muscle of CHF patients. In previous training studies in heart failure patients elderly individuals were mostly underrepresented, making it difficult to extrapolate the beneficial effects of training interventions in the age stratum in which heart failure is most prevalent.
The study documents a specific pattern of the catabolic-anabolic imbalance in the skeletal muscle with reduced local IGF-I expression, increased MuRF-1 and TNF-α expression and unchanged levels of MAFbx and myostatin. Four weeks of endurance training were effective in reducing the catabolic TNF-α and MuRF-1 expression while increasing IGF-I levels. Myostatin and MAFbx expression were unchanged by training.

Understanding the specific changes causing the catabolic-anabolic imbalance in CHF is an essential first step in the development of pharmaceutical intervention strategies aimed at blocking muscle catabolism in CHF. For the time being endurance training provides an excellent anabolic stimulus in both younger and elderly patients with heart failure and should be regarded as a key component of an anticatabolic treatment approach in heart failure patients of all age groups.

**Funding Sources:** The study was funded by the German Research Foundation (DFG grant Gi535/1-1 to S. Gielen & V. Adams).

**Conflict of Interest Disclosures:** None

**References:**


Table 1: Baseline Characteristics

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Data as mean±SEM. (*p<0.05 vs. healthy controls; #p<0.05 vs. <55 years) Abbreviations: LV: left-ventricular; VO2max: maximal oxygen consumption; ICD: implantable cardioverter defibrillator; ACE inhibitor: angiotensin converting enzyme inhibitor; ARB: angiotensin II subtype I receptor blocker
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Data as mean±SEM, *p<0.05 vs. begin; # p<0.05 vs. control subjects ≤55 years at begin. Abbreviations: Fmax: Maximal force of the right quadriceps muscle; CSA: cross-sectional area.
Figure Legends:

**Figure 1.** CONSORT flow diagram illustrating patient screening, treatment allocation, and follow-up.

**Figure 2.** The local expression of the muscle-specific E3-ligase MuRF-1 was significantly increased in CHF patients. No difference was seen between older and younger CHF patients indicating that age did not additively contribute to ubiquitin-proteasome-mediated proteolysis in the age-range studied. Four weeks of exercise training reduced MuRF-1 expression by -32.8% (p=0.02) in CHF patients ≤55 years and by -37.0% (p<0.05) in CHF patients ≥65 years. Expression levels were decreased to the levels observed in HC indicating that the training intervention effectively blocked the disease-related catabolic UPS activation. § p<0.05 vs. HC, * p<0.05 vs. control, # p<0.05 vs. baseline.

**Figure 3.** The relative change in ubiquitinylated proteins was highly significant in the CHF training group regardless of age indicating the functional relevance of the reduced MuRF-1 expression on intracellular protein degradation. No change was observed in HC and control groups. * p<0.05 vs. control.

**Figure 4.** IGF-I mRNA expression in the vastus lateralis muscle biopsies was significantly reduced in heart failure patients as compared to healthy controls. After four weeks of exercise training local IGF-I expression increased approximately twofold in CHF patients of both age strata. No effect of endurance training on local IGF-I expression was observed in HC, who had normal IGF-I levels at baseline. § p<0.05 vs. HC, * p<0.05 vs. control, # p<0.05 vs. baseline.
250 Healthy Subjects Assessed for Eligibility

107 Not met Inclusion Criteria
83 Refused to Participate

60 Healthy Subjects Randomized

30 Below 55 years
15 Assigned to Training
15 Assigned to Control
0 Did Not Receive Assigned Intervention
0 Lost to Follow-up
0 Discontinued Intervention
60 Included in Analysis
0 Excluded From Analysis

30 Above 65 years
15 Assigned to Training
15 Assigned to Control
60 Included in Analysis
0 Excluded From Analysis

303 CHF-Patients Assessed for Eligibility

189 Not met Inclusion Criteria
54 Refused to Participate

60 CHF-Patients Randomized

30 Below 55 years
15 Assigned to Training
15 Assigned to Control
0 Did Not Receive Assigned Intervention
0 Lost to Follow-up
0 Discontinued Intervention
60 Included in Analysis
0 Excluded From Analysis

30 Above 65 years
15 Assigned to Training
15 Assigned to Control
60 Included in Analysis
0 Excluded From Analysis
Exercise Training Attenuates MuRF-1 Expression in the Skeletal Muscle of Patients with Chronic Heart Failure Independent of Age: The Randomized Leipzig Exercise Intervention in Chronic Heart Failure and Aging (LEICA) Catabolism Study
Stephan Gielen, Marcus Sandri, Irina Kozarez, Jürgen Kratzsch, Daniel Teupser, Joachim Thiery, Sandra Erbs, Norman Mangner, Karsten Lenk, Rainer Hambrecht, Gerhard Schuler and Volker Adams

Circulation. published online May 7, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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Supplemental Material

Attachment 1

Supplemental Methods

18S-rRNA Primer:
5'-ATACAGGACTCTTTTCGAGGCC-3' and 5'-CGGGACACTCAGCTAAGAG CAT-3' at 61°C annealing.

MuRF1 Primer:
5'-AGAACATCATCGACATCTACA-3' and 5'-ACTCACTTTTCTTCTCATCCA-3' at 55°C annealing.

MuRF1 TaqMan Probe:
5'-GAGGTGCCACCTGCTCCAT-3'

MAFbx Primer:
5'-GAAGAGCGGCAGTTTCGT-3' and 5'-TGCCACTCAGGGATGTGA-3' at 55°C annealing.

MAFbx TaqMan Probe:
5'-TCCAACAGCCGGACCACGT-3'

TNF-α Primer:
5'-CTTCTTCTTCTGATCGTGGC -3' and 5'-GGGTTTGCTACAACATGGGC-3' at 58°C annealing.

TNF-α TaqMan Probe:
5'-CGCCACCACGCTTCTTCTGCC-3'
**Catepsin-L Primer:**

5´-GTCAGTGTGGTTCTTGTTGG3´.

**IGF1 Primer:**

IGF1-1 5´-CACCATGTCTCTCTCGCATCT-3´, IGF1-2 5´-ATCCACGATGCTGTCT
GAGG-3´,

**Myostatin Primer:**

5´-TCTTCACATCAATACTCTGCA-3´; 5´-ATTCGCTCA CCGAGTCTGACTT-3´ 55°C
annealing

**Myostatin Probes:**

5´-LC640-GTGCAAATCCTGAGACTCATCAAACCATG-PH-3´;
5´-GAGAGCCGTCAAGACTCTACACACAGTG-FL-3´.

**Attachment 2 – Supplemental Results**

**Cardiopulmonary Exercise Testing**

**Age-Related Baseline Differences**

In both younger and older patients with CHF maximal exercise capacity (in Watts) and maximum oxygen uptake were significantly reduced versus HC. No age-dependent differences in exercise capacity were observed between the younger and older CHF patients (Table Suppl. 1a). As expected, elderly HC achieved a significantly lower maximal workload and maximum oxygen uptake as compared to younger HC (Table Suppl. 1a).

**Training Effects**

All CHF patients randomized to exercise training increased their maximal exercise capacity (younger CHF by 31% from 66±3 to 86±2 Watt; p=0.02; older CHF by 37% from 60±2 to
82±2 Watt; p=0.0001; Table Suppl. 1a) and their maximal oxygen uptake (young by 26% from 13.3±1.6 to 18.1±1.5 ml/kg*min; p=0.01; old by 27% from 12.9±1.4 to 17.1±1.1ml/kg*min; p=0.008; Table Suppl. 1a). We detected no age-related differences between younger and older training patients with respect to training response (young vs. old training patients p=0.1 for ∆Pmax and p=0.74 for ∆VO2 max). Notably, the maximal heart rates during cardiopulmonary exercise testing did not differ between CHF patients ≤55 years and ≥65 years, most likely as a result of the uptitrated beta-adrenergic blockade.

After four weeks of endurance training maximal exercise capacity increased by 30% from 129±3 to 152±4 Watt (p=0.003) in the younger and by 31% from 91±4 to 119±3 Watt (p=0.001) in the older HC group. Maximal oxygen uptake increased by 14% from 23.9±2.1 to 27.9±2.9 ml/kg*min (p=0.01) in the younger and by 19% from 21.1±1.7 to 26.1±2.2 ml/kg*min in the older training group (p=0.004; Table Suppl. 1b). There were no detectable age-dependent differences in the training response (young vs. old training patients p=0.84 for change of maximal workload (∆Pmax) and p=0.62 for ∆VO2 max).

In all untrained control groups no significant changes in exercise capacity or VO2 max were observed.

Left Ventricular Dimensions and Systolic LV Function

Age-Related Baseline Differences

Both younger and older patients with CHF had severely reduced LV-EF and increased left atrial and LV diameters with no age-related differences (Table Suppl. 2a). No age-related differences were observed between younger and older HC regarding LV-EF, LV-volumes, or LV diameters. Left atrial size was slightly increased among older HC (42±2 vs. 46±2mm; p=0.04; Table Suppl. 2a).
Training Effects

In both younger and older patients with CHF randomized to training, a significant improvement in LV-EF was observed (young CHF by +19% from 27±2% to 34±2%, older CHF by +17% from 29±2% to 35±2%; p<0.05 vs. control; Table Suppl. 2a). In parallel, LV end-diastolic and end-systolic diameters were reduced after training (Table Suppl. 2a). No age-dependent attenuation of the training effects was noted.

Four weeks of exercise training did not affect LV-EF or left ventricular diameters in either young or old healthy subjects as compared to controls (Table Suppl. 2a).

Attachment 3 – Supplemental Discussion

1. Functional Changes

There are a few of reports of no or limited improvement of exercise capacity following training interventions in elderly patients with CHF: Just recently, Brubaker did not observe a significant improvement in peak oxygen uptake, 6-minute walk distance, LV ejection fraction, and quality of life after a low-moderate intensity (three trainings sessions of 30-40 min endurance exercise) 16-week supervised training program.(1) His patients had a mean age of 70±5 years (range 60-80). In the HF-ACTION study a minor improvement of just +4.2% (+0.6 L/min kg in peak oxygen consumption) after 3 months of a moderate intensity training program was measured, which resulted from poor adhesion to prescribed training duration. (2)

The effects of the present high-intensity endurance training program with a total exercise time of 460 min/week (including one group training session per week) on exercise capacity were surprisingly pronounced – given the short intervention time of only 4 weeks: a significant 26% increase of VO\textsubscript{2\ max} was recorded in CHF patients ≤55 years and a non-attenuated increase of 27% among elderly CHF patients ≥65 years. The effects our training
program were comparable to the effects we previously observed after more prolonged training interventions, either after 3 months (3) or after 6 months (4) (also compare the ExtraMATCH metaanalysis (5)). A major reason for the effectiveness of the training intervention was the complete supervision of the patients and probably the application of multiple short training sessions with 1-2h intervals between them. New studies suggest that high-intensity interval training may be even more effective in regard to increasing exercise capacity (6), however, we did not want to introduce a high degree of resistive exercise components in the training program of the current study, which could make training completion difficult for the elderly patients.

Based on the HF-ACTION database of 2,331 CHF patients Forman described that age was the strongest predictor of peak VO\(_2\) with a significant decline in peak VO\(_2\) after the age of 40. (7) In line with previous publications he hypothesized that the decrease in heart rate reserve with increasing age may be the key factor for the age-related decline. (8–10) In the present study VO\(_2\)\(_{\text{max}}\) did not differ significantly between the CHF patient \(\leq 55\) years and \(\geq 65\) years. Three factors are important to interpret the lack of any baseline difference:

1. Patients were recruited based on the clinical inclusion criteria of a left ventricular ejection fraction \(\leq 40\%\) and a minimal exercise capacity of 25W. The resulting sample may not be representative of the entire patient cohort due to the small patient number.

2. The proportion of patients in more advanced stages of CHF is higher in the LEICA study (43.3% NYHA III \(\leq 55\) years and 50% NYHA III \(\geq 65\) years) as compared to the HF-ACTION cohort.(7) The selection of sicker patients in both age groups could explain that the exercise-limiting effect of the disease may be dominant over age.

3. All our patients were on maximally uptitrated betablocker medication, which was not interrupted for the exercise test. This could have reduced the heart rate reserve in both patient age groups to a similar level.
In line with previous studies from our group (3,4) we found a small but significant improvement of left ventricular ejection fraction in CHF patients, accompanied by a decrease in LV enddiastolic and endsystolic diameters. These findings are also consistent with a previously published meta-analysis on training effects on left ventricular remodelling in chronic heart failure: Haykowsky and colleagues collected a total of 14 prospective randomized clinical trials with LV ejection fraction as a clinical endpoint. (11) Of these studies, nine investigated aerobic endurance training as an exercise intervention and included a total of 292 training patients and 246 control patients. In combination, these nine trials with a total of 538 patients showed a weighted mean difference in ejection fraction in the training group of +2.59% (confidence interval +1.44% - +3.74%; range -0.05% - +5.00%). Trials testing strength training alone or in combination with aerobic training were inconclusive with regard to the effects on LV ejection fraction. (11) It is noteworthy, however, that the present study is the first to document that the reverse remodeling induced by the training intervention is visible as early as four weeks after the initiation of exercise training.

2. Anabolic Activation:

GH: Based on aging research a decline in circulating growth hormone (GH) levels at a rate of 1% per year after the age of 30 is expected. (12) GH contributes to muscle hypertrophy by promoting the fusion of myogenic precursor cells into myotubes, a process which is independent from IGF-I. In the present study GH serum levels were significantly lower in CHF patients as compared to HC in all age groups. However, GH serum concentrations were not affected by the training intervention, neither in HC nor in CHF patients. Most likely, the sample size or the age difference were too small to detect the expected 20-25% decrease in GH serum levels between the younger and the older HC.
**IGF-I:** It has been previously shown, however, that the induction of IGF-I is sufficient to induce skeletal muscle hypertrophy (13,14) and to block the age-related loss of muscle mass (15). IGF-I induces muscle hypertrophy partly via the phosphatidylinositol-3 kinase (PI3K)/Akt and the RAPTOR/TORC1 pathway. In addition, treatment with IGF-I was able to antagonize the upregulation of MuRF-1 and MAFbx in dexamethasone-induced muscle atrophy models via Akt-mediated phosphorylation of FOXO transcription factors, which are excluded from the nucleus when phosphorylated thus inhibiting their ability to upregulate MuRF-1 and MAFbx (16). Therefore, the finding of a significant increase in local IGF-I expression is a key component to explain the normalization of MuRF-1 after the training intervention – together with the reduced TNF-α expression.

The present study extends the results of our previous work on the effects of CHF and endurance training on local expression of IGF-I and IGF-I receptor (17,18) in the sense that already 4 weeks of high-intensity endurance training are sufficient to induce a significant increase in IGF-I expression in the vastus lateralis muscle. Additionally, no attenuation in the training-induced increase IGF-I of local IGF-I expression was observed in elderly patients with CHF or elderly HC.

**3. Inflammatory Activation:**

TNF-α regulation is of special interest for studies of catabolic-anabolic imbalance due to its established role for induction of the UPS (19), most likely via Foxo4 as described by Moylan et al. (20)

We have previously shown that 6 months of endurance exercise training reduced local mRNA expression of TNF-α, IL1-β, and IL-6 in skeletal muscle biopsies (4). To confirm the anti-inflammatory training effects in the LEICA study we measured local mRNA expression in skeletal muscle biopsies obtained from the vastus lateralis muscle before and after training.
A -40.5% and a -33.3% decline of local TNF-α mRNA expression was measured in CHF patients ≤55 years and ≥65 years of age, respectively. The extent of the local TNF-α reduction was similar to the reduction observed after 6 months of endurance training (-36.8% (4)).

Apart from the study by Gielen 2003(4) only animal data are available on training effects on local expression of pro-inflammatory cytokines: Battista showed a reduction of TNF-α mRNA and protein expression after 8-10 weeks of treadmill training in the soleus and the extensor digitorum longus (EDL) muscle of rats with LAD-ligation induced heart failure.(21) The anti-inflammatory effect as assessed by IL-10/TNF-α ratio was more pronounced in the soleus than in the EDL, suggesting a fiber composition dependent response. Of note, Adams showed a similar training-induced decrease in TNF-α expression in the non-infarcted remote myocardium, which was related to a reduced MuRF-1 and MAFbx mRNA expression.(22)

Muscle biopsy studies in older male HC documented a significant 2.8-fold increase in local TNF-α expression as compared to younger HC. (23) However, the age difference in this study was 50 years so that the differences are much more pronounced as compared to our study with an age difference of only 22-25 years, which was probably too small to detect age-related differences in muscle TNF-α expression.
Supplemental References:


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Data as mean±SEM; Abbreviations: VO$_2$\text{max}: maximal oxygen consumption; VO$_2$\text{VT}: oxygen consumption at ventilatory threshold; HR: heart rate; Sys: systolic; Dia: diastolic; BP: blood pressure.

* p<0.05 versus begin; # p<0.05 versus control subjects <55 years at begin.
### Table Suppl. 1b ANOVA p-Values to Table Suppl. 1a

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Data as mean±SEM; Abbreviations: LV-EF: left ventricular ejection fraction; LVESD: left ventricular end-systolic diameter; LV-EDD: left ventricular end-diastolic diameter.

* P<0.05 versus begin; # p<0.05 versus control subjects <55 years.
Table Suppl. 2b ANOVA p-Values to Table Suppl. 2a

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