**Article:**
Schmederer, Z, Rolim, N, Scott Bowen, T et al. (2018) Endothelial function is disturbed in a hypertensive diabetic animal model of HFpEF: Moderate continuous vs. high intensity interval training. International Journal of Cardiology, 273. pp. 147-154. ISSN: 0167-5273

https://doi.org/10.1016/j.ijcard.2018.08.087

**Endothelial function is disturbed in a hypertensive diabetic animal model of HFpEF: moderate continuous vs. high intensity interval training.**

Zelia Schmederer[1], Natale Rolim, PhD[2], T Scott Bowen, PhD[3], Axel Linke, MD[4], Ulrik Wisloff, PhD[2,5], Volker Adams, PhD[4] - On behalf of the OptimEx study group

1 University Clinic of Cardiology, Heart Center Leipzig, Leipzig, Germany

2 K.G. Jebsen Center of Exercise in Medicine, Department of Circulation and Medical Imaging, Faculty of Medicine, NTNU, Trondheim, Norway

3 School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom

4 Department of Molecular and Experimental Cardiology, TU Dresden, Heart Center Dresden, Dresden, Germany.

5 School of Human Movement & Nutrition Science, University of Queensland, Australia.

**Short title:** Effects of exercise training on endothelial dysfunction in HFpEF

Total word count: 3480

**Address for correspondence:**

Volker Adams, PhD

Laboratory for experimental and molecular cardiology

TU Dresden, Heart Center Dresden,

Fetscherstrasse 74

01307 Dresden

Tel: +49 351 458 6627

Email: volker.adams@mailbox.tu-dresden.de

**Abstract**

**Background:** Heart failure with preserved ejection fraction (HFpEF) is associated with endothelial dysfunction, but the molecular mechanisms still remain unclear. Whether exercise training (ET) along with which optimal modality can improve endothelial function is controversial. The present study used a hypertensive, diabetic-driven HFpEF animal model (ZSF1 rats) to determine whether different training modalities (moderate-continuous (MCT) and high-intensity interval training (HIIT)) could reverse endothelial dysfunction and to understand the underlying molecular mechanisms.

**Methods and Results:** The development of HFpEF in ZSF1 obese animals was confirmed by echocardiography and hemodynamic measurements. Thereafter, animals were randomized into following groups: 1) sedentary, 2) 8 weeks of MCT, 3) 8 weeks of HIIT. ZSF1 lean animals served as control. In vitro measurement of endothelial function in aortic rings revealed significantly impaired endothelial-dependent and -independent vasodilation in HFpEF, which was reversed by MCT and HIIT. At the molecular level, the development of endothelial dysfunction was associated with a reduced expression / activation of endothelial nitric oxide synthase (eNOS), an increase in NADPH and activation of c-Jun N-terminal protein kinase (JNK), a reduced collagen I/III ratio and a reduced lining of the vessel wall by endothelial cells. ET primarily decreased NADPH oxidase expression, and JNK activation, elevated collagen I/III ratio while further improving aortic endothelial cell coverage.

**Conclusions:** The present study provides evidence that endothelial dysfunction occurs in experimental HFpEF and that ET, independent of the studied training modality, reverses endothelial dysfunction and specific molecular alterations. ET may therefore provide an important therapeutic intervention for HFpEF patients.

**Introduction**

Heart failure (HF) is the most frequent cause of hospitalization in patients above 65 years with increasing incidence. Approximately 50 % of the HF patients have a preserved ventricular ejection fraction (HFpEF)[1], which is associated with several comorbidities such as arterial hypertension, diabetes, obesity and renal dysfunction. Classic treatment regimens that have proven effective in HF patients with reduced ejection fraction (HFrEF) have failed to improve survival in HFpEF.[2-5] However, exercise training (ET) might be an alternative treatment option, since several studies[6-10] and meta-analyses[11-13] in HFpEF reported beneficial effects on exercise intolerance, which is the main symptom associated with this disease. Further, findings from a small pilot study in HFpEF patients also suggested that high-intensity interval training (HIIT) exerts greater benefits than moderate-intensity continuous exercise training (MCT).[14] In contrast to HFrEF (reviewed in [15]), however, the mechanisms responsible for the benefits of ET in HFpEF remain largely unclear.

Some years ago, Paulus and Tschöpe[16] presented a new paradigm for the development of HFpEF where coronary microvascular inflammation-triggered endothelial dysfunction initiated a cascade leading to eventual fibrosis and stiffening of the cardiomyocytes. Hence, endothelial dysfunction might play a crucial role in the pathogenesis as well as the outcome of HFpEF, making it a potential target for prevention and treatment of HFpEF. Yet in HFpEF patients data with respect to endothelial dysfunction remains controversial, with some[17-20] but not others[21;22] reporting impaired endothelial function when compared to controls. This controversy is further complicated in that a gold-standard experimental model of HFpEF is still missing to directly confirm patient findings and, of the few models developed only a limited number have investigated endothelial function.[23;24] Using a nutritive hypertensive rat model of HFpEF, we recently demonstrated that HIIT prevented the development of endothelial dysfunction in the setting of primary prevention.[23] However, no data are yet available in HFpEF investigating either the impact of ET on endothelial function in the setting of secondary prevention or on the effects of different training modalities.

The present study, therefore, used a genetic hypertensive, diabetic-related rat model (ZSF1 lean and obese rats) characterized by cardiorenal metabolic syndrome. The ZSF1 model was developed by crossing rat strains with two separate leptin receptor mutations (fa and facp), the lean female ZDF rat (+/fa) and the lean male SHHF rat (+/facp), derived from the obese spontaneously hypertensive rat carrying the corpulent facp gene).[25] After the ZSF1 obese rats developed signs of HFpEF we assessed 1) the degree of endothelial dysfunction developed; and 2) the efficacy of various exercise training regimes (HIIT or MCT) to reverse endothelial dysfunction. We hypothesized that the development of HFpEF would be associated with endothelial dysfunction triggered by a cascade of molecular alterations, which would be rescued by both exercise training regimes.

## Methods

### Animals and study design

Male ZSF1 lean (control, n=19) and ZSF1 obese (n=47) rats were included into the study, as previously published.[26] [27]At the age of 20 weeks the development of HFpEF was confirmed by echocardiography / invasive hemodynamic measurements and tissue material from a subset of animals were collected (control n=8; ZSF1 obese n=12). The remaining control rats (control, n=11) were kept sedentary for another 8 weeks whereas the remaining ZSF1 obese animals were randomized into the following groups: (1) sedentary (HFpEF-sedentary, n=13); (2) HIIT exercise training (HFpEF-HIIT, n=11) or (3) MCT exercise training (HFpEF-MCT, n=11). Rats were exposed to identical conditions in a 12 h light/dark cycle, with food and water provided ad libitum. Eight weeks after randomization, echocardiography and invasive hemodynamic measurements were performed to elucidate the degree of diastolic dysfunction. Rats were subsequently sacrificed (opening of the chest in deep anesthesia) and the aorta was removed and

sliced, and either transferred in the organ bath, fixed in formalin or snap frozen in liquid nitrogen for molecular analyses. All procedures and experiments were approved by the Norwegian Council for Animal Research, which was in accordance with Use of Laboratory Animals by the European Commission Directive 86/609/EEC.

**$VO_{2max}$ measurement and training intervention**

Maximal oxygen uptake ($VO_{2max}$) testing was performed with the animals running until exhaustion on a treadmill at 25° inclination in a metabolic chamber as previously described.[28] Rats performed HIIT 3x per week over 8 weeks on a treadmill at an inclination of 25° as previously described.[28] Other rats performed MCT for 5x per week over 8 weeks at an inclination of 25° on the treadmill for 40 min at 60 % $VO_{2peak}$ followed by 10 min of running at 40-50 % $VO_{2peak}$.[26]

**Heart function**

Transthoracic echocardiography (Vevo 2100; VisualSonics, Ontario, Canada) and invasive hemodynamic pressure measurements were performed as recently described in detail.[29]

**Measurement of endothelial function**

Endothelial function of aortic rings was analyzed in vitro, which provided a standardized environment not affected by external factors (i.e., hormones or inflammatory cytokines). For the measurement of endothelial function, the aorta was rapidly excised and aortic rings (0.3 cm) mounted between a hook

and a force transducer and endothelial-dependent and –independent vasodilation was measured as recently described.[23]

**Vessel wall thickness**

Sections (3 µm) from formalin fixed, paraffin-embedded aorta were stained with hematoxylin and eosin. The thickness of the vessel wall (an index of aortic hypertrophy) was then quantified using imaging software (Analysis Five, Olympus, Münster, Germany) and related to the vessel lumen.

**Endothelial cell coverage**

Immunohistochemical staining was used to quantify the percentage of the aortic lumen covered with endothelial cells. Paraffin-embedded sections (3 µm) were incubated over-night at 4°C with anti-eNOS antibody (1:20; Santa Cruz). After extensive washing steps the sections were incubated with an anti-rabbit biotin (Sigma, 1:500) followed by streptavidin coupled to horseradish peroxidase (1:200; Thermo Scientific) and visualized with diaminobenzidine (DAKO). The endothelial cell coverage was then quantified in percent of the lumen using imaging software (Analysis Five, Olympus, Münster, Germany).

**mRNA expression**

Total RNA was isolated from frozen aortic rings using RNAeasy (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using random hexamer and sensiscript reverse transcriptase (Qiagen). cDNA was used for quantitative RT-PCR using specific primers for hypoxanthine guanine phosphoribosyl transferase (5`-CTCATGGACTGATTATGGACAGGAC-3´ and 5`-GCAGGTCAGCAAAGAACTTATAGCC-3´) collagen I (5´-

TCAAGATGGTGGCCGTTACT-3´ and 5´-CATCTTGAGGTCACGGCATG-3´), collagen-III (5´-ATGAATTGGGATGCAACTAC-3´ and 5´-TCTAGTGGCTCATCATCACA-3´) and tumor necrosis factor alpha (TNF-$\alpha$ rat, Biorad PrimePCR assay).

**Protein expression**

Protein was extracted from frozen aortic samples by incubation at 4°C over-night in lysis buffer (50 mmol/L Tris, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1% NP-40, 0.25% sodium-deoxycholate, 0.1% SDS, 0.1% Triton X-100; pH 7.4), which contained a protease inhibitor mix (Inhibitor mix M, Serva, Heidelberg, Germany). Western blot analyses were performed as recently described[22, 24] using the following antibodies: anti-eNOS (1:200; Santa Cruz), anti p-eNOS-Ser$^{1177}$ (1:2000, BD Biosciences, Heidelberg, Germany) anti gp91phox, anti-LOX-1 (both 1:1000, Abcam, Cambridge, UK), anti-nitrotyrosine (1:1000; Abcam), anti-SAPK/JNK and anti-phospho-SAPK/JNK (both 1:1000; both Cell Signaling, Frankfurt, Germany). Samples were normalized to glyceraldehyde 3-phosphate dehydrogenase (1:30000, HyTest Ltd, Turku, Finland), which served as the loading control.

**Statistical analyses**

Data are presented as mean ± SEM. Between-group differences were assessed by independent ANOVA, while endothelial-dependent and -independent vasodilation were assessed by two-way repeated measures ANOVA using SPSS version 22 (SPSS Inc., Chicago, USA). Statistical significance was accepted as $p<0.05$.

**Results:**

**Animal characteristics**

20 weeks of age: As shown in Table 1(see supplement) the ZSF1 obese animals exhibited signs of HFpEF when compared to control rats. This was documented by a significant increase in E/E´ and LVEDP, whereas $VO_{2peak}$ was significantly reduced. Despite a significant increase in body weight, LVEF was still preserved in both groups (above 60%) with no difference in systolic blood pressure.

28 weeks of age: LVEDP and E/E´ were still significantly increased in the ZSF1 obese animals when compared to lean controls, and neither MCT nor HIIT had an impact on those parameters (supplement Table 1). As expected, $VO_{2peak}$ increased in both training groups when compared to the sedentary animals.

**Endothelial function at 20 weeks**

Vasodilation was measured in the aorta of all animals sacrificed at 20 weeks of age (ZSF1 obese n=12; ZSF1 lean n=8). The development of HFpEF (ZSF1 obese group) was associated with impaired endothelium-dependent vasodilation when compared to control group, such that maximal dilation upon ACh was significantly impaired in the HFpEF group when compared to lean controls ($p<0.05$; Figure 1A). The ACh concentration needed to induce a 50% relaxation ($EC_{50}$) also showed a significant difference between controls and HFpEF animals (66.2±12.0 vs. 109.7±4.9 nmol/L ACh; $p<0.01$).

Comparing endothelium-independent vasodilation, no significant difference was detected between HFpEF and control animals (Figure 1B), and hence no difference in $EC_{50}$ for SNP was seen (control: 42±16.0, HFpEF: 42±10 nmol/L SNP).

**Endothelial function after intervention at the age of 28 weeks**

*Endothelial-dependent vasodilation*

Endothelial dependent (Figure 1C,D) and endothelial independent (Figure 1E,F) vasodilation were assessed in all animals after finishing the 8 weeks of intervention. ZSF1 lean animals served as healthy controls. Endothelial-dependent vasodilation of the aorta induced by increasing concentrations of ACh was significantly impaired in the HFpEF-sedentary animals when compared to the controls (Figure 1C). Besides the reduced maximal endothelium-dependent vasodilation in the HFpEF-sedentary animals (Figure 1D), a significantly higher ACh concentration was also necessary to induce a 50% vasodilation when compared to controls (93.3±10.7 vs. 46.7±4.4 nmol/L, $p<0.01$). These impairments in endothelial dependent vasodilation improved in both training groups (Figure 1C) and as such maximal dilation improved significantly in the training groups with both training modalities being equally effective (Figure 1D).

*Endothelial-independent vasodilation*

Comparing endothelial-independent vasodilation after 8 weeks of exercise training, an impairment in vasodilation was also observed in the HFpEF sedentary animals when compared the control animals ($p<0.05$, Figure 1E,F). Both training interventions equally effectively reversed this impairment in endothelial-independent vasodilation (Figure 1E,F). This impairment in endothelial-independent vasodilation and the effect of exercise training is also evident when analyzing the $EC_{50}$ values for SNP, which is significantly elevated in the HFpEF-sedentary animals and reduced by MCT or HIIT (control: 15±0.8 nmol/L; HFpEF-sedentary: 50±6 nmol/L; HFpEF-MCT: 25±6 nmol/L; HFpEF-HIIT: 27±5 nmol/L; $p<0.001$ control vs. HFpEF-sedentary; $p<0.05$ HFpEF-sedentary vs. HFpEF-MCT and HFpEF-HIIT).

**Protein expression of endothelial nitric oxide synthase (eNOS)**

Protein expression of eNOS and phosphorylated eNOS (ph-eNOS-Ser$^{1177}$) was analyzed in homogenates of the aorta harvested from animals before and after 8 weeks of intervention at an age of 20 and 28 weeks.

At 20 weeks of age, the expression of eNOS was not significantly different between controls and HFpEF animals (control: 1.0±0.22 vs. HFpEF: 0.78±0.07 arb. Units; $p>0.05$), whereas the ratio of ph-eNOS-Ser$^{1177}$ (the activated form of eNOS by phosphorylation at Ser$^{1177}$) over total eNOS was significantly reduced in the HFpEF group (1.0±0.22 vs. 0.22±0.04 arb. Units; $p<0.001$).

At 28 weeks the expression of eNOS was significantly reduced in the HFpEF-sedentary animals when compared to the controls ($p<0.01$, Figure 2A). Exercise training had no impact on the level of eNOS protein expression (Figure 2A). Calculating the ratio of ph-eNOS-Ser$^{1177}$ over total eNOS expression also a significant reduction was observed in the HFpEF-sedentary animals compared to the controls, with no impact of exercise training (Figure 2B).

**Protein expression of NADPH oxidase and nitrotyrosine modified proteins**

Protein expression of the gp91$^{phox}$ subunit of the NADPH oxidase tended to be, but was not significantly elevated in HFpEF animals at the age of 20 weeks when compared to controls (1.25±0.07 vs. 1.0±0.19 arb. units, $p=0.16$). Analyzing the samples at the age of 28 weeks, the expression of gp91$^{phox}$ was significantly elevated in the aorta from HFpEF-sedentary animals when compared to controls ($p<0.05$; Figure 2C). This significantly elevated gp91$^{phox}$ expression was not detected in the aorta of animals either performing MCT or HIIT (Figure 2C). To investigate if the altered expression of gp91phox is effective in

modulating NADPH oxidase activity, and therefore reactive oxygen production, nitro-tyrosine modified proteins, an indirect marker of peroxynitrite formation, a reaction product of NO and ROS, were quantified by western blot. As shown in figure 2D,E, the amount of nitro-tyrosine modified proteins was significantly elevated by 50% in HFpEF-sedentary animals when compared to controls at 28 weeks. This rise in nitro-tyrosine modified proteins was not observed in the HFpEF trained animals, independent of training modality (Figure 2D).

With respect to the protein expression of LOX-1, a receptor protein involved in the pathogenesis of atherosclerosis[27], a trend ($p=0.07$) towards an increase in the HFpEF-sedentary animals by 34% was seen when compared to controls at 28 weeks. This trend was not observed in the HFpEF animals performing either MCT or HIIT.

**Endothelial cell coverage and vessel wall hypertrophy**

The lining by endothelial cells (endothelial cell coverage) was significantly reduced in the HFpEF animals at 20 (88.1±1.1 vs. 79.4±1.5 %, $p<0.001$) and 28 weeks ($p<0.001$; Figure 3A) when compared to control animals. Exercise training, either MCT or HIIT, resulted in significantly higher endothelial cell coverage as in the sedentary HFpEF animals (Figure 3A). Compared with controls, aortic wall thickness normalized to vessel lumen was found to be significantly increased in HFpEF-sedentary animals when compared to control, HFpEF-MCT and HFpEF-HIIT animals, indicative of aortic wall hypertrophy (Figure 3B).

**Expression of collagen I and III**

Vessel stiffening has been documented to be partially determined by a reduced collagen I/III ratio. Assessing gene expression of the collagen I/III ratio in aortic tissue at 28 weeks of age demonstrated a

reduction in the HFpEF-sedentary animals when compared to control ($p<0.05$, Figure 3C). This difference was not observed in the animals from the training groups (Figure 3C).

**Expression of TNF-α and activation of SAPK/JNK**

Quantification of TNF-α mRNA expression in the aorta showed a 43% elevation in the HFpEF-sedentary compared to control which did not reach statistical significance ($p=0.09$, Figure 3D). To investigate if the c-Jun N-terminal protein kinase (JNK) pathway was activated in aortas from HFpEF animals and if exercise training modulates the activation western blots analyses were performed. As shown in figure 4 A,B the development of HFpEF is associated with an increased activation/phosphorylation of JNK. Especially the phosphorylation of the p54 splice isoform of JNK was increased by around 58% (Figure 4B), whereas only a trend (increased by 22%, $p=0.06$) was noted for the p46 splice isoform (Figure 4A).This increased phosphorylation was attenuated in the animals of the exercise training groups, with no difference between the training modalities (Figure 4A,B).

**Discussion**

The key findings emerging from the present study can be summarized as follows: 1) endothelial dysfunction was present in the aorta of HFpEF animals and this was associated with numerous molecular alterations including reduced expression and activation (phosphorylation) of eNOS, an increase in expression of NADPH oxidase and nitrotyrosine modifications and activation of JNK, reduced coverage of the vessel wall by endothelial cells, and a reduced collagen I/III ratio; 2) ET, independent of the modality, reversed endothelial dysfunction and this was associated with improvements in endothelial cell coverage, an increase in the collagen I/III ratio, and a reduction in JNK activation and NADPH oxidase

protein expression and nitrosative modifications. Taken together our findings support data in HFpEF reporting that endothelial dysfunction occurs and that ET (MCT and HIIT) should be considered as a treatment strategy to reverse endothelial dysfunction.

**Endothelial dysfunction in HFpEF**

The development of HFpEF in the obesity–driven ZSF1 animal model was clearly associated with the development of endothelium dysfunction. At the age of 20 and 28 weeks, when the animal exhibit clear signs of HFpEF such as elevated E/E´ and LVEDP, reduced exercise capacity but still preserved LVEF, endothelial-dependent vasodilation was significantly impaired. Similarly, endothelial-independent vasodilation was also impaired in HFpEF, but this was only apparent in the 28 week old rats. The observation of impaired endothelial function in this hypertension, diabetes-driven HFpEF model supports observations in other HFpEF animal models. Our group recently reported endothelial dysfunction is developed in a hypertension rat model of HFpEF (Dahl salt-sensitive rat)[23], whereas Gevaert and colleagues reported endothelial dysfunction in an aging mouse model of HFpEF.[24] Besides endothelium-dependent vasodilation, also endothelium-independent vasodilation was impaired in all three models. This implies that not only the endothelial cell layer is affected by HFpEF, but that alterations also occur in the media or adventitia. This is clearly different to what is observed in HFrEF animal models, where in most studies only endothelium-independent vasodilation is impaired.[30-32] The occurrence of endothelial dysfunction in HFpEF patients is still controversial with some[17-20] but not all[21;22] describing endothelial dysfunction. As recently discussed by Gevaert and colleagues[33], this may be due to differences of the vascular bed where the measurements were performed (microvascular vs. macrovascular bed), different methodologies (reactive hyperemic index vs. flow-mediated dilation) and the heterogeneity of the control groups investigated.

**Molecular mechanisms responsible for endothelial dysfunction**

The molecular mechanisms responsible for the endothelial dysfunction in HFpEF are poorly investigated. This lack of knowledge is partially due to lack of established animal models. In the present study, the development of endothelial dysfunction was associated with a reduced expression of eNOS, a reduction in $Ser^{1177}$ phosphorylated eNOS, an increased activation of JNK and an elevation in $gp91^{phox}$ expression, a subunit of the NADPH oxidase. Based on studies performed in HFrEF models, we know that bioavailability of NO is the crucial factor in regulating vascular tone (reviewed in[34]) and that the bioavailable NO is regulated by the expression/activation of eNOS and by the production of reactive oxygen species (ROS) scavenging NO. The significance of alterations in the NO system in HFpEF was recently supported by a study documenting that circulating NO metabolites were significantly reduced in HFpEF when compared to HFrEF patients.[35] In line with this observation, supplementation with inorganic nitrate ($NO_3$-rich beetroot juice) improved exercise capacity in HFpEF patients by modulating peripheral vascular function.[36] The reduction of NO in HFpEF is probably due to a reduced expression and activation of eNOS via phosphorylation at $Ser^{1177}$ residue and by an increase in ROS generated via NADPH oxidase. It is well documented that elevated ROS reduce bioavailable NO and mediates progressive endothelial damage (reviewed in[37]). The elevation of ROS in the situation of HFpEF is further supported by the observation that in the sedentary HFpEF animals nitrotyrosine modification of proteins is significantly elevated when compared to controls. With respect to the signaling pathway leading to an elevated expression of NADPH-oxidase we may speculate that an increased inflammation-mediated activation of JNK (activation via phosphorylation) finally results in an increased expression and activity of NADPH-oxidase and finally ROS concentration. This activation pathway was recently documented in porcine aortic cells, where the angiotensin-II mediated elevation of NADPH-oxidase expression was associated with the activation of JNK.[38] Our observation of reduced eNOS expression/activation in the present

HFpEF model is in line with earlier studies in other animal models such as hypertensive rats[23] and senescence-accelerated mice.[24] What is the molecular trigger leading to a reduced expression of eNOS and an elevation of NADPH oxidase expression? According to Paulus and Tschöpe[16], a systemic inflammation is the initiating event leading to endothelial dysfunction. Indeed, in the present study a trend towards increased TNF-$\alpha$ expression was evident, while in other recent studies a significant elevation of inflammatory cytokines have been reported in HFrEF and HFpEF from either animal[39] or patient[27] tissue. Besides influencing NO bioavailability, a reduced coverage of the vessel wall by endothelial cells, a possible indicator for impaired intima repair mechanisms, may further contribute to endothelial dysfunction.

Besides the impairment of endothelium-dependent vasodilation, also endothelium-independent vasodilation was impaired in HFpEF, suggesting a stiffening of the aorta. Arterial stiffness is well established as an independent marker of cardiovascular risk in hypertension. The molecular analysis of collagen expression either in hypertensive patients[40] or animals[41] has revealed increased collagen I degradation without changes in collagen III. Analyzing the ratio of collagen I/III, a clear reduction in our HFpEF animals was seen suggesting that also in this animal model of HFpEF a stiffer and less compliant aorta is present. This is in line with observations in Dahl salt-sensitive rats exhibiting the phenotype of HFpEF, where a stiffer aorta was postulated.[23] This increased stiffening may have further been aggravated by the increase in aortic wall hypertrophy as seen in this and other animal models of HFpEF[23] as well as in patients.[42] Furthermore, in the present study we observed a hypertrophy of the vessel wall, which might further contribute to the endothelial-independent impairment of vasodilation. This goes in line with previous observations by our group [23]

**Effects of exercise training in HFpEF**

ET is a well-established treatment strategy for HFrEF patients, but data on HFpEF are limited. With respect to the impact of ET in HFpEF on endothelial function, only a limited number of studies are available and these have shown discordant results.[14;23;43] Two human studies[14;43] used either HIIT or MCT for 4 to 16 weeks as training intervention. Despite improved exercise capacity, no positive effect on endothelial function could be documented. In an animal study[23], HIIT in the setting of primary prevention attenuated the development of endothelial dysfunction. In the present study both modalities of ET (MCT and HIIT) significantly reversed endothelial dysfunction over a period of 8 weeks when compared to the HFpEF sedentary animals. While the discrepancy between the human and animal studies with respect to the effectiveness of ET on endothelial dysfunction is unclear, further research in this field is warranted. Most likely, data from the ongoing OptimEx multicenter trial investigating different training regimes in HFpEF patients will help to clarify this discrepancy.[44] Nevertheless, when designing new training studies in HFpEF investigating endothelial function it may be more important to keep the compliance of the patient high instead of modulating the training modalities. Another effect of improving endothelial function by ET could be a direct impact on myocardial force-frequency relation (FFR). This speculation is supported by a report from Shen and colleagues that impairing endothelial function negatively impacts on FFR.[45]

At the molecular level, ET seems to affect mainly the protein expression of NADPH oxidase and subsequently ROS production, the endothelial cell coverage, vessel wall hypertrophy and the expression of collagen I/III. Taken together ET may influence NO bioavailability via directly modulating ROS generation (attenuation of $gp91^{phox}$ expression and nitro-tyrosine modification of proteins), thereby elevating NO bioavailability, and improving compliance of the aorta. This is in line with several studies documenting that a ROS-driven NO inactivation impairs endothelial-dependent vasodilation. [46-49] The impact of exercise training on ROS is further supported by an earlier study from our group[50] where 4 weeks of ET significantly reduced the expression of several subunits of NADPH oxidase and lowered the

level of ROS in left internal mammary artery of patients with stable coronary artery disease. Both training interventions were equally effective in beneficially modulating the functional, molecular and histological alterations observed in this HFpEF model. Therefore, one may speculate that a repetitive increase in shear stress evoked by ET is important to improve endothelial function, while the exact training modality (with different duration and total extent of shear stress increase) is of minor importance. Unfortunately, no further studies investigating the impact of ET at the molecular level on endothelial function in HFpEF are available, and more studies are clearly warranted.

**Study limitations**

Given the heterogeneity of comorbidities in HFpEF patients, the present hypertension, diabetes-driven animal model may only reflect a specific subgroup of HFpEF patients and the conclusion of endothelial dysfunction in HFpEF may only be limited to this specific cohort. Unfortunately, animal models of HFpEF are scarce and the existing ones can be divided into three groups according to HFpEF risk factors including hypertension, diabetes/obesity, and age. Analyzing endothelial function in animal models resembling these risk factors, endothelial dysfunction was present in all of them – hypertension (Dahl salt –sensitive rat)[23], diabetes/obesity (ZSF1 lean/obese rats, present study), and age (senescence-accelerated mouse).[24] Therefore, the conclusion that endothelial dysfunction is present in HFpEF seems to be valid and the discrepancy to some human studies has to be clarified in future but may reflect the indirect measures of endothelial function performed. In the present study, we only analyzed the expression of $gp91^{phox}$ by western blot as marker for the generation of ROS. Indeed, NADPH oxidase is only one potential source of ROS in addition to the ROS generated by mitochondrial and eNOS uncoupling. Using the Dahl salt-sensitive HFpEF model, a study found a significant eNOS uncoupling by measuring the dimer/monomer ratio of eNOS.[5]

## Conclusions

Our data provide additional evidence that endothelial function (endothelial-dependent and endothelial-independent) is impaired in HFpEF, which was associated with a reduced expression of eNOS (NO production), an elevated activation of JNK and expression of the NADPH oxidase subunit $gp91^{phox}$ (oxidative stress reducing NO bioavailability) and a reduction in endothelial cell coverage and collagen I/III ratio (vascular stiffness impeding endothelial-independent vasodilation). ET, initiated after the onset of HFpEF and endothelial dysfunction, improved endothelial function independent of the training modality (HIIT or MCT) by modulating specific molecular markers. However, further research in this field is still required and the results from the ongoing OptimEx multicenter trail[44] will help to confirm whether animal findings translate to the clinical environment.

## Sources of funding

Support for this study was provided by the European Commission, Framework Program 7, grant number: EU 602405-2. The authors are supported by grants from the K.G. Jebsen Foundation (UW, NR) Norwegian Research Council (UW), and the Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology (UW) and the Medical Faculty of the University Leipzig (ZS).

## Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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**Figure Legends.**

**Fig. 1:** At 20 (A,B) and 28 weeks of age (C-F), endothelium-dependent (A,C) and independent (B,E) vasodilation of the aorta was analyzed in pre-constricted rings in response to increasing concentrations of acetylcholine (ACh) or sodium nitroprusside (SNP). Comparing the maximal acetylcholine-induced (D) and the sodium nitroprusside-induced (F) vasodilation a significantly reduced response was detected in the HFpEF-sedentary animals (n=13) when compared to the other three groups (controls. n=11; HFpEF-MCT: n=11; HFpEF-HIIT: n=11). *p<0.05, ***p<0.001 vs. control; #p<0.05 vs. HFpEF-sedentary.

**Fig. 2:** Protein expression of endothelial nitric oxide synthase (eNOS) (A), the ratio of $Ser^{1177}$ phosphorylated eNOS over total eNOS (B), the subunit $gp91^{phox}$ of the NADPH oxidase (C) and nitrotyrosine (D) was quantified in the aorta of control, HFpEF-sedentary, HFpEF-MCT and HFpEF-HIIT animals at the age of 28 weeks. Representative examples of Western blots are depicted (E). *p < 0.05, ** p<0.01, *** p<0.001 vs. ZSF1 lean.

**Fig. 3:** Endothelial cell coverage (A) and aortic wall thickness (B) were determined in sections of the aorta either by immunohistochemical staining for eNOS (A) or hematoxylin and eosin staining (B). Representative images are depicted on top of the bar graphs. Quantitative RT-PCR analysis was used to quantify the expression of collagen I/III (C) and TNF-$\alpha$ (D). * p<0.05 vs. control; *** p<0.001 vs. ZSF1 lean; §§ p<0.01, §§§ p<0.001 vs. HFpEF-sedentary. In the representative image → marks an area of the aorta where no endothelial cell layer is present.

**Fig. 4:** Protein expression of JNK and phosphorylated JNK splice isoforms p46 and p54 was quantified by western blot analyses in the aorta of control, HFpEF-sedentary, HFpEF-MCT and HFpEF-HIIT animals at

the age of 28 weeks. Representative examples of western blots are depicted on top of the bar graphs.

*** $p<0.001$ vs. ZSF1 lean, §§ $p<0.01$, §§§ $p<0.001$ vs. HFpEF-sedentary.