Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure With Preserved Ejection Fraction in a Rat Metabolic Risk Model

Nazha Hamdani, PhD*; Constantijn Franssen, MD*; André Lourenço, MD*; Inês Falcão-Pires, PhD; Dulce Fontoura, PhD; Sara Leite, PhD; Luisa Plettig, PhD; Begoña López, PhD; Coen A. Ottenheijm, PhD; Peter Moritz Becher, MD; Arantxa González, PhD; Carsten Tschöpe, MD, PhD; Javier Díez, MD, PhD; Wolfgang A. Linke, PhD; Adelino F. Leite-Moreira, MD, PhD; Walter J. Paulus, MD, PhD

Background—Obesity and diabetes mellitus are important metabolic risk factors and frequent comorbidities in heart failure with preserved ejection fraction. They contribute to myocardial diastolic dysfunction (DD) through collagen deposition or titin modification. The relative importance for myocardial DD of collagen deposition and titin modification was investigated in obese, diabetic ZSF1 rats after heart failure with preserved ejection fraction development at 20 weeks.

Methods and Results—Four groups of rats (Wistar-Kyoto, n=11; lean ZSF1, n=11; obese ZSF1, n=11, and obese ZSF1 with high-fat diet, n=11) were followed up for 20 weeks with repeat metabolic, renal, and echocardiographic evaluations and hemodynamically assessed at euthanization. Myocardial collagen, collagen cross-linking, titin isoforms, and phosphorylation were also determined. Resting tension (F\textsubscript{passive})–sarcomere length relations were obtained in small muscle strips before and after KCl–KI treatment, which unanchors titin and allows contributions of titin and extracellular matrix to F\textsubscript{passive} to be discerned. At 20 weeks, the lean ZSF1 group was hypertensive, whereas both obese ZSF1 groups were hypertensive and diabetic. Only the obese ZSF1 groups had developed heart failure with preserved ejection fraction, which was evident from increased lung weight, preserved left ventricular ejection fraction, and left ventricular DD. The underlying myocardial DD was obvious from high muscle strip stiffness, which was largely (±80%) attributable to titin hypophosphorylation. The latter occurred specifically at the S3991 site of the elastic N2Bus segment and at the S12884 site of the PEVK segment.

Conclusions—Obese ZSF1 rats developed heart failure with preserved ejection fraction during a 20-week time span. Titin hypophosphorylation importantly contributed to the underlying myocardial DD.

Key Words: diabetes mellitus ■ diastole ■ heart failure ■ myocardium ■ obesity

Heart failure with preserved ejection fraction (HFPEF) is currently observed in 50% of patients with heart failure.\(^1\) The incidence of HFPEF relative to heart failure with reduced ejection fraction continues to rise, and its prognosis fails to improve partly because of lack of a specific HFPEF therapy.\(^2\)

Editorial see p 1112

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Prevalence of comorbidities is higher in HFPEF than in heart failure with reduced ejection fraction.\(^3\) Comorbidities, such as obesity and diabetes mellitus, are key constituents of metabolic risk and known to be associated with the progressive left ventricular (LV) remodeling and dysfunction characteristically observed in HFPEF.\(^4,5\) In HFPEF, body mass index has a U-shaped relation to mortality in contrast to heart failure with reduced ejection fraction where it displays an inverse relation with mortality.\(^6\) Diabetes mellitus has long been recognized to be associated with LV diastolic dysfunction.\(^7\) In HFPEF, heart failure with reduced ejection fraction, and aortic stenosis, diabetes mellitus worsens diastolic LV stiffness through a variety of mechanisms, such as myocardial fibrosis, advanced glycation endproduct deposition, and high cardiomyocyte stiffness.\(^8,9\) High cardiomyocyte stiffness was especially evident in patients with HFPEF and in patients with aortic stenosis and diabetes mellitus, was associated with hypophosphorylation of the giant cytoskeletal protein titin.\(^9,10\)

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From the Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands (N.H., C.F., C.A.O., W.J.P.); Department of Cardiovascular Physiology, Ruhr University Bochum, Bochum, Germany (N.H., L.P., W.A.L.); Department of Physiology and Cardiothoracic Surgery, University of Porto, Porto, Portugal (A.L., I.F.-P., D.F., S.L., A.F.L.-M.); Division of Cardiovascular Sciences, Centre for Applied Medical Research, University of Navarra, Pamplona, Spain (B.L., A.G., J.D.); and Department of Cardiology and Pneumology, Charité-Universitätsmedizin Berlin, Berlin, Germany (P.M.B., C.T.).

*Dr Hamdani, Franssen, and Lourenço contributed equally to this article.

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Correspondence to Walter J. Paulus, MD, PhD, Department of Physiology, Institute for Cardiovascular Research VU (ICaR-VU), VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. E-mail w.j.paulus@vumc.nl

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and corrected in vitro by administration of protein kinase A (PKA) or PKG.\textsuperscript{9,11} Furthermore, patients with high metabolic risk frequently experience salt-sensitive hypertension, which is like obesity associated with systemic oxidative stress.\textsuperscript{12}

To elucidate the mechanisms underlying myocardial dysfunction in metabolic risk–related HFPEF, the present study investigated (1) LV hemodynamics; (2) myocardial histology; (3) in vitro stiffness of small muscle strips; (4) cardiomyocyte stiffness, and (5) myocardial titin phosphorylation in hypertensive ZSF1 rats, which became morbidly obese and diabetic, during a 20-week period, because of the absence of satiation and unlimited access to a regular (ZSF1-obese) or high-fat diet (ZSF1-obese+HFD).

Methods
An expanded Methods section is available in the online-only Data Supplement.

Animal Model
Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22), and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1-week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection, and echocardiographic evaluation. To assess diastolic function, peak velocity of early (E) and late (A) mitral inflow signals and the ratio of E over E′ (peak velocity of early diastolic lateral mitral annular motion) were measured as an indication of LV filling pressure.\textsuperscript{13} From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc, #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks, animals underwent hemodynamic evaluation under anesthesia and were subsequently euthanized with procurement of myocardial tissue samples for histological, biochemical, and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h light/dark cycle at 22°C room temperature and had unlimited access to a regular (ZSF1-obese) or high-fat diet (ZSF1-obese+HFD).

Histology and Collagen Gene Expression
Collagen volume fraction was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picrosirius red.\textsuperscript{8,9} A sircol-based assay was performed to calculate the amount of soluble collagen. The degree of cross-linking was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between soluble and insoluble collagen. Gene expression of collagen 1A1 and collagen 3A1 was performed using real-time polymerase chain reaction.

Force Measurements on Small Muscle Strips and Cardiomyocytes
Cardiomyocytes and muscle strips were incubated for 5 and 30 minutes, respectively, in relaxing solution supplemented with 0.2% Triton X-100 to remove all membrane structures and subsequently attached by force transducer and length motor. Resting tension (F\textsubscript{rest}) was recorded between 1.9 and 2.5 μm sarcomere length (SL). F\textsubscript{stiffness} of cardiomyocytes was measured before and after PKG incubation. In muscle strips, thick and thin filaments were extracted by immersing the preparation in relaxing solution containing 0.6 mol/L KCl (45 minutes at 20°C) followed by relaxing solution containing 1.0 mol/L KI (45 minutes at 20°C). After the extraction procedure, the muscle bundles were stretched again and the passive force remaining after KCl/KI treatment was assumed to be extracellular matrix (E-matrix) based. Titin-based passive force was determined as total passive force minus E-matrix–based passive force.\textsuperscript{15}

Titin Analysis
Titin Isoform Separation
Homogenized myocardial samples were analyzed by 1.8% SDS-PAGE. Protein bands were visualized by Coomassie blue or SYPRO Ruby, scanned, and analyzed densitometrically.\textsuperscript{15,16}

Titin Phosphorylation Assays
After 1.8% SDS-PAGE, gels were stained with Pro-Q Diamond for 1 hour and subsequently with Sypro Ruby overnight. Phosphorylation signals on Pro-Q Diamond-stained gels were indexed to Sypro Ruby–stained titin signals.\textsuperscript{9,10,15,16}

Titin/Phosphotitin Immunoblots
SDS-PAGE (1.8%) and Western blot were performed to measure site-specific phosphorylation and expression of titin using custom-made, affinity-purified, phosphosite-specific antibodies against phospho-S3991 (N2Bus), phospho-S12742 (PEVK), and phospho-S12884 (PEVK; positions in mouse [Mus musculus] titin according to UniProtKB identifier A2ASS6), and antibodies recognizing the corresponding nonphosphorylated sequence around these sites.\textsuperscript{16}

Statistical Analysis
Groups were compared by 2-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by 1-way ANOVA for single acquisitions. Pressure–volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a P value of <0.05 was considered significant. Single comparisons were assessed by an unpaired Student t test. Bonferroni-adjusted t tests were used subsequently for multiple comparisons after repeated measures ANOVA. Statistical analysis was performed with SPSS (version 15.0; SPSS Inc, Chicago, IL).

Results
Cardiometabolic Risk in Obese ZSF1 Rats
ZSF1-Obese and ZSF1-Obese+HFD rats had higher weight gain at 20 weeks (Figure 1A). Energy intake was initially also higher in these animals but leveled off at 20 weeks (Figure 1A). In both obese groups, glycemia levels, glucose tolerance, and insulin resistance were higher (Figure 1B; Tables 1 and 2). Hyperglycemia caused glycosuria, increased urine output, and compensatory water intake. Proteinuria suggested presence of diabetic nephropathy, despite preserved creatinine clearance and plasma protein levels (Tables 1 and 2).

Echocardiography, Hemodynamics, and Morphometrics
Serial echocardiographic studies at 10, 14, and 18 weeks demonstrated normal systolic function in all groups (Figure 2A and 2B; Tables 3 and 4). Concentric LV remodeling was present throughout the entire study in both obese groups (Figure 2A and 2B; Tables 3 and 4), which progressively developed diastolic LV dysfunction, evident from a restrictive LV inflow signal, higher E/E′, and increased left atrial area at 14 and 18 weeks (Figure 2B; Tables 3 and 4).

At 20 weeks, hemodynamic evaluation confirmed normal LV systolic performance, evident from LV ejection fraction,
LV $\frac{dP}{dT_{\text{max}}}$ and end-systolic pressure–volume relationship $E_{\text{es}}$ (slope of linear end-systolic pressure–volume relationship for indexed volumes; Figure 2C; Table 5). Diastolic LV dysfunction was again evident from a prolonged $\tau$, elevated LV end-diastolic pressure, an upward shift of the LV diastolic pressure–volume relationship and a higher LV diastolic chamber stiffness constant ($\beta$) (Figure 2C; Table 5).

At euthanization, lung and heart weights were increased in both obese groups (Figure 3A; Table 6). There was evidence of visceral adiposity with more perirenal and perigonadal fat (Table 6).

Histology
Cardiomyocyte hypertrophy was confirmed histologically in both ZSF1-Obese groups (Figure 3B). Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), and procollagen carboxyl-terminal proteinase type I enhancer were similar in all groups (Figure 4). In line with these findings, the relative mRNA expression of collagen 1A1 and collagen 3A3 was also similar among all groups (Figure 4). No significant differences of Lysyl oxidase expression were observed between all groups

Table 1. Metabolism and Renal Function at 10, 14, and 18 Weeks in WKY and ZSF1-Lean Groups

<table>
<thead>
<tr>
<th></th>
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<td>ZSF1-Lean</td>
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<td>5.7±0.1</td>
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<td>6.3±0.1</td>
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<td>Glycemia, mg/dL</td>
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<td>Plasma creatinine, mg/dL</td>
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<td>Urine output, ml/kg per day</td>
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<tr>
<td>$C_{\text{cr}}$, ml/min per kilogram</td>
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<td>8.1±0.2</td>
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<td>Water intake, ml/kg per day</td>
<td>135±6</td>
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<td>85±3*</td>
<td>76±5†</td>
<td>52±3†</td>
<td>41±5*†</td>
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</tbody>
</table>

Values are mean±SEM (n=11, each group). AUC indicates area under curve; $C_{\text{cr}}$, creatinine clearance; IR, insulin resistance; OGT, oral glucose tolerance; and WKY, Wistar-Kyoto rats.

*P<0.05 vs 10th wk; and †P<0.05 vs WKY.
The relative contributions of collagen and titin were determined in small muscle strips (Figure 5). \( F_{\text{passive}} \)–SL relations were constructed for SL ranging from 1.9 to 2.3 \( \mu \text{m} \). \( F_{\text{passive}} \) was higher in both obese groups from a SL of 2.075 \( \mu \text{m} \) onward (Figure 5A). To discern the contribution of E-matrix, \( F_{\text{passive}} \)–SL relations were also constructed after extraction with KCl/KI (Figure 5B). The contribution of titin was calculated by subtracting E-matrix–based \( F_{\text{passive}} \) from total \( F_{\text{passive}} \) at each SL (Figure 5C). \( F_{\text{passive}} \) attributable to E-matrix and titin was higher in both obese groups from a SL of 2.175 and 2.025 \( \mu \text{m} \) onward, respectively. At the upper limit of the physiological SL range (2.2 \( \mu \text{m} \)), titin accounted for 82% and 78% of \( F_{\text{passive}} \) in ZSF1-Obese and ZSF1-Obese+HFD groups, respectively.

\( F_{\text{passive}} \)–SL relations of isolated skinned cardiomyocytes were steeper in ZSF1-Obese and ZSF1-Obese+HFD (Figure 5D). Incubation with PKG \( \alpha \) returned the \( F_{\text{passive}} \)–SL relations to control levels (Figure 5D). No significant differences of active tension were observed between groups in single skinned small strips and skinned cardiomyocytes.

### Table 2. Metabolism and Renal Function at 10, 14, and 18 Weeks in ZSF1-Obese and ZSF1-Obese+HFD Groups

<table>
<thead>
<tr>
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<tbody>
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<td>ZSF1-Obese+HFD</td>
<td>ZSF1-Obese+HFD</td>
<td>ZSF1-Obese</td>
<td>ZSF1-Obese+HFD</td>
<td>ZSF1-Obese+HFD</td>
</tr>
<tr>
<td>Plasma proteins, g/L</td>
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<td>8.7±0.3†‡</td>
<td>9.7±0.4†‡</td>
<td>7.2±0.2†‡</td>
<td>6.9±0.2†‡</td>
<td>8.3±0.2†‡§</td>
</tr>
<tr>
<td>Proteinuria, mg/d</td>
<td>92±9†‡</td>
<td>115±9†‡</td>
<td>174±10†‡</td>
<td>105±13†‡</td>
<td>88±14†‡§</td>
<td>126±13†‡§</td>
</tr>
<tr>
<td>Glycemia, mg/dL</td>
<td>113±7</td>
<td>112±5†‡</td>
<td>134±10†‡</td>
<td>121±8</td>
<td>156±16†‡§</td>
<td>178±16†‡§</td>
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<tr>
<td>OGT AUC, mg/dL per hour</td>
<td>223±13†‡</td>
<td>226±14†‡</td>
<td>224±10†‡</td>
<td>228±16†‡</td>
<td>260±24†‡</td>
<td>259±17†‡</td>
</tr>
<tr>
<td>IR AUC, mg/dL per hour</td>
<td>111±12†‡</td>
<td>123±6†‡</td>
<td>152±9†‡</td>
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<td>154±13†‡§</td>
<td>185±17†‡§</td>
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<tr>
<td>Glycerol, mg/d</td>
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<td>730±90†‡</td>
<td>405±45†‡</td>
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<td>1070±93†‡§</td>
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<tr>
<td>Plasma creatinine, mg/dL</td>
<td>0.34±0.02</td>
<td>0.31±0.02†‡</td>
<td>0.35±0.03</td>
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<td>0.40±0.03</td>
</tr>
<tr>
<td>Urine output, mL/kg per day</td>
<td>187±23†‡</td>
<td>98±17†‡</td>
<td>79±18†‡</td>
<td>186±24†‡</td>
<td>179±19†‡§</td>
<td>177±18†‡§</td>
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<tr>
<td>( C_\text{cr} ), mL/min per kilogram</td>
<td>10.1±0.4</td>
<td>9.2±0.8</td>
<td>7.4±0.8*</td>
<td>9.3±0.5</td>
<td>5.6±0.2†‡§</td>
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<tr>
<td>Water intake, mL/kg per day</td>
<td>203±23†‡</td>
<td>110±16†‡</td>
<td>91±16†‡</td>
<td>208±24†‡</td>
<td>179±20†‡§</td>
<td>182±17†‡§</td>
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</tbody>
</table>
| Values are mean±SEM (n=11, each group). AUC indicates area under curve; \( C_\text{cr} \), creatinine clearance; HFD, high-fat diet; IR, insulin resistance; and OGT, oral glucose tolerance.

\* \( P < 0.05 \) vs 10th wk; † \( P < 0.05 \) vs WKY; ‡ \( P < 0.05 \) vs ZSF1-Lean; and § \( P < 0.05 \) vs ZSF1-Obese.

(WKY [1.75±0.44], ZSF1-Lean [1.76±0.19], ZSF1-Obese [1.50±0.35], and ZSF1-Obese+HFD [1.70±0.12]).

**F\text{passive} in Small Muscle Strips and Cardiomyocytes**

The relative contributions of collagen and titin were determined in small muscle strips (Figure 5). \( F_{\text{passive}} \)–SL relations were constructed for SL ranging from 1.9 to 2.3 \( \mu \text{m} \). \( F_{\text{passive}} \) was higher in both obese groups from a SL of 2.075 \( \mu \text{m} \) onward (Figure 5A). To discern the contribution of E-matrix, \( F_{\text{passive}} \)–SL relations were also constructed after extraction with KCl/KI (Figure 5B). The contribution of titin was calculated by subtracting E-matrix–based \( F_{\text{passive}} \) from total \( F_{\text{passive}} \) at each SL (Figure 5C). \( F_{\text{passive}} \) attributable to E-matrix and titin was higher in both obese groups from a SL of 2.175 and 2.025 \( \mu \text{m} \) onward, respectively. At the upper limit of the physiological SL range (2.2 \( \mu \text{m} \)), titin accounted for 82% and 78% of \( F_{\text{passive}} \) in ZSF1-Obese and ZSF1-Obese+HFD groups, respectively.

\( F_{\text{passive}} \)–SL relations of isolated skinned cardiomyocytes were steeper in ZSF1-Obese and ZSF1-Obese+HFD (Figure 5D). Incubation with PKG \( \alpha \) returned the \( F_{\text{passive}} \)–SL relations to control levels (Figure 5D). No significant differences of active tension were observed between groups in single skinned small strips and skinned cardiomyocytes.
Titin Hypophosphorylation

N2B titin isoform expression relative to WKY was similar in all groups (WKY, 100±9.4%; ZSF1-Lean, 100.5±9.1%; ZSF1-Obese, 95.0±11.4%; and ZSF1-Obese+HFD, 71.5±11.4%), but titin phosphorylation decreased by 67% and 82% in ZSF1-Obese and ZSF1-Obese+HFD rats, respectively (Figure 6A). Ex vivo phosphorylation by PKG significantly increased all-titin phosphorylation in ZSF1-Obese and ZSF1-Obese+HFD, up to the level measured in WKY and ZSF1-Lean (Figure 6B). Using affinity-purified phosphospecific antibodies, we assessed phosphorylation status by Western blot at a conserved serine within the N2Bus segment (S3991 of full-length mouse titin) and at 2 conserved serines within the PEVK segment (S12742 and S12884; Figure 6C–6E). In the obese groups, significant hypophosphorylation was observed at the phospho-N2Bus S3991 site and at the phospho-PEVK S12884 site but comparable phosphorylation at the phospho-PEVK S12742 site. Protein loading was checked by a sequence-specific antibody that corresponded to the phosphospecific antibody.

Table 3. Echocardiographic Studies at 10, 14, and 18 Weeks in WKY and ZSF1-Lean Groups

<table>
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<th>10th Wk</th>
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<tr>
<td>HR, beats per minute</td>
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<td>dLVPW, mm</td>
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<td>LV mass, mg</td>
<td>447±24</td>
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</tr>
<tr>
<td>CI, µL/min per cm²</td>
<td>99±16</td>
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<td>214±18</td>
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<tr>
<td>FS, %</td>
<td>37±2</td>
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<tr>
<td>EF, %</td>
<td>72±3</td>
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</table>

Values are mean±SEM (n=6, each group). CI indicates cardiac index; dLVPW, left ventricular posterior wall measured in diastole; E/A, ratio between peak E and A waves of pulsed-wave Doppler mitral flow velocity; E/E′, ratio between peak E wave velocity of pulsed-wave Doppler mitral flow and peak E′ wave velocity of tissue Doppler at the lateral mitral annulus; EDVI, end-diastolic volume indexed for body surface area; EF, ejection fraction; FS, fractional shortening; HR, heart rate; LAA, left atrial area; LV, left ventricular; MPI, myocardial performance or Tei index; S, peak systolic tissue Doppler velocity; and WKY, Wistar-Kyoto rats.

Table 4. Echocardiographic Studies at 10, 14, and 18 Weeks in ZSF1-Obese and ZSF1-Obese+HFD Groups

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<tr>
<th></th>
<th>10th Wk</th>
<th>14th Wk</th>
<th>18th Wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats per minute</td>
<td>344±11†‡</td>
<td>347±11†</td>
<td>328±16</td>
</tr>
<tr>
<td>dLVPW, mm</td>
<td>1.56±0.04†</td>
<td>1.50±0.05†</td>
<td>1.49±0.06†</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>698±45</td>
<td>686±42†</td>
<td>714±33†</td>
</tr>
<tr>
<td>CI, µL/min per cm²</td>
<td>136±23</td>
<td>215±20†</td>
<td>255±22*</td>
</tr>
<tr>
<td>FS, %</td>
<td>36±2</td>
<td>37±2</td>
<td>39±2</td>
</tr>
<tr>
<td>EF, %</td>
<td>71±3</td>
<td>73±3</td>
<td>75±2</td>
</tr>
<tr>
<td>S, mm/s</td>
<td>66±4</td>
<td>67±4</td>
<td>63±6</td>
</tr>
<tr>
<td>E/A</td>
<td>1.33±0.03</td>
<td>1.34±0.09†</td>
<td>1.32±0.08†</td>
</tr>
<tr>
<td>E/E′</td>
<td>12.4±0.4</td>
<td>14.2±0.5††</td>
<td>17.2±0.8††</td>
</tr>
<tr>
<td>MPI (Tei)</td>
<td>0.78±0.05</td>
<td>0.77±0.06</td>
<td>0.78±0.02</td>
</tr>
<tr>
<td>EDVI, µL/cm²</td>
<td>1.58±0.13</td>
<td>1.59±0.79</td>
<td>1.86±0.07*</td>
</tr>
<tr>
<td>LAA, mm²</td>
<td>2.5±0.2</td>
<td>3.0±0.2</td>
<td>3.6±0.2††</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=6, each group). CI indicates cardiac index; dLVPW, left ventricular posterior wall measured in diastole; E/A, ratio between peak E and A waves of pulsed-wave Doppler mitral flow velocity; E/E′, ratio between peak E wave velocity of pulsed-wave Doppler mitral flow and peak E′ wave velocity of tissue Doppler at the lateral mitral annulus; EDVI, end-diastolic volume indexed for body surface area; EF, ejection fraction; FS, fractional shortening; HFD, high-fat diet; HR, heart rate; LAA, left atrial area; LV, left ventricular; MPI, myocardial performance or Tei index; and S, peak systolic tissue Doppler velocity.

*P<0.05 vs 10th wk; †P<0.05 vs WKY; ‡P<0.05 vs ZSF1-Lean; and §P<0.05 vs ZSF1-Obese.
The present study identified cardiac titin hypophosphorylation to be associated with high myocardial stiffness and HFPEF in an obese ZSF1 rat model with high metabolic risk.

Table 5. Hemodynamics at the Age of 20 Weeks

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA, cm²</td>
<td>468±5</td>
<td>508±7*</td>
<td>651±5†</td>
<td>616±4‡</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>117±4</td>
<td>146±6*</td>
<td>181±6†</td>
<td>170±6†</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>94±6</td>
<td>127±6*</td>
<td>149±5†</td>
<td>140±5*</td>
</tr>
<tr>
<td>DAP, mmHg</td>
<td>75±7</td>
<td>106±7*</td>
<td>125±6*</td>
<td>113±5*</td>
</tr>
<tr>
<td>HR, beats per minute</td>
<td>346±10</td>
<td>391±9*</td>
<td>354±12†</td>
<td>336±8†</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>5±0</td>
<td>4±1</td>
<td>9±1*†</td>
<td>7±1*†</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>9430±770</td>
<td>11700±964</td>
<td>13000±680*</td>
<td>11000±497</td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>−7880±838</td>
<td>−12600±686*</td>
<td>−11700±541*</td>
<td>−11700±429*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>8.3±0.3</td>
<td>7.6±0.4</td>
<td>10.5±0.6*</td>
<td>9.5±0.4†</td>
</tr>
<tr>
<td>EF, %</td>
<td>60±4</td>
<td>55±3</td>
<td>59±4</td>
<td>63±3</td>
</tr>
<tr>
<td>ESPVR Eₛₒ, mmHg/µL per cm²</td>
<td>0.64±0.15</td>
<td>2.49±0.71*</td>
<td>2.00±0.39†</td>
<td>2.04±0.39*</td>
</tr>
<tr>
<td>EDV/τ, µL/cm²</td>
<td>0.016±0.002</td>
<td>0.023±0.002*</td>
<td>0.028±0.002*</td>
<td>0.029±0.004*</td>
</tr>
</tbody>
</table>

For the purpose of volume indexation, BSA was estimated as $9.1 \times (\text{body weight [g]})^{2/3}$. Values are mean±SEM (n=11, each group). $\beta_I$ indicates chamber stiffness constant for indexed volumes, derived from exponential EDPVR; $\tau$, time constant of isovolumetric relaxation; BSA, body surface area; $dP/dt_{\text{max}}$, maximum rate of pressure rise; $dP/dt_{\text{min}}$, maximum rate of pressure fall; DAP, diastolic arterial pressure; $E_S$, slope of linear end-systolic pressure–volume relationship for indexed volumes; EDP, end-diastolic pressure; EDPVR, end-diastolic pressure–volume relationship; EF, ejection fraction; HFD, high-fat diet; HR, heart rate; MAP, mean blood pressure; SAP, systolic arterial pressure; and WKY, Wistar-Kyoto rats.

*P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; and ‡P<0.05 vs ZSF1-Obese.

Discussion

The present study identified cardiac titin hypophosphorylation to be associated with high myocardial stiffness and HFPEF in an obese ZSF1 rat model with high metabolic risk.

Metabolic Risk–Related HFPEF Model

At the time of euthanization at 20 weeks, high metabolic risk was evident clearly in the obese ZSF1 rats fed either regular diet or high-fat diet. Compared with lean ZSF1 rats or WKY
rats, obese ZSF1 rats showed many features of high metabolic risk such as visceral obesity evident from elevated perirenal and perigonadal fat, insulin resistance, hyperglycemia, and physical inactivity evident from striated muscle wasting. Arterial blood pressure was elevated in both obese and lean ZSF1 rats. At the time of euthanization, HFPEF was, however, only present in the obese ZSF1 rats, and high metabolic risk, therefore, seemed to be a prerequisite in this model for HFPEF development. As such, the current model differs from previous experimental HFPEF models, which largely disregarded metabolic risk as they were performed in old, hypertensive dogs or in Dahl salt-sensitive hypertensive rats. The current model, however, closely resembles clinical HFPEF where metabolic risk is highly prevalent as evident from numerous HFPEF registries or large outcome trials.

The HFPEF presentation observed in this metabolic risk model also shares characteristic features with clinical HFPEF presentation. After 18 weeks, during closed chest echocardiographic evaluation, the E/E' ratio was diagnostic of diastolic LV dysfunction (ZSF1-Obese, 17.2±0.8; ZSF1-Obese+HFD, 15.8±1.1). At euthanization, lung weight was 60% higher in obese animals. The latter probably resulted from episodes of pulmonary edema occurring during physical activity. A similar situation occurs in patients with HFPEF who frequently have

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**Table 6. Morphometrics at the Age of 20 Weeks**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>ZSF1-Lean</th>
<th>ZSF1-Obese</th>
<th>ZSF1-Obese+HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL, mm</td>
<td>41.5±0.1</td>
<td>42.1±0.4</td>
<td>40.9±0.4†</td>
<td>39.6±0.3†‡</td>
</tr>
<tr>
<td>LV+IVS weight/TL, mg/mm</td>
<td>15.4±0.6</td>
<td>16.6±0.8</td>
<td>19.7±1.1*</td>
<td>20.2±1.3†</td>
</tr>
<tr>
<td>RV weight/TL, mg/mm</td>
<td>4.8±0.4</td>
<td>4.5±0.3</td>
<td>5.5±0.3</td>
<td>5.6±0.2†</td>
</tr>
<tr>
<td>Lung weight/TL, mg/mm</td>
<td>51±2</td>
<td>53±2</td>
<td>75±3†</td>
<td>75±2†</td>
</tr>
<tr>
<td>Liver weight/TL, mg/mm</td>
<td>252±9</td>
<td>314±12</td>
<td>906±55†</td>
<td>769±29†</td>
</tr>
<tr>
<td>Kidney weight/TL, mg/mm</td>
<td>23.3±0.3</td>
<td>26.0±0.8</td>
<td>37.1±0.9†</td>
<td>33.9±1.5†</td>
</tr>
<tr>
<td>Perirenal fat weight/TL, mg/mm</td>
<td>64±4</td>
<td>58±6</td>
<td>362±12†</td>
<td>415±14†</td>
</tr>
<tr>
<td>Perigonadal fat weight/TL, mg/mm</td>
<td>62±3</td>
<td>56±5</td>
<td>161±6†</td>
<td>145±5†‡</td>
</tr>
<tr>
<td>Gastrocnemius weight/TL, mg/mm</td>
<td>56±1</td>
<td>63±2</td>
<td>53±1†</td>
<td>48±1†</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=11, each group). HFD, high-fat diet; IVS, interventricular septum; LV, left ventricular; RV, right ventricle; TL, tibial length; and WKY, Wistar-Kyoto rats.

*P<0.05 vs WKY; †P<0.05 vs ZSF1-Lean; and ‡P<0.05 vs ZSF1-Obese.

**Figure 4.** Collagen volume fraction and cross-linking. A, Representative images of myocardial fibrosis (picrosirius red; magnification, x200) in all groups. B to G, Collagen volume fraction, collagen cross-linking, procollagen carboxyl-terminal proteinase type I (PCP), PCP enhancer (PCPE), relative mRNA expressions of collagen 1A1 and collagen 3A1 in all groups. Nine-week-old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22), and Wistar-Kyoto rats (WKY, n=11).
moderate abnormalities in diastolic LV function at rest but striking elevations of LV filling pressure during exercise because of a steep diastolic LV pressure–volume relation. Steep diastolic LV pressure–volume and myocardial passive–length relations were also present in the ZSF1 obese rats. The limited elevation of LV end-diastolic pressure in ZSF1 obese rats during open chest hemodynamic evaluation probably resulted from a reverse effect: thoracotomy and anesthesia reduced venous return to the heart, which led to a prompt fall in LV filling pressures because of steep diastolic LV pressure–volume and myocardial Fpassive–length relations. Apart from elevated E/E′, high diastolic LV chamber stiffness, and high myocardial stiffness, obese ZSF1 rats also had other evidence of diastolic LV dysfunction such as progressive LA enlargement and a significant increase in τ. The latter could, however, also be partially accounted for by the higher systolic arterial pressure in ZSF1-obese rats.

Systolic LV function in the ZSF1 obese rats closely resembled systolic LV function of patients with HFPEF as global indices of LV systolic performance (LV dP/dtmax, LV ejection fraction, and EES) were all preserved. In ZSF1 obese rats, Ees was even higher than in control WKY rats because of a steep end-systolic LV pressure–volume relation. The simultaneous presence of steep end-systolic and end-diastolic LV pressure–volume relations forces the left ventricle to function as a fixed stroke volume pump and explains the swings from pulmonary edema to low output frequently observed in patients with HFPEF.

**Titin Versus E-Matrix**

ZSF1-Obese rats had a steeper myocardial Fpassive–SL relation (Figure 5A). After extraction of the cardiac muscle strips with KCl/KI, which depolymerized thick and thin filaments thereby leaving titin unanchored, the contribution of the E-matrix to myocardial Fpassive became evident (Figure 5B). Subsequently, the contribution of titin could be calculated by subtracting the contribution of the E-matrix from the measured Fpassive at each SL (Figure 5C). For SLs ranging from 2.0 to 2.2 μm, the contribution of titin greatly exceeded the contribution of E-matrix. At 2.0 μm, the contribution of titin was 6.8 and 9.3× larger than of E-matrix for ZSF1-obese and ZSF1-obese+HFD rats. At 2.2 μm, the contribution of titin was still 4.6 and 3.6× larger. SLs ranging from 2.0 to 2.2 μm covered the physiological range of LV filling pressures (from 5 to 40 mm Hg). Using a thick wall ellipsoid model of the LV and the measured LV end-diastolic pressure of 5 mm Hg (Table 5; WKY rats), the calculated LV end-diastolic wall stress (1.25 kN/m²) corresponded with measured Fpassive (1.26 kN/m²) at a 2.0 μm SL. Similarly, after adjusting the values of LV end-diastolic volume index and diastolic LV posterior wall thickness for a 2.2 μm SL and substituting LV end-diastolic wall stress by the measured Fpassive at 2.2 μm SL (13.70 kN/m² in ZSF1-obese+HFD), the same thick wall ellipsoid model yielded a LV end-diastolic pressure of 41 mm Hg. Hence, up to filling pressures exceeding 40 mm Hg, titin accounted for 82% and 78% of Fpassive in ZSF1-obese and ZSF1-obese+HFD rats, respectively. High titin-based stiffness is, therefore, the main contributor to high myocardial stiffness and likely also to HFPEF development in this metabolic risk–related rat HFPEF model. The importance of intrinsic cardiomyocyte stiffness was also evident from the Fpassive–SL relation of isolated skinned cardiomyocytes (Figure 5D), which was steeper and shifted upward in both ZSF1-obese and ZSF1-obese+HFD rats. Furthermore, in vitro administration of PKG.

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**Figure 5.** Fpassive of small muscle strips and cardiomyocytes. A, Fpassive-total vs sarcomere length (SL) curves for all groups. B, Fpassive-E matrix vs SL curves for all groups. C, Fpassive-titin vs SL curves for all groups. D, Fpassive-cardiomyocyte vs SL curves for all groups before (solid curves) and after protein kinase G (PKG) treatment (dashed curves). Curves are second-order polynomial fits to the mean values (n=16/4 muscle strips/group and n=15/4 cardiomyocytes/group). A to C, *P<0.05 ZSF1-Obese+high-fat diet (HFD) vs Wistar-Kyoto rats (WKY); †P<0.05 ZSF1-Obese vs WKY; and ††P<0.05 ZSF1-Obese+HFD vs ZSF1-Lean. D, *P<0.05 ZSF1-Obese+HFD vs WKY and ZSF1-Obese; †P<0.05 ZSF1-Obese vs WKY and ZSF1-Obese+HFD; ††P<0.05 effect of PKG.
to the isolated cardiomyocytes corrected the $F_{\text{passive}} - \text{SL}$ relations. This in vitro reversibility suggests the high $F_{\text{passive}}$ of cardiomyocytes of obese ZSF1 rats to result more from altered phosphorylation status than from structural changes of titin, such as isoform shifts or oxidative damage. In vitro reversibility of high $F_{\text{passive}}$ was also observed in the ZSF1-obese+HFD rats, which were exposed to the highest systemic oxidative stress.

Limited involvement of the E-matrix in the high $F_{\text{passive}}$ of the obese ZSF1 rats was evident also from histological/biochemical analyses of myocardial tissue. Global myocardial collagen volume fraction, collagen cross-linking, collagen 1A1 or collagen 3A3 gene expression, and procollagen carboxyl-terminal proteinase type I or procollagen carboxyl-terminal proteinase type I enhancer activity were unaltered in the obese ZSF1 rats (Figure 4). Despite these findings, there was a small increase in myocardial $F_{\text{passive}}$ attributable to the E-matrix at SL $>2.175 \mu m$ in ZSF1-obese and ZSF1-obese+HFD rats (Figure 5B). This increase could have resulted from subtle alterations in endomysial collagen, which remained undetected by histological analysis or by components of the E-matrix other than collagen.

**Titin Hypophosphorylation**

Titin stiffness can be modulated mainly through isoform shifts or alterations of the phosphorylation status. In patients presenting with eccentric LV remodeling after myocardial infarction or with dilated cardiomyopathy, a titin isoform shift from the stiff N2B to the compliant N2BA isoform has been reported. In patients with concentric LV remodeling related to HFPEF or aortic stenosis, most studies failed to observe a major shift in titin isoform expression. Rat hearts predominantly express N2B titin isoform, the proportion of which remained unaffected in the present study by the concentric LV remodeling observed in lean and obese ZSF1 rats. As previously observed in patients with HFPEF, patients with aortic stenosis and type 2 diabetes mellitus, and old hypertensive dogs with HFPEF, overall N2B titin isoform phosphorylation was greatly reduced in the obese ZSF1 rats, especially when exposed to HFD (Figure 6A). Using site-specific antibodies, the S3991 site and the S12884 of the N2Bus and PEVK segments of titin were identified as being hypophosphorylated (Figure 6C and 6E). The S3991 site can be phosphorylated by both PKA and ERK2 (extracellular-regulated kinase 2) and recently was also shown to be hypophosphorylated in old hypertensive HFPEF dogs. Phosphorylation of the S12742 site of the PEVK segment was unaltered compared to young hypertensive HFPEF dogs where it was hyperphosphorylated. Increased phosphorylation of the N2Bus segment is reported after PKA or PKG administration and shown to lower $F_{\text{passive}}$, whereas increased phosphorylation of the S12742-PEVK occurs after PKCα and calmodulin-dependent protein kinase II can phosphorylate the S12884-PEVK site. Phosphorylation of the S12884-PEVK site
by calmodulin-dependent protein kinase II leads to a reduction of F\textsubscript{passive}.\textsuperscript{37} The elevated F\textsubscript{passive} observed in the present study in the obese ZSF1 rats is consistent with the observed hypophosphorylation of the S3991 site within the N2Bus segment and the observed hypophosphorylation of the S12884 site within the PEVK segment.

Conclusions
Obese ZSF1 rats with a high metabolic risk profile developed HFPEF at 20 weeks. The diagnosis of HFPEF was based on lung congestion, preserved global LV systolic function, and diastolic LV dysfunction. The latter was evident from elevated E/E\textprime, LA enlargement, high LV diastolic chamber stiffness, and high myocardial stiffness. High myocardial stiffness was largely (≥80%) attributable to high cardiomyocyte stiffness, which resulted from hypophosphorylation of titin.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Heart failure with preserved ejection fraction (HFPEF) accounts for >50% of all heart failure cases. Both arterial hypertension and metabolic comorbidities, such as overweight/obesity and type 2 diabetes mellitus, are prevalent in HFPEF. Hitherto, experimental studies mainly tried to reproduce HFPEF in arterial hypertension models such as old dogs with bilateral renal wrapping or Dahl salt-sensitive rats and largely overlooked the prominent involvement of metabolic comorbidities. The present experimental study, therefore, investigated ZSF1 rats that are first generation hybrids between the Zucker diabetic fatty and spontaneously hypertensive heart failure rats. Lean and obese ZSF1 rats are hypertensive as they inherited the hypertension gene from male spontaneously hypertensive heart failure rats. Obese ZSF1 rats also inherited 2 different leptin receptor mutations from female Zucker diabetic fatty and male spontaneously hypertensive heart failure rats. At 20 weeks, the obese, but not the lean ZSF1, rats had developed HFPEF, which was evident from increased lung weight, preserved left ventricular ejection fraction, normal left ventricular end-diastolic volume index, elevated left ventricular filling pressures, left atrial enlargement, and a high diastolic left ventricular stiffness modulus. High myocardial stiffness was also obvious in isolated cardiac muscle strips and could be attributed after myofilamentary extraction to stiffer titin and not to collagen deposition. High titin stiffness resulted from hypophosphorylation of its elastic segments. Titin hypophosphorylation was, therefore, identified as the main contributor to HFPEF in an experimental animal model that comes close to the clinical HFPEF presentation as it combines metabolic comorbidities with arterial hypertension.
Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure With Preserved Ejection Fraction in a Rat Metabolic Risk Model


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Supplemental Material

Myocardial Titin Hypophosphorylation Importantly Contributes to Heart Failure with Preserved Ejection Fraction in a Rat Metabolic Risk Model
METHODS AND MATERIALS

Animal model
Nine-week old male ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11) were obtained from Charles River (Barcelona, Spain) and fed with Purina Diet (#5008). After a 1 week laboratory adaptation period, animals underwent phenotypic evaluation consisting of metabolic cage studies, blood sample collection and echocardiographic evaluation. From this point onward, a subgroup of ZSF1 obese rats (ZSF1-Obese+HFD, n=11) was randomly allocated to receive HFD (Research Diet Inc. #D12468). Weight gain and energy intake were recorded every third day. Phenotypic evaluation was repeated at the 14th and 18th week of life. At 20 weeks of age, animals underwent hemodynamic evaluation under anaesthesia and were subsequently sacrificed with procurement of myocardial tissue samples for histological, biochemical and biomechanical studies. Animals were kept in individually ventilated chambers in a controlled environment with a 12-h-light/-dark cycle at 22ºC room temperature and had unlimited access to food. Investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication no. 85–23, revised 1996) and was approved by the ethics committee of the Faculty of Medicine of Porto.

ZSF1 rats (Charles River, Barcelona, Spain) are first generation hybrids between the ZDF (Zucker diabetic fatty) and SHHF (spontaneously hypertensive heart failure) rats, which carry two different leptin receptor mutations (fa and fa°C). A female ZDF (+/fa) is crossbred with a male SHHF (+/fa°C) rat, leading to either lean ZSF1 or obese ZSF1 rats (fa/fa°C).1 Both ZSF1 rats are hypertensive, since they inherit the hypertension gene from the SHHF rats but only obese ZSF1 rats develop diabetes and dyslipidemia.2

Metabolic studies and renal function
After a 24h acclimatization period, water and energy intake, weight gain and urine output were recorded, and a 24h urine sample was collected in metabolic cages (Techniplast, Buguggiate). After successive 24h rest intervals, all rats underwent oral glucose and insulin resistance testing, at the end of 12h feed-deprivation periods. Glycemia was recorded at baseline and 15, 30, 60, 90 and 120 min
(Freestyle-Mini) after a 1 g.Kg\(^{-1}\) glucose gavage or a 0.5 U.Kg\(^{-1}\) intraperitoneal insulin injection, respectively. Echocardiographic studies were conducted, after another 24h rest period, and a blood sample (1.5 mL) was collected from the subclavian vein under anaesthesia, at the end of the procedure.

**Echocardiography**

Rats (n=11 per group) were anaesthetized by inhalation of 8% sevoflurane in vented containers, orotracheally intubated and mechanically ventilated. Anaesthesia was maintained with sevoflurane (1-2.5%) titrated to avoid the toe pinch reflex. Rats were placed in left-lateral decubitus on a heating pad, the ECG was monitored (lead II) and their temperature was kept at 38°C. The skin was shaved and depilated. After applying prewarmed echocardiography gel a linear 15MHz probe (Sequoia 15L8W) was gently positioned on the thorax. Systolic and diastolic wall thickness and cavity dimensions were recorded, in M-mode and 2D echocardiography, at the level just above the papillary muscles in the parasternal short axis view. The long axis diastolic dimensions of the left ventricle and transverse aortic root diameter were recorded by 2D and M-mode echocardiography, respectively, in the parasternal long axis view. Aortic flow velocity was recorded by pulsed-wave Doppler just above the aortic valve. Mitral flow velocity tracings were obtained with pulsed-wave Doppler just above the mitral leaflets, peak systolic tissue velocity and E’ were measured with tissue Doppler at the medial mitral annulus and lateral mitral annulus, respectively, and left atrial dimensions were measured, at their maximum, by 2D echocardiography in the four chamber view. Acquisitions were done while transiently suspending mechanical ventilation and recordings were averaged from three consecutive heartbeats (Siemens Acuson Sequoia C512). Left ventricular (LV) mass and volumes were calculated by the 2D area-length method. Myocardial performance index was retrieved from the mitral flow pattern.

**Haemodynamic evaluation**

After sedation (100 µg.kg\(^{-1}\) and 5 mg.kg\(^{-1}\) intraperitoneal fentanyl and midazolam, respectively), anaesthesia (8 and 2.5–3% sevoflurane for induction and maintenance, respectively; Penlon Sigma Delta), endotracheal intubation, mechanical ventilation (TOPO, Kent scientific), 8 mL.kg\(^{-1}\).h\(^{-1}\)
intravenous warm Ringer’s solution infusion (NE-1000, New Era Pump Systems), temperature maintenance at 38°C on a heating pad, left thoracotomy, LV and right ventricular (RV) pressure-volume catheter insertion through the apex (SPR-838 and PVR-1045 Millar Instruments, respectively), and ascending aorta probe placement (Transonic) that allowed CO measurement (Active Redirection Transit Time Flowmeter, Triton Technology), signals were continuously acquired (MPVS 300, Millar Instruments), recorded at 1000 Hz (ML880 PowerLab 16/30, ADinstruments), and analyzed (PVAN 3.5, Millar Instruments). Recordings were obtained at suspended end-expiration. The LV catheter was advanced to record systemic arterial pressure. Parallel conductance was assessed with hypertonic saline. After euthanasia (100 mg.kg⁻¹ intravenous pentobarbital), blood (4mL) was collected for storage (-80°C) and for volume calibration (910–1048, Millar instruments). LV volumes were varied using transient inferior vena cava constrictions by adjusting a sling around the inferior vena cava. Organs were weighed, RV and LV + interventricular septum (IVS) were weighed after dissection, and tibia length (TL) was measured. Samples were either snap-frozen and stored at -80°C or processed for histology.Weights were normalized to TL due to the large body weight differences between groups.

Histomorphological analysis was performed on elastica-von-Giesson and hematoxylin-eosin stained, 4 μm thick, sections of tissue placed in 4% buffered formaldehyde solution. As previously validated, MyD was determined perpendicularly to the outer contour of the cell membrane at nucleus level.

Collagen- volume fraction and cross-linking

The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system in sections stained with collagen-specific picro-sirius red, as previously reported in ZSF1 lean (ZSF1-Lean, n=11), ZSF1 obese (ZSF1-Obese, n=22) and Wistar-Kyoto rats (WKY, n=11). All measurements were performed in duplicate by 2 independent observers. The inter- and intra-observer coefficients of variation were <4%. To distinguish between cross-linked (insoluble) and non-cross-linked (soluble) collagen a colorimetric procedure was employed. First, a fast green-sirius red assay was performed to identify and quantify total collagen. In a second step, a sircol-based assay was performed to obtain and quantify soluble collagen. The amount of insoluble
collagen was calculated by subtracting the amount of soluble collagen from the amount of total collagen. The degree of cross-linking was calculated as the ratio between the insoluble and the soluble forms of collagen. All measurements were performed in duplicate. The inter- and intra-assay coefficients of variation were 5 and 3%, respectively.3-5

**RNA isolation and gene expression analysis**

Frozen tissue sections ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=11) and Wistar-Kyoto rats (WKY, n=5) were minced in Trizol and further disrupted during 10 minutes of vigorous shaking. To extract the RNA, chlorophorm was added, mixed, and centrifuged. The aqueous phase containing the RNA was collected in a separate tube, and isopropanol was added. For precipitation, the RNA solution was centrifuged 15 minutes at 4°C at high speed. The RNA pellet was then further purified using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. One μg of RNA was reverse transcribed into cDNA using the High Capacity Kit (Applied Biosystems) and then further diluted to a final concentration of 5 ng/μL cDNA.

The relative quantification of mRNA levels was carried out on a 7900 HT (Applied Biosystem). To assess the mRNA expression of the target genes, real-time PCR was performed using 5 μL of the gene expression master mix (Applied Biosystems) and 0.5 μL of the gene expression assay for Col1A1 (Rn01463848_m1) and Col3A1 (Rn01437683_m1) (each includes forward and reverse primers as well the fluorescently FAM-labelled probe) from Applied Biosystems, and 1 μL of cDNA in a final volume of 10 μL. Quantification of the house keeping gene GAPDH (Rn99999916_s1) as an internal control was performed for each sample. Data were normalized to 18S RNA level as an endogenous control and are expressed using the formula $2^{-\Delta\Delta Ct}$ in comparison to the corresponding untreated controls.

**Force measurements on skinned cardiomyocytes and strips**

**Single skinned cardiomyocytes**

Force measurements were performed on single skinned cardiomyocytes as described.3,4,6 Cardiomyocytes were isolated from WKY, ZSF1-Lean, ZSF1-Obese and ZSF1-Obese+HFD rat hearts
(n=15/4 cardiomyocytes/group). Briefly, samples were defrozen in relaxing solution (in mmol/L: free Mg, 1; KCl, 100; EGTA, 2; Mg-ATP, 4; imidazole, 10; pH 7.0), mechanically disrupted and incubated for 5 min in relaxing solution supplemented with 0.5% Triton X-100. The cell suspension was washed 5 times in relaxing solution. Single cardiomyocytes were selected under an inverted microscope (Zeiss Axiovert 135, 40x objective) and attached with silicone adhesive between a force transducer and a piezoelectric motor as part of a "Permeabilized Myocyte Test System" (1600A; with force transducer 403A; Aurora Scientific, Aurora, Ontario, Canada).

Cardiomyocyte $F_{\text{passive}}$ was measured in relaxing buffer at room temperature within a sarcomere-length range between 1.9 and 2.3 µm. Force values were normalized to myocyte cross-sectional area calculated from the diameter of the cells, assuming a circular shape. As a test of cell viability, each cardiomyocyte was also transferred from relaxing to maximally activating solution (pCa4.5), at which isometric force developed. Once a steady state force was reached, the cell was shortened within 1 ms to 80% of its original length to determine baseline force. Only cells developing active forces >20 kN/m² were included in the analysis. The passive tension was measured under steady state shear (viscous and elastic properties). Subsequently cardiomyocytes were incubated in relaxing solution supplemented with the PKG1$\alpha$ (0.1 U/mL; Sigma, batch 034K1336), guanosine cGMP (10 µmol/L, Sigma) and dithiothreitol (6 mmol/L; Sigma). After 40-min-long incubation with PKG1$\alpha$, $F_{\text{passive}}$ measurements were again performed in relaxing solution at SL 1.9-2.3 µm.

**Skinned muscle strips**

Left papillary muscles were dissected after sacrificing the animals (n=16/4 muscle strips/group). Small muscle strips were created and chemically permeabilized in a 1% Triton X-100 solution for 30 minutes. After clipping both ends, the strips were mounted between a length motor and a force transducer on top of an inverted microscope. Strips were activated at 20°C with solutions containing a saturating Ca²⁺ concentration to determine maximal active tension (force/cross sectional area) at a sarcomere length (SL) of 2.0 µm. Subsequently, strips were stretched from SL 1.9 µm to 2.3 µm, with a velocity of 10% muscle length per second and while in a solution with low Ca²⁺ (pCa9), to determine passive tension generation. Afterwards, the strips underwent an extraction protocol of 45 minutes in
0.6M KCl followed by 45 minutes in 1.0M KI to depolymerize thick and thin filaments respectively, leaving titin unanchored, and the strips were again passively stretched as described above. The remaining tension after this procedure is extraction-insensitive and caused by extracellular matrix, i.e. collagen. The passive tension was measured under steady state shear (viscous and elastic properties).

**Titin-isoform separation and titin phosphorylation by Pro-Q Diamond/Sypro Ruby**

Titin isoforms were separated as described. Briefly, tissue samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were solubilized in 50 mM Tris-sodium dodecyl sulfate (SDS) buffer (pH 6.8) containing 8 µg/mL leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 µL/mL; Sigma). Samples were heated for 3 minutes at 96°C and centrifuged. Then, samples (20 µg; equal concentration checked by spectroscopic methods) were separated by agarose-strengthened 1.8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 5 mA constant current for 16 hours. The phosphorylation state of cardiac titin was determined using Pro-Q Diamond phosphoprotein. Titin gels were stained for one hour with Pro-Q Diamond, and then overnight with Sypro Ruby (Molecular Probes). Staining was visualized using the LAS-4000 Image Reader (Fuji Science Imaging Systems) and signals were analyzed using Multi Gauge V3.2 or AIDA software. Finally signals obtained from Pro-Q Diamond staining were normalized to signals obtained from Sypro Ruby staining.

**Titin and phospho-titin analysis by Western blot**

1.8% SDS-PAGE followed by Western blot was performed to measure expression and site-specific phosphorylation of titin. Samples from ZSF1 lean (ZSF1-Lean, n=5), ZSF1 obese (ZSF1-Obese, n=10) and Wistar-Kyoto rats (WKY, n=5) were applied at a concentration that was within the linear range of the detection system (30 µg dry weight; checked by spectroscopic methods). Sequence-specific or phosphosite-specific anti-titin antibodies were custom-made by Eurogentec, Belgium (positions in mouse (*Mus musculus*) titin according to UniProtKB identifier A2ASS6)). The following affinity-purified antibodies were used.
- Anti-titin- mouse N2Bus against EEGKSLSFPLA (rabbit polyclonal; 1:1000).

- Anti-phospho-N2Bus (S3991 in mouse titin and S4010 in human titin) against EEGK(PO3H2)L SFPLA (rabbit polyclonal; 1:500).

- Anti-PEVK-domain against (cross-species conserved) sequence EVVLKSVLRK (1:1000)

- Anti-phospho-PEVK-domain (S12742 in mouse titin and S11878 in human titin) against EVVLKS(PO3H2)VL RK (1:500)

- Anti-PEVK-domain against (cross-species conserved) sequence KLRPGSGGEKPP (1:100)

- Anti-phospho-PEVK-domain (S12884 in mouse titin and S12022 in human titin) against KLRPGS(PO3H2)GGEKPP (1:500)

The amino acid sequences of rat are identical to the amino acid sequences of mouse.9;10

Titin antibodies gave specific signals on Western blots with cardiac tissue. Following SDS-PAGE, proteins were transferred to Hybond ECL nitrocellulose membranes. Blots were pre-incubated with 3% bovine serum albumin in Tween Tris-buffered saline (TTBS); 10 mmol/L Tris-HCl; pH 7.6; 75 mmol/L NaCl; 0.1% Tween) for 1 hour at room temperature. Then, blots were incubated overnight at 4°C with the primary antibodies against the respective (phospho) protein. After washing with TTBS, primary antibody binding was visualized using secondary horseradish peroxidase-labeled, goat-anti-rabbit/mouse antibodies (dilution 1:1000; DakoCytomation) and enhanced chemiluminescence (ECL Western blotting detection; Amersham Biosciences). Staining was visualized using the LAS-4000 Image Reader and analyzed with Multi Gauge V3.2 or AIDA software. PVDF stains were saved for comparison of protein load. Loading was also controlled by comparing signals of the phospho-specific antibodies with those of the respective sequence-specific antibodies. Finally signals obtained from phospho-specific antibodies were normalized to signals obtained from sequence-specific antibodies.

**Data analysis**

Circumferential LV end-diastolic wall stress (σ) was computed using a thick wall ellipsoid model of the LV:
\[
\sigma = \frac{PD}{2h} \times \left[ 1 - \frac{h}{D} - \frac{D^2}{2L^2} \right]
\]

where \( P \) is LV end-diastolic pressure, \( h \) is LV wall thickness, and \( D \) and \( L \) are LV short axis diameter and long axis length at the midwall.

Groups were compared by two-way repeated measures ANOVA whenever serial acquisitions were obtained for the same animal, and by one-way ANOVA for single acquisitions. Pressure-volume loop analysis was analyzed using LabChart 7 Pro v7.3.1. Values are given as mean±SEM. A 2-tailed test with a probability of value <0.05 was considered significant. Single comparisons were assessed by an unpaired Student \( t \) test. Bonferroni-adjusted \( t \) tests were used subsequent for multiple comparisons after repeated measure ANOVA. Statistical analysis was performed with SPSS (Version 15.0; SPSS Inc,Chicago,Ill).

**Supplemental References**