Heart rate reduction by \( I_f \)-inhibition improves vascular stiffness and left ventricular systolic and diastolic function in a mouse model of heart failure with preserved ejection fraction

Jan-Christian Reil\(^{1}\)*, Mathias Hohl\(^{1}\), Gert-Hinrich Reil\(^{2}\), Henk L. Granzier\(^{3}\), Mario T. Kratz\(^{1}\), Andrey Kazakov\(^{1}\), Peter Fries\(^{4}\), Andreas Müller\(^{4}\), Matthias Lenski\(^{1}\), Florian Custodis\(^{1}\), Stefan Gräber\(^{5}\), Gerd Fröhlig\(^{1}\), Paul Steendijk\(^{6}\), Hans-Ruprecht Neuberger\(^{†,†}\), and Michael Böhm\(^{†,†}\)

\(^{1}\)Klinik für Innere Medizin III, Kardiologie, Angiologie und Internistische Intensivmedizin, Universitätsklinikum des Saarlandes, Kirrberger Straße D 66421, Homburg/Saar, Germany; 
\(^{2}\)Klinik für Innere Medizin I, Kardiologie, Klinikum Oldenburg, Germany; 
\(^{3}\)Department of Physiology, University of Arizona, Tucson, AZ, USA; 
\(^{4}\)Klinik für Radiologie, Universitätsklinikum des Saarlandes, Homburg/Saar, Germany; 
\(^{5}\)Institut für Medizinische Biometrie, Universität des Saarlandes, Homburg/Saar, Germany; and 
\(^{6}\)Department of Cardiothoracic Surgery, Leiden University Medical Center, Leiden, The Netherlands

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Aims
In diabetes mellitus, heart failure with preserved ejection fraction (HFPEF) is a significant comorbidity. No therapy is available that improves cardiovascular outcomes. The aim of this study was to characterize myocardial function and ventricular-arterial coupling in a mouse model of diabetes and to analyse the effect of selective heart rate (HR) reduction by \( I_f \)-inhibition in this HFPEF-model.

Methods and results
Control mice, diabetic mice (db/db), and db/db mice treated for 4 weeks with the \( I_f \)-inhibitor ivabradine (db/db-Iva) were compared. Aortic distensibility was measured by magnetic resonance imaging. Left ventricular (LV) pressure–volume analysis was performed in isolated working hearts, with biochemical and histological characterization of the cardiac and aortic phenotype. In db/db aortic stiffness and fibrosis were significantly enhanced compared with controls and were prevented by HR reduction in db/db-Iva. Left ventricular end-systolic elastance \( (E_{es}) \) was increased in db/db compared with controls \((6.0 \pm 1.3 \text{ vs. } 3.4 \pm 1.2 \text{ mmHg/\mu L, } P < 0.01)\), whereas other contractility markers were reduced. Heart rate reduction in db/db-Iva lowered \( E_{es} \) \((4.0 \pm 1.1 \text{ mmHg/\mu L, } P < 0.01)\), and improved the other contractility parameters. In db/db active relaxation was prolonged and end-diastolic capacitance was lower compared with controls \((28 \pm 3 \text{ vs. } 48 \pm 8 \text{ \mu L, } P < 0.01)\). These parameters were ameliorated by HR reduction. Neither myocardial fibrosis nor hypertrophy were detected in db/db, whereas titin N2B expression was increased and phosphorylation of phospholamban was reduced both being prevented by HR reduction in db/db-Iva.

Conclusion
In db/db, a model of HFPEF, selective HR reduction by \( I_f \)-inhibition improved vascular stiffness, LV contractility, and diastolic function. Therefore, \( I_f \)-inhibition might be a therapeutic concept for HFPEF, if confirmed in humans.

Keywords
Heart rate reduction • Ventricular-arterial coupling • Diastolic dysfunction • HFPEF • Vascular stiffness

* Corresponding author. Tel: +49 6841 1623000, Fax: +49 6841 1623369, Email: jan.reil@uniklinikum-saarland.de

† H.R.N. and M.B. share senior authorship.

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Introduction

Population-based epidemiological studies reported that almost half of the patients with congestive heart failure have preserved ejection fraction (HFPEF).1,2 Thirty to 40% of these HFPEF patients have diabetes mellitus (DM). Previous non-invasive studies have reported a close relation between DM and diastolic dysfunction in humans.3 Beside delayed left ventricular (LV) relaxation and disturbed compliance, combined ventricular systolic, and arterial stiffening with corresponding abnormal ventricular-arterial coupling are considered important pathophysiological features in patients with HFPEF.4–8 Based on theoretical considerations, heart rate (HR) reduction could beneficially influence LV diastolic dysfunction.9 Selective HR reduction improves diastolic filling by prolonging diastolic period without inducing negative lusitropy compared with beta-blockade.10 Heart rate reduction was shown to reduce elevated arterial elastance thereby possibly improving abnormal ventricular-arterial coupling.11

Ivabradine (iva) is a selective inhibitor of the cardiac pacemaker I\textsubscript{f}-current12 lowering HR without modifying atrioventricular or intraventricular conduction and contractility in humans and animals.13,14 It was the aim of the study to characterize myocardial intraventricular conduction and contractility in a mouse model of DM using diabetes type 2 (db/db) mice.15 Additionally, the effect of selective HR reduction on this model was analysed. Besides magnetic resonance imaging (MRI) measurements in vivo to assess aortic distensibility, haemodynamics were investigated by pressure–volume (PV) analyses of isolated working hearts. Moreover, molecular mediators of haemodynamic changes like titin isofoms and Ca\textsuperscript{2+}-handling proteins were characterized.

Methods

For detailed Methods, please refer to the Supplementary material online. The study was approved by the animal ethics committee of the Saarland University and animal handling was performed according to the European directive on laboratory animals (86/609/EEC). Animals were housed under standard conditions with a 12 h light/dark cycle and free access to water and chow. Male leptin receptor-deficient C57BL/KsJleprdb/leprdb type 2 diabetic mice (db/db, n = 31) and their non-diabetic heterozygote littermates (db/+; controls; n = 33) (Charles River Laboratories, Sulzfeld, Germany) were used for haemodynamic measurements at the age of 7 and 11 weeks. A group of 7-week-old male db/db mice were fed for 4 weeks with Iva (20 mg/kg body weight/day) dissolved in the drinking water (db/db-iva). n = 23).

Non-invasive blood pressure measurements in vivo

Heart rate and blood pressure were measured by a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC, USA) in conscious animals between week 7 and 11 (n = 6 per group).

Magnetic resonance imaging studies in vivo

The MRI studies were used to determine aortic distensibility16 in db/db mice and performed on a 9.4 Tesla horizontal bore animal scanner (Bruker Biospec 94/20, Ettlingen, Germany). Eighteen animals (six controls, six db/db mice, and six db/db-iva mice) were included.

Working heart preparation and data analyses of pressure–volume measurements

Eight db/db mice and eight controls were investigated at an age of 7 weeks. Nine db/db, 9 db/db-iva, and 11 control mice were analysed at an age of 11 weeks as reported elsewhere (see Supplementary material online for details).17

Data analyses of pressure–volume measurements

In PV loops LV pressure was plotted as function of instantaneous LV volume (Figure 3). The end-systolic pressure volume relationship (ESPVR) and its slope E\textsubscript{es} (end-systolic ventricular elastance) were assessed by a sudden increase in afterload pressure from 60 to 150 mmHg while preload was kept constant at 10 mmHg and a HR of 400 b.p.m.

When ventricles were made to eject against increasing levels of afterload (‘aortic occlusion’), end-systolic PV coordinates are indicated by solid circles and fit to the equation: \( P_{\text{es}} = E_{\text{es}} (V_{\text{es}}-V_0) \) (a linear relation in the measured range), where \( P_{\text{es}} \) is end-systolic pressure, \( E_{\text{es}} \) is end-systolic volume, and \( V_0 \) is volume axis intercept. End-systolic elastance is a measure of LV contractility and systolic stiffness.18 During ‘aortic occlusion’ the maximal developed peak LV end-systolic pressure was measured. The pressure value minus 60 mmHg defined the preload-dependent maximal pressure amplitude (PA\textsubscript{max}). PA\textsubscript{max} is a contractile parameter defining contractility reserve. V10 is the ventricular capacitance indicating the end-diastolic volume of left ventricle at a preload of 10 mmHg. Ejection fraction (EF) was calculated by the equation \( EF = \frac{V_{\text{ed}}-V_{\text{es}}}{V_{\text{ed}}-V_0} \times 100 \% \), where \( V_0 \) is end-diastolic volume. Transmitral flow profile (E- and A-wave) was measured by a conductance catheter.

Biochemical analysis

For morphological and histological analysis of the left ventricle, eight animals per group were investigated. Western blot analysis was performed by standard methods. For titin protein analysis, sodium dodecyl sulfate–agarose electrophoresis was performed in six mice per group.19 For histological examinations of the ascending aorta (n = 6 per group) Sirius Red staining for collagen content was performed, while superoxide production was measured by dihydroethidium fluorescence and NO content was calculated by diaminofluorescein diacetate fluorescence.

Reverse transcription and real-time polymerase chain reaction

RNA isolation and real-time polymerase chain reaction analysis (n = 8 per group) was performed as described elsewhere.17 Primer sequences were as follows: GAPDH forward: cctgctcactcctgggtct; GAPDH reverse: cattgagaacttcagccac; connective tissue growth factor (CTGF) forward: aagcgactttagaactctg; CTGF reverse: gctcttgtgaagacact; titin isoform N2BA forward: gca-gatctgtccgcttct; titin isoform N2BA reverse: gatcttcaagagctgtgc;
Statistical analysis
Continuous variables are presented as mean ± SD. Group differences at an age of 7 weeks were calculated by the Mann–Whitney U test. Group differences between all three groups at an age of 11 weeks were first determined by the Kruskal–Wallis test. If the result was a P value <0.05, differences between single groups were calculated by the Mann–Whitney test as a post hoc test with a corrected level of significance. P values <0.05 resp. <0.017 (corrected) were considered to reflect statistically significant differences. Calculated P values are two-sided. All analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results
Metabolism and haemodynamics in vivo
Fasting glucose levels and plasma insulin concentration were significantly increased in db/db mice compared with controls at an age of 11 weeks. Glucose and insulin levels of db/db-Iva and db/db mice did not differ significantly (Figure 1A and B). Serum cystatine C levels were similar in all three groups excluding renal failure (Table 3).
Systolic blood pressure did not differ significantly between week 7 and 11 in all groups (Figure 1C). The diastolic blood pressure of db/db mice was significantly lower compared with controls at week 11. In db/db-Iva mice diastolic blood pressure was comparable to controls (Figure 1D). After 11 weeks pulse pressure was significantly increased in db/db mouse compared with controls, whereas pulse pressure of db/db-Iva mice was similar to controls (Figure 1E). In db/db mice HR was significantly elevated compared with controls during the 4 weeks of observation whereas HR in db/db-Iva mice did not differ compared with controls (Figure 1F). At week 11, HR of db/db mice was significantly higher compared with the HR at week 7.

**Magnetic resonance imaging of aortic area in vivo**

At week 11, cardiac cycle-dependent change in aortic area and apparent distensibility were significantly reduced in db/db mouse compared with controls (0.008 ± 0.003 vs. 0.03 ± 0.006 mmHg, \( P < 0.01 \), Figure 2, Table 1) indicating increased stiffness of the aortic wall. Interestingly, this reduction was not seen in db/db-Iva mice (0.03 ± 0.009 mmHg, \( P = 0.54 \)). Figure 2A shows representative images of the aortic end-systolic and end-diastolic area of a control, a db/db, and a db/db-Iva mouse. The difference of aortic area between end-systole and end-diastole is decreased in db/db mouse compared with controls, while in db/db-Iva mouse it was higher and comparable to controls (Figure 2B and Table 1). Reduced aortic area change in db/db mice was mainly related to increased diastolic aortic area, although diastolic blood pressure was significantly lower in these animals (Table 1).

**Pressure–volume analyses of isolated working hearts**

End-systolic elastance was significantly increased in db/db mouse compared with controls at 7 weeks of age (Table 2). Other parameters of LV systolic and diastolic function did not differ significantly between db/db and controls (Table 2).

At 11 weeks a representative example of PV-analyses of a db/db mouse, a control and a db/db-Iva mouse is given in Figure 3A. The different slopes of ESPVR (i.e. \( E_{es} \)) in the three groups are evident (coloured straight lines). Additionally, there was a leftward shift of the PV-loops of db/db and db/db-Iva mice. End-systolic elastance was significantly elevated in db/db mouse compared with controls (6.0 ± 1.3 vs. 3.4 ± 1.2 mmHg/\( \mu L \), \( P < 0.01 \), Figure 3B). Ivabradine reduced \( E_{es} \) in db/db-Iva compared with db/db (4.0 ± 1.1 mmHg/\( \mu L \), \( P < 0.017 \)). Interestingly, \( dp/dt_{max} \) (4848 ± 481 vs. 5798 ± 965 mmHg/s, \( P < 0.017 \)) and PA max (52 ± 14 mmHg, \( P < 0.01 \)) were significantly reduced in db/db mouse compared with controls (Figure 3C and D, Table 3). In db/db-Iva mouse \( dp/dt_{max} \) (6000 ± 529 mmHg/s, \( P < 0.01 \)) and PA max (42 ± 9 mmHg, \( P < 0.01 \)) were significantly higher than in db/db mice. Ejection fraction was similar in all groups (Table 3).

At week 11, the diastolic parameters of ventricular function were severely impaired in db/db mice (Table 3). The relaxation constant \( \tau \) was significantly prolonged, and LV capacitance (\( V_{10} \) = end-diastolic volume at a preload of 10 mmHg) was significantly decreased compared with controls (28 ± 3 \( \mu L \), \( P < 0.01 \)).

**Figure 2** (A) Representative magnetic resonance imaging analyses of aortic end-systolic and end-diastolic area in controls, db/db, and db/db-Iva mice after 11 weeks. (B) Aortic distensibility. \( *P < 0.017 \).

Ivabradine improved diastolic function by reducing \( \tau \) and increasing \( V_{10} \) significantly compared with db/db mice (37 ± 4 \( \mu L \), \( P < 0.01 \), Figure 3E). Transmitral flow profile revealed a significantly increase of E/A ratio in db/db mouse compared with controls indicating a restrictive filling pattern of LV (Figure 3F). E/A ratio of db/db-Iva mice, however, were similar to controls. As a result of decreased \( V_{10} \) and contractility stroke volume was significantly lower in db/db mouse compared with controls. Stroke volume of db/db-Iva mouse was significantly higher compared with db/db mice but lower than controls (Table 3).

**Histology**

Collagen content of the aorta of db/db mice was significantly increased compared with controls and db/db-Iva mice. Accordingly,
augmented in the aorta of db/db mice. Oxidative stress indicated by an increase of superoxide content was similar to db/db mice compared with controls while body weight of db/db mice was excluded by MRI.

Heart rate reduction in mice with HFPEF was similar to db/db mice compared with controls while body weight of db/db mice was reduced compared with controls and db/db-Iva mice (Figure 4, Table 1). Severe aortic calcification or plaque formation was not seen in db/db-Iva mice. Phosphorylation at serine 16 was reduced in db/db-Iva mice compared with controls. Capillary density of db/db-Iva mice tended to be higher compared with db/db mice (Table 3).

**Biochemical analyses**

The mRNA expression of the transcription variant titin N2B was significantly elevated in db/db mice compared with controls, while in db/db-Iva mice N2B was lower than in db/db mice. The mRNA expression of the transcription variant titin N2BA was similar in all groups (Figure 5C). These results are supported by an altered protein expression of the titin-isoforms. The ratio of titin N2AB to titin N2B was significantly lower in db/db mice compared with controls indicating increased expression of the stiffer N2B-isoform (Figure 5D). In db/db-Iva mice this ratio was higher than in db/db mice and comparable to controls. Total titin protein was similar in all groups (not shown).

Myocardial relaxation is tightly linked to regulation of Serca2A activity via the phosphorylation status of phospholamban. While total protein expression of phospholamban was comparable in all groups, phosphorylation at threonine 17 was significantly lower in db/db mice compared with controls. This reduction was not seen in db/db-Iva mice. Phosphorylation at serine 16 was decreased only by trend in db/db mice (Figure 6A). Serca2A protein expression was similar in all groups (Figure 6B).

**Discussion**

The aim of the study was to characterize myocardial function and ventricular-arterial coupling in a diabetic mouse model and to investigate the influence of HR reduction on LV function in this HFPEF model. Selective HR reduction by Iva improved vascular and diastolic function, preserved systolic contractility, and reduced systolic stiffness in db/db mice by influencing aortic oxidative stress indicated by an increase of superoxide content was augmented in the aorta of db/db mice while NO synthesis was reduced compared with controls and db/db-Iva mice (Figure 4, Table 1). Severe aortic calcification or plaque formation was excluded by MRI.

Total body weight was significantly increased in db/db mice compared with controls while body weight of db/db-Iva mice was similar to db/db mice. Heart weight and heart weight-to-tibia length ratio did not differ between the groups (Table 3). Eleven weeks old db/db mice did neither show myocardial hypertrophy nor fibrosis compared with controls (Figure 5A, Table 3). Gene expression of CTGF was also comparable in all groups (Figure 5B). However, the density of capillaries was significantly reduced in db/db mice compared with controls. Capillary density of db/db-Iva mice tended to be higher compared with db/db mice (Table 3).

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**Table 1** Results of magnetic resonance imaging measurements at week 11

<table>
<thead>
<tr>
<th></th>
<th>Control ( n=6 )</th>
<th>db/db ( n=6 )</th>
<th>P value (KWT)</th>
<th>db/db vs. db/db-Iva ( n=6 )</th>
<th>P value db/db vs. control ( n=6 )</th>
<th>P value db/db-Iva vs. control ( n=6 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>End-systolic aortic area (mm²)</td>
<td>2.39 ± 0.09</td>
<td>2.41 ± 0.13</td>
<td>0.48</td>
<td>2.49 ± 0.13</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>End-diastolic aortic area (mm²)</td>
<td>1.72 ± 0.17</td>
<td>1.94 ± 0.15</td>
<td>0.4</td>
<td>1.77 ± 0.16</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Change in aortic area (mm²)</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.46 ± 0.05</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Aortic distensibility (1/mmHg)</td>
<td>0.03 ± 0.006</td>
<td>0.008 ± 0.003</td>
<td>&lt; 0.01</td>
<td>0.03 ± 0.009</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Pulse pressure (mmHg)</td>
<td>15.6 ± 4.7</td>
<td>34.6 ± 9.8</td>
<td>&lt; 0.01</td>
<td>15.4 ± 5.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>92.2 ± 5</td>
<td>75.6 ± 8.1</td>
<td>&lt; 0.01</td>
<td>90.0 ± 4.0</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure/ end-diastolic aortic area (mmHg/mm²)</td>
<td>53.8 ± 3.1</td>
<td>39.3 ± 6.7</td>
<td>&lt; 0.01</td>
<td>51.8 ± 5.4</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>262 ± 51</td>
<td>303 ± 53</td>
<td>0.69</td>
<td>0.36</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

KWT, Kruskal–Wallis test.

**Table 2** Systolic and diastolic haemodynamic parameters in control and db/db mice at an age of 7 weeks

<table>
<thead>
<tr>
<th></th>
<th>Control ( n=8 )</th>
<th>db/db ( n=8 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( E_{es} ) (mmHg/µL)</td>
<td>3.4 ± 0.8</td>
<td>5.3 ± 0.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>( V_0 ) (µL)</td>
<td>9.2 ± 29.0</td>
<td>-13.8 ± 20.6</td>
<td>0.22</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>8.2 ± 1.3</td>
<td>7.3 ± 0.8</td>
<td>0.20</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>19.0 ± 3.7</td>
<td>17.0 ± 2.1</td>
<td>0.27</td>
</tr>
<tr>
<td>EF (%)</td>
<td>53.3 ± 7.8</td>
<td>55.4 ± 9.0</td>
<td>0.67</td>
</tr>
<tr>
<td>dP/dt_(max) (mmHg/s)</td>
<td>4883 ± 612</td>
<td>4557 ± 763</td>
<td>0.43</td>
</tr>
<tr>
<td>PA_(max) (mmHg)</td>
<td>39.8 ± 6.9</td>
<td>32.3 ± 18.8</td>
<td>0.46</td>
</tr>
<tr>
<td>Diastolic parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t (ms)</td>
<td>11.7 ± 1.3</td>
<td>12.2 ± 2.4</td>
<td>0.64</td>
</tr>
<tr>
<td>dP/dt_(max) (mmHg/s)</td>
<td>-3450 ± 407</td>
<td>-3029 ± 403</td>
<td>0.20</td>
</tr>
<tr>
<td>V_(10) (µL)</td>
<td>36.6 ± 9.6</td>
<td>31.0 ± 4.1</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\( E_{es} \), end-systolic elastance; \( V_0 \), volume axis intercept; CO, cardiac output; SV, stroke volume; PA\_(max), maximal pressure amplitude; EF, ejection fraction; V\_(10), end-diastolic volume at a preload of 10 mmHg.
structure, the myocardial contractile apparatus, and Ca^{2+} handling proteins.

**Db/db mice show typical features of heart failure with preserved ejection fraction**

Db/db mice showed signs of metabolic syndrome associated with increased body weight, blood glucose, and insulin serum concentrations. This was associated with an elevated heart rate, disturbed ventricular-arterial coupling, and diastolic dysfunction. Blood pressure and MRI measurements in vivo indicated increased afterload. Db/db mice showed increased vascular stiffness demonstrated by reduced aortic distensibility in MRI studies in the presence of widened blood pressure amplitude compared with controls. The latter was caused by a lack of aortic elastic recoil in diastole. Interestingly, systolic hypertension was not present in db/db mice aged 7–11 weeks. This constellation could contribute to an increased afterload primarily due to enhanced pulsatile load. Patients with HFPEF, however, are characterized by both, increased systolic blood pressure and decreased aortic distensibility. Our results may therefore indicate that increased pulsatile load is the main trigger for increased systolic stiffness and subsequent disturbed ventricular-arterial coupling as suggested by another study. This study demonstrated a strong inverse correlation between vascular compliance and systolic stiffness ($E_{es}$).

**Figure 3** (A) Original pressure–volume-loops and end-systolic pressure volume relationship measurements in 11-week-old controls, db/db, and db/db-Iva mice. (B) End-systolic elastance, (C) $dP/dt_{max}$, (D) $P_{A_{max}}$, (E) end-diastolic volume ($V_{10}$) in controls, db/db, and db/db-Iva mice at week 11. (F) Representative transmitral flow profile in controls, db/db, and db/db-Iva mice at week 11. *$P < 0.017$. J-C. Reil et al. 2844
Heart rate reduction in mice with HFPEF

Table 3  Basic characteristics, systolic, and diastolic haemodynamic parameters at an age of 11 weeks

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>db/db</th>
<th>P value control vs. db/db</th>
<th>db/db vs. db/db-Iva</th>
<th>P value control vs. db/db-Iva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>&lt;0.01</td>
<td>24.8 ± 1.6</td>
<td>&lt;0.01 41.2 ± 3.8</td>
<td>0.03 37.9 ± 4.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>0.11</td>
<td>155.3 ± 13.4</td>
<td>146.8 ± 10.5</td>
<td>138.7 ± 16.5</td>
<td></td>
</tr>
<tr>
<td>Tibia length (cm)</td>
<td>0.72</td>
<td>2.0 ± 0.06</td>
<td>2.0 ± 0.07</td>
<td>2.0 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Heart weight/tibia length</td>
<td>0.24</td>
<td>77.8 ± 7.5</td>
<td>73.8 ± 5.4</td>
<td>70.1 ± 9.6</td>
<td></td>
</tr>
<tr>
<td>Total collagen content (LV) (%)</td>
<td>0.16</td>
<td>2.1 ± 0.7</td>
<td>1.4 ± 0.6</td>
<td>1.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Myocyte diameter (µm)</td>
<td>0.52</td>
<td>11.9 ± 1.0</td>
<td>12.7 ± 1.5</td>
<td>12.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Amount of capillaries/mm²</td>
<td>&lt;0.01</td>
<td>22078 ± 2645</td>
<td>&lt;0.01 16877 ± 2176</td>
<td>0.64 18126 ± 2224</td>
<td>0.02</td>
</tr>
<tr>
<td>Cystatin C (mg/mL)</td>
<td>0.43</td>
<td>0.13 ± 0.05</td>
<td>0.09 ± 0.03</td>
<td>0.12 ± 0.04</td>
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</table>

Haemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>db/db</th>
<th>P value control vs. db/db</th>
<th>db/db vs. db/db-Iva</th>
<th>P value control vs. db/db-Iva</th>
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<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eₚ (mmHg/mL)</td>
<td>&lt;0.01</td>
<td>3.4 ± 1.2</td>
<td>&lt;0.01 6.0 ± 1.3</td>
<td>&lt;0.01 4.0 ± 1.1</td>
<td>0.27</td>
</tr>
<tr>
<td>V₀ (µL)</td>
<td>0.35</td>
<td>-10.2 ± 31</td>
<td>-36.5 ± 36.1</td>
<td>-17.3 ± 36.1</td>
<td></td>
</tr>
<tr>
<td>dP/dtₘₚₚ (mmHg/s)</td>
<td>&lt;0.01</td>
<td>5798 ± 965</td>
<td>&lt;0.017 4848 ± 481</td>
<td>&lt;0.01 6000 ± 529</td>
<td>0.97</td>
</tr>
<tr>
<td>PAₘₚₚ (mmHg)</td>
<td>&lt;0.01</td>
<td>52 ± 14</td>
<td>&lt;0.01 25 ± 16</td>
<td>&lt;0.01 42 ± 9</td>
<td>0.13</td>
</tr>
<tr>
<td>EF (%)</td>
<td>0.17</td>
<td>57 ± 7</td>
<td>54 ± 11</td>
<td>61 ± 5</td>
<td></td>
</tr>
<tr>
<td>Stroke volume (µL)</td>
<td>&lt;0.01</td>
<td>26 ± 5</td>
<td>15 ± 3</td>
<td>21 ± 2</td>
<td>0.03</td>
</tr>
<tr>
<td>Stroke work (mmHg µL)</td>
<td>&lt;0.01</td>
<td>1998 ± 513</td>
<td>&lt;0.017 1167 ± 276</td>
<td>&lt;0.017 1516 ± 233</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Diastolic parameters

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>db/db</th>
<th>P value control vs. db/db</th>
<th>db/db vs. db/db-Iva</th>
<th>P value control vs. db/db-Iva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V₁₀ (µL) at preload 10 mmHg</td>
<td>&lt;0.01</td>
<td>48 ± 8</td>
<td>&lt;0.01 28 ± 3</td>
<td>&lt;0.01 37 ± 4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Relaxation constant τ (ms)</td>
<td>&lt;0.01</td>
<td>9.9 ± 1.3</td>
<td>&lt;0.01 14.3 ± 4.1</td>
<td>0.04 11.6 ± 1.3</td>
<td>0.04</td>
</tr>
<tr>
<td>dP/dtₘₚₚ (mmHg/s)</td>
<td>&lt;0.01</td>
<td>-3900 ± 681</td>
<td>&lt;0.01 2984 ± 517</td>
<td>0.27 -3231 ± 391</td>
<td>0.03</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>&lt;0.01</td>
<td>1.2 ± 0.3</td>
<td>&lt;0.017 2.1 ± 0.6</td>
<td>&lt;0.017 1.4 ± 0.4</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Db/db mice showed significantly elevated values of Eₚ as a consequence of chronically elevated afterload that indicates ‘abnormal’ ventricular-arterial coupling. End-systolic elastance and vascular stiffening strongly correlate with each other. End-systolic elastance comprises active (‘contractility’) and passive LV-constitutive properties (‘chamber stiffness’), but nevertheless does not indicate the relative contribution of one of these. Increased Eₚ is generally assumed to primarily demonstrate ‘contractility’ but hypertrophy and fibrosis per se can also increase Eₚ, indicating major increase in LV stiffness. Here, we evaluated the interrelation of both components of Eₚ. This approach became feasible by using dP/dtₘₚₚ and PAₘₚₚ as two additional inotropic parameters. Db/db mice showed elevated Eₚ values compared with controls, whereas dP/dtₘₚₚ and PAₘₚₚ were significantly reduced. The divergence of Eₚ and both other contractile parameters demonstrated compromised LV contractility with increased chamber stiffness as a composed cause of increased Eₚ. Differences in Eₚ values were measured at identical afterload conditions in the working heart apparatus. Thus, increased Eₚ of db/db mice persisted in vitro being probably induced by chronically increased afterload in vivo. As myocardial fibrosis and hypertrophy were not detected in db/db mice, ‘fixation’ of Eₚ has to be explained by other structural myocardial abnormalities (see below).

Diastolic function was also compromised in db/db mice aged 11 weeks as indicated by prolonged relaxation and a leftward shift in the PV-diagram in db/db mice. Other haemodynamic changes were derived from LV cavity reduction, because the smaller LV cavity induced a fall of LV stroke volume and cardiac output. In the presence of a stiff aorta, however, systolic blood pressure remained unaltered while pulse pressure rose. Additionally, transmitral flow demonstrated high E/A ratios showing a restrictive flow pattern in db/db mice. Diastolic filling mainly occurred in early diastole. Therefore, the significant increase in HR of db/db mice during the observation period may be interpreted as a compensatory mechanism to maintain cardiac output by increasing the number of cardiac filling cycles.
Selective heart rate reduction improves vascular and myocardial function in the heart failure with preserved ejection fraction model

Ea is proportional to the product of total peripheral resistance (TPR) and HR (Ea ≈ TPR × HR), explaining its HR dependency. In clinical and experimental studies, lowering HR was shown to improve ventricular-arterial coupling, in part by decreasing Ea.\textsuperscript{11,25} In our study, Iva treatment improved vascular compliance in \( \text{db/db} \) mice as indicated by increased aortic distensibility and decreased pulse pressure amplitudes. These changes reduced the total afterload in \( \text{db/db-Iva} \) mice.

Afterload reduction by Iva resulted in significantly lower \( E_{\text{es}} \) values compared with \( \text{db/db} \) mice whereas \( \text{dP/dt}_{\text{max}} \) and \( \text{PA}_{\text{max}} \) values remained significantly higher. This pattern reflects higher

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**Figure 4** Photomicrographs from histological sections of the ascending aorta of a control, a \( \text{db/db} \), and a \( \text{db/db-Iva} \) mouse (A) Sirius Red was used to stain collagen. (B) Diaminoflavorensein diacetate represents NO content. (C) Dihydroethidium revealed hydroxide ions as a measure of oxidative stress. (D) Collagen fraction, (E) NO content in controls, and (F) oxidative stress, \( \text{db/db} \), and \( \text{db/db-Iva} \) mice at week 11. *\( P < 0.017 \).
LV contractility with lower systolic chamber stiffness compared with \( \text{db/db} \) mice. In particular, it was demonstrated that an increase in contractility is not necessarily linked with an increase in \( E_{\text{es}} \). Thus, increased LV systolic stiffness can be identified by a change of \( E_{\text{es}} \) and other contractile parameters \( (dP/dt_{\text{max}}, PA_{\text{max}}) \) towards opposite directions. These results are in accordance with echocardiographic measurements of patients with HFPEF.\(^{18}\)

If ischaemia or myocardial infarction would have been present in our diabetes model a pattern of impaired contractility would have been identified in \( \text{db/db} \) mice by a common reduction of \( E_{\text{es}}, E_{\text{F}}, \) and \( dP/dt_{\text{max}} \) values as well as a rightward shift in the PV-diagram.\(^{26}\) That was actually not the case. Strikingly, after Iva treatment \( E_{\text{es}} \) decreased due to improved vascular compliance despite an increased \( dP/dt_{\text{max}} \). Therefore, the changing slope of \( E_{\text{es}} \) considered with alterations in \( dP/dt_{\text{max}} \) can distinguish between haemodynamic patterns of changed LV systolic stiffness and contractility.

Diastolic dysfunction of \( \text{db/db} \) was attenuated in \( \text{db/db-Iva} \) mice showing faster relaxation and an increase in LV capacitance and stroke volume indicated by a rightward shift in the PV-diagram compared with \( \text{db/db} \) mice. Nevertheless, both parameters did not reach control values. Since LV systolic and diastolic stiffness also affect each other,\(^{8,20}\) reduction of systolic stiffness is accompanied by a decrease in diastolic stiffness\(^{20}\) with a subsequent increase in diastolic filling resulting in a higher stroke volume. Of note, in a rat model of transaortic constriction with LV hypertrophy and signs of HFPEF, the impact of HR reduction on vascular function, and ventricular-arterial interaction was emphasized by the failure of Iva to improve ventricular systolic and diastolic function. In this model afterload was ‘mechanically fixed’ and therefore could not be modified by HR reduction.\(^{27}\)

**Biochemical and histological findings in \( \text{db/db} \) mice**

Ivabradine did not alter metabolism in diabetic mice. The ascending aorta of \( \text{db/db} \) mice showed increased fibrosis and enhanced oxidative stress resulting in an increased aortic stiffness. Heart rate reduction by Iva reduced collagen content and oxidative stress to the level of controls thereby improving vascular compliance and aortic recoil. In contrast, at an age of 11 weeks, \( \text{db/db} \) mice showed neither myocardial fibrosis nor ventricular hypertrophy. Accordingly, expression of profibrotic CTGF mRNA in control, \( \text{db/db} \), and \( \text{db/db-Iva} \) mice was comparable. These results are in line with previously described observations in \( \text{db/db} \) mice.\(^{28}\) Nevertheless, these findings are remarkable because LV hypertrophy and fibrosis had been shown to be morphological characteristics associated with diastolic dysfunction and increased systolic stiffness.\(^{12,20,24,29}\) Interestingly, rarification of capillaries was observed and appeared to be the only patho-histological substrate in the left ventricles of \( \text{db/db} \) mice compared with controls. Reduced capillary density is a core feature of maladaptive remodelling.

**Figure 5** (A) Photomicrographs from histological sections of left ventricle of a control, a \( \text{db/db} \) and a \( \text{db/db-Iva} \) mouse. Sirius Red was used to stain collagen. (B) Expression of connective tissue growth factor mRNA normalized to GAPDH expression. (C) mRNA expression of titin transcription variant N2BA and N2B normalized to GAPDH. (D) Representative sodium dodecyl sulfate-gel of titin protein and cumulative analysis and titin-isoforms N2BA/N2B ratio, obtained from left ventricular tissue samples in control, \( \text{db/db} \), and \( \text{db/db-Iva} \) mice. *\( P < 0.017. \)**
contributing to LV systolic and diastolic dysfunction\textsuperscript{30} that tended to be improved by HR reduction with Iva.

Ventricular passive distensibility mainly depends on the intracellular protein titin, whereas extracellular matrix plays a minor role.\textsuperscript{31} Titin, spanning the whole sarcomere, acts as a bidirectional spring responsible for early diastolic recoil and late diastolic resistance to stretch. Two titin-isoforms exist, the more compliant N2BA and the stiffer isoform N2B.\textsuperscript{32} \textit{Db/db} mice showed a significant increase in mRNA and protein expression of N2B. This may at least partially explain impaired LV compliance and relaxation.\textsuperscript{32} Heart rate reduction by Iva treatment, however, reduced expression of the titin N2B isoform and was associated with beneficial effects on systolic and diastolic myocardial function.

Interestingly, a recent clinical study demonstrated that patients with HFPEF have higher ratios of the myocardial N2B/N2BA titin-isoform compared with patients with systolic heart failure and healthy controls.\textsuperscript{29} Since LV systolic and diastolic stiffness strongly correlate with each other\textsuperscript{8,20} one may speculate that titin-isoform switching may also contribute to increased systolic stiffness in our model: Titin is only relaxed in its resting position, so that mechanical force is needed to expand this spring during diastole or to compress it in systole.\textsuperscript{33} The stiffer isoform may develop abnormally increased resistance thereby impairing the flexibility of the spring in both directions and contributing to increased systolic stiffness in \textit{db/db} mice, as well. The same mechanism may also account for the observed ‘fixation’ of \(E_s\) in \textit{db/db} mice under in vitro conditions. However, this hypothesis has not been proven yet and has to be confirmed by further studies.

Besides, impaired relaxation could also be explained by an impaired calcium handling in \textit{db/db} mice. Our data revealed a significant reduction of threonine 17 phosphorylation of phospholamban in \textit{db/db} mice, resulting in Serca2a inhibition.\textsuperscript{34} Ivabradine treatment significantly improved phosphorylation of threonine 17 in \textit{db/db} mice potentially accelerating myocardial relaxation.

**Beta-blockers vs. ivabradine in heart failure with preserved ejection fraction**

It was recently shown, that HR reduction in HFPEF with the beta-blocker nebivolol failed to increase patients’ exercise tolerance (ELANDD-trial).\textsuperscript{35} The success of beta-blockers and Iva to achieve beneficial outcomes in patients may probably depend on high-baseline heart rates (>80 b.p.m.) and the extent of HR reduction (10–15 b.p.m.).\textsuperscript{9} Probably, baseline HR of the ELANDD trial was too low (about 73 b.p.m.) and the amount of HR reduction too small [about 6 b.p.m. (8%) from baseline] to influence HFPEF. Interestingly, an older study comparing the effect of atenolol and nebivolol in patients with diastolic dysfunction was successful,\textsuperscript{36} when baseline HR was reduced by 17 b.p.m. (20%, atenolol) and 11 b.p.m. (14.5%, nebivolol). In the presented study HR of \textit{db/db} mice was decreased by nearly 100 b.p.m. (about 19% of baseline value).

**Conclusions**

Diabetic \textit{db/db} mice showed abnormal ventricular-arterial coupling due to decreased vascular compliance, reactively elevated \(E_s\) and disturbed diastolic dysfunction. Thus, \textit{db/db} mice can be regarded as a model of HFPEF. Selective HR reduction by Iva decreased vascular and LV systolic stiffness thereby significantly improving LV contractility and diastolic function in this model. It is speculated that LV systolic and diastolic stiffness are induced by a common subcellular mechanism involving a higher titin-isoform N2B expression while myocardial hypertrophy and fibrosis were missing.
Finally, our results suggest that selective HR reduction might be a therapeutic step for disturbed ventricular-arterial coupling and diastolic dysfunction, particularly in HFPEF patients. However, this concept has to be confirmed in human disease.

Supplementary material
Supplementary material is available at European Heart Journal online.

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References