Diabetes Mellitus Worsens Diastolic Left Ventricular Dysfunction in Aortic Stenosis Through Altered Myocardial Structure and Cardiomyocyte Stiffness

Inês Falcão-Pires, PhD; Nazha Hamdani, PhD; Attila Borbély, MD, PhD; Cristina Gavina, MD; Casper G. Schalkwijk, PhD; Jolanda van der Velden, PhD; Loek van Heerebeek, MD; Ger J.M. Stienen, PhD; Hans W.M. Niessen, MD, PhD; Adelino F. Leite-Moreira, MD, PhD; Walter J. Paulus, MD, PhD

Background—Aortic stenosis (AS) and diabetes mellitus (DM) are frequent comorbidities in aging populations. In heart failure, DM worsens diastolic left ventricular (LV) dysfunction, thereby adversely affecting symptoms and prognosis. Effects of DM on diastolic LV function were therefore assessed in aortic stenosis, and underlying myocardial mechanisms were identified.

Methods and Results—Patients referred for aortic valve replacement were subdivided into patients with AS and no DM (AS; n = 46) and patients with AS and DM (AS-DM; n = 16). Preoperative Doppler echocardiography and hemodynamics were implemented with perioperative LV biopsies. Histomorphometry and immunohistochemistry quantified myocardial collagen volume fraction and myocardial advanced glycation end product deposition. Isolated cardiomyocytes were stretched to 2.2-µm sarcomere length to measure resting tension (F_{passive}). Expression and phosphorylation of titin isoforms were analyzed with gel electrophoresis with ProQ Diamond and SYPRO Ruby stains. Reduced LV end-diastolic distensibility in AS-DM was evident from higher LV end-diastolic pressure (21 ± 1 mm Hg for AS versus 28 ± 4 mm Hg for AS-DM; P = 0.04) at comparable LV end-diastolic volume index and attributed to higher myocardial collagen volume fraction (AS, 12.9 ± 1.1% versus AS-DM, 18.2 ± 2.6%; P < 0.001), more advanced glycation end product deposition in arterioles, venules, and capillaries (AS, 14.4 ± 2.1 score per 1 mm² versus AS-DM, 31.4 ± 6.1 score per 1 mm²; P = 0.03), and higher F_{passive} (AS, 3.5 ± 1.7 kN/m² versus AS-DM, 5.1 ± 0.7 kN/m²; P = 0.04). Significant hypophosphorylation of the stiff N2B titin isoform in AS-DM explained the higher F_{passive} and normalization of F_{passive} after in vitro treatment with protein kinase A.

Conclusions—Worse diastolic LV dysfunction in AS-DM predisposes to heart failure and results from more myocardial fibrosis, more intramyocardial vascular advanced glycation end product deposition, and higher cardiomyocyte F_{passive}, which was related to hypophosphorylation of the N2B titin isoform. (Circulation. 2011;124:00-00.)

Key Words: aortic valve stenosis ■ myocytes, cardiac ■ diabetes mellitus ■ diastole ■ fibrosis ■ titin ■ myofilamentary proteins

Diabetes mellitus (DM)-induced diastolic left ventricular (LV) dysfunction is increasingly recognized as an important determinant of morbidity and mortality in heart failure. In patients with DM, high diastolic LV stiffness hinders LV remodeling after myocardial infarction2-2 and raises LV filling pressures at similar LV filling volumes in both heart failure (HF) with reduced LV ejection fraction (HFREF) and HF with normal LV ejection fraction (HFNEF).3 As a consequence, patients with DM have a higher incidence of HF after myocardial infarction1,2,4,5 and a poorer prognosis on either HFREF or HFNEF development.6-8 Mechanisms responsible for raising myocardial stiffness in DM consist of excessive fibrosis,9 deposition of advanced glycation end products (AGEs),10 and high cardiomyocyte stiffness, evident from an elevated in vitro cardiomyocyte resting tension (F_{passive}).3 Their relative contributions to myocardial stiffness differ in HFREF and HFNEF; fibrosis and AGEs are more important in HFREF and high F_{passive} is more prominent in HFNEF.3 Like macrovascular complications,11,12 DM-related diastolic LV dysfunction failed to improve during intensified glycemic control,13 and because of this “hyperglyc-
mic memory,3 involvement of epigenetic processes was recently suspected.14,15

Clinical Perspective on p 666

In aortic stenosis (AS), studies on interactions with DM focused mainly on the progression of sclerocalcific aortic valve disease, which appeared to be accelerated by the presence of DM.16–20 Recently, experimental studies have started to address the combined effect of DM and pressure overload on diastolic LV function. In insulin-resistant mice, transverse aortic constriction resulted in more diastolic LV dysfunction and less survival.21 Suprarenal aortic banding induced similar results in diabetic rats with slowing of LV relaxation and blunting of the LV lusitropic response to acute β-adrenergic stimulation.22 The clinical relevance of these experimental findings has so far been confirmed in the metabolic syndrome but not in DM. When asymptomatic mild AS was associated with the metabolic syndrome, diastolic LV dysfunction was more pronounced.23 The present study extends these observations and investigates whether there is more diastolic LV dysfunction when symptomatic AS is associated with DM. Furthermore, by using perioperative LV myocardial biopsies, the present study explores the myocardial mechanisms responsible for the more severe diastolic LV dysfunction that occurs when AS and DM are comorbidities. Here, we compare fibrosis, AGE deposition, cardiomyocyte F\textsubscript{passive}, titin isoform composition, and titin isoform phosphorylation in LV myocardium of AS patients in the presence or absence of DM.

Methods

Patients

The study population consisted of 62 patients with symptomatic AS referred for surgical valve replacement and operated on between January 2006 and December 2008. Symptoms were dyspnea (n = 30), angina (n = 15), and syncope (n = 17). Patients with atrial fibrillation, significant valvular lesions other than AS, or significant coronary stenosis (>50%) were excluded. In 16 patients, AS was associated with DM (AS-DM). A patient had DM if he or she used a glucose-lowering medication and/or insulin or had a fasting plasma glucose ≥7.0 mmol/L.24 No patient was using thiazolidinediones. Perioperative LV myocardial biopsies were procured during aortic valve replacement. In all patients, LV biopsy material consisted of endomyocardial tissue resected from the LV outflow tract (Morrow procedure) because of concomitant LV outflow tract narrowing. In each patient, biopsy material was split into 5 to 6 samples (±5 mg each); 3 were formalin-fixed and 2 to 3 were snap-frozen in liquid nitrogen. The formalin-fixed biopsies were used for quantitative histomorphometry; the snap-frozen biopsies were used for cardiomyocyte force measurements and myofilamentary proteomics. The local ethics committee approved the study protocol, and written informed consent was obtained from all patients.

The control group consisted of 11 patients and was composed of 2 subgroups: 3 patients with normal LV function and major ventricular arrhythmias in whom transvascular LV biopsies were procured and 8 explanted donor hearts. Histomorphometric and cardiomyocyte functional data observed in the AS and AS-DM patients were also compared with similar data derived from previously reported HFREF and HFNEF patients.8 The HFREF patient population (LV ejection fraction, 60%–70%) consisted of 28 patients hospitalized for worsening HF and without significant coronary artery disease or active lymphocytic infiltration in the LV endomyocardial biopsy. The HFNEF patient population (LV ejection fraction, 29%–32%) consisted of 28 patients hospitalized for worsening HF, no signifi-
cant coronary artery disease, and no amyloid deposits in the LV endomyocardial biopsy.

Quantitative Histomorphometry

Light Microscopy

Light microscopic quantification of cardiomyocyte dimensions and collagen volume fraction (CVF) has previously been described and validated.25,26 The histomorphometric analysis of the biopsy samples was performed on Elastica van Gieson-, hematoxylin and eosin-, and Picrosirius Red–stained 4-μm-thick sections of tissue (≥5 sections of each sample). Images of these sections were acquired with a projection microscope (×50). Subsequent image analysis with Slidebook 4.0 software (3I, Denver, CO) was performed to determine cardiomyocyte diameter (MyD; μm) and extent of reactive interstitial fibrosis, which was expressed as CVF (%). Areas of reparative and perivascular fibrosis were excluded. We determined MyD perpendicular to the outer contour of the cell membrane at the nucleus level in 15 representative myocytes of the section in 24 AS and 9 AS-DM patients. We calculated CVF as the sum of all connective tissue areas divided by the sum of connective tissue and muscle areas averaged over 4 to 6 representative fields of the section in 23 AS and 9 AS-DM patients. In our laboratory, normal values of MyD and CVF for LV endomyocardial biopsy material are 13.1±0.3 μm and 5.4±2.2%, respectively.

Immunohistochemistry

Deposition of AGEs was inferred from measurement of the AGE N\textsuperscript{-carboxymethyl}lysine (CML) in 7 AS and 7 AS-DM patients. In each patient, 4 to 6 representative fields in 5 tissue sections were analyzed. Development of the anti-CML monoclonal antibody used and the immunohistochemical staining technique have previously been described.27,28 An immunohistochemical AGE score per square millimeter is reported and was derived as follows: Each positively stained vessel was given an intensity grade (1 = weak staining, 2 = moderate staining, 3 = intense staining), and the sum of all positively stained vessels multiplied by their intensity grade was subsequently divided by the slide area to yield an AGE score per square millimeter.

Force Measurements in Isolated Cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously.29,30 Biopsy samples (5- to 10-g wet weight) of 25 AS and 14 AS-DM patients were defrosted in relaxing solution, mechanically disrupted, and incubated for 5 minutes in relaxing solution supplemented with 0.2% Triton X-100 to remove all membrane structures. Single cardiac myocytes were subsequently attached with silicone adhesive between a force transducer and a piezoelectric motor (3.1±0.3 cardiomyocytes per patient). F\textsubscript{passive} was measured at sarcomere lengths ranging from 1.6 to 2.2 μm. To assess the reversibility of elevated F\textsubscript{passive}, myocytes were also incubated in relaxing solution supplemented with the catalytic subunit of protein kinase A (PKA; 100 U/mL; Sigma; batch-12K7495) and 6 mmol/L dithiothreitol (MP-Biochemicals). After 40 minutes of incubation with PKA, F\textsubscript{passive} measurements were repeated. Force values were normalized for myocyte cross-sectional area. In our laboratory, F\textsubscript{passive} of normal human cardiomyocytes is 3.5±0.4 kN/m².25

Myofilamentary Protein and Titin Isoform Phosphorylation

Phosphorylation of troponin I, troponin T, myosin light chain-2, desmin, and myosin binding protein-C was assessed as previously described29 in 8 AS and 6 AS-DM patients. Myocardial tissue was washed 3 times in acetone and dissolved in 1D sample buffer (62.5 mmol/L TRIS [pH 6.8], 15% glycerol, 1% SDS, and 1.5% hydroxyethyl disulfide) with 100 mmol/L dithiothreitol, heated (5 minutes at 80°C), and centrifuged (20 minutes at 12 000g and 20°C), and a sample (∼30 μg dry weight in 12 μL) was applied on a 4% to 15% gradient gel (Criterion, BioRad). The gel was run at 100 V for 30 minutes followed by 200 V for 50 minutes and was stained for 1
hour with ProQ Diamond (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Staining was analyzed with a LAS-3000 system (Fuji Science Imaging Systems) and AIDA Image analyzer software (Isotopenmellegeräte GmbH, Staufenhardt, Germany). Subsequently, the gel was washed and stained overnight with Sypro Ruby (Molecular Probes) according to the manufacturer’s instructions and finally analyzed.

For titin isoform phosphorylation, myocardial tissue samples from 8 AS and 6 AS-DM patients were homogenized in 50 to 100 μL Tris-SDS buffer (pH 6.8) containing 8 μg/ml leupeptin (Peptin Institute, Japan). Protein samples (~30 μg dry weight in 6 μL) were applied on the wells, and titin isoforms were separated on agarose-strengthened 2% SDS–polyacrylamide gels and stained for 1 hour with ProQ Diamond and Sypro Ruby as described above.

Data Analysis

Echocardiographic Data

We derived LV end-systolic volume, LV end-diastolic volume, LV posterior wall thickness, and interventricular septal thickness from 2-dimensional echocardiograms, and we calculated LV mass index (LVMI) in accordance with the recent recommendations for cardiac chamber quantification.\(^{36}\) In our laboratory, the normal values for LVMI and the ratio of LVMI to LV end-diastolic volume index are 92±3 g/m² and 1.27±0.04, respectively.\(^{36}\) Peak aortic valve velocity, mean aortic transvalvular pressure gradient, and aortic valve area index were derived from Doppler echocardiographic examination of the aortic valve. Mean aortic transvalvular pressure gradient was obtained with the modified Bernoulli echocardiographic equation: $\text{P} = 4\text{V}^2$.

Statistics

Values are given as mean±SEM. Single comparisons between AS and AS-DM were assessed by an unpaired Student t test. Significance for categorical variables was determined by the Fisher exact test. Effects of PKA and sarcomere length in AS and AS-DM were analyzed by 2-factor repeated-measures ANOVA. Bonferroni-corrected t tests served as subsequent multicomparison tests. Rela-

tions between 2 continuous variables were assessed with linear regression analysis. To assess the relation between LVEDP and CVF, a multiple regression analysis that included both AS status (AS versus AS-DM) and CVF was used. Statistical analysis was performed with SPSS (version 9.0).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Left Ventricular Diastolic Dysfunction in Aortic Stenosis and Aortic Stenosis With Diabetes Mellitus

The Table compares clinical and hemodynamic characteristics of AS and AS-DM patients. As evident from the aortic valve area index and mean aortic transvalvular pressure gradient, the severity of AS was comparable in AS and AS-DM patients. Concentric LV hypertrophy was present in both groups as evident from an LVMI that was significantly larger than normal ($P<0.001$ for both AS and AS-DM) and from a ratio of LVMI to LV end-diastolic volume index that also was significantly larger than normal ($P<0.001$ for both AS and AS-DM). The extent of concentric LV hypertrophy was comparable in AS and AS-DM. Left ventricular volumes and LV ejection fraction were also similar in AS and AS-DM, but end-diastolic LV distensibility was lower in AS-DM as evident from a higher LVEDP ($P=0.04$) at a comparable LV end-diastolic volume index (Figure 1).

Table. Clinical and Hemodynamic Characteristics of Aortic Stenosis and Aortic Stenosis–Diabetes Mellitus Patients

<table>
<thead>
<tr>
<th></th>
<th>AS (n=46)</th>
<th>AS-DM (n=16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64.8±2.9</td>
<td>66.9±2.6</td>
<td>0.58</td>
</tr>
<tr>
<td>% by Male, n</td>
<td>23/46</td>
<td>6/16</td>
<td>0.56</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.7±0.7</td>
<td>28.5±0.9</td>
<td>0.57</td>
</tr>
<tr>
<td>Obesity, n</td>
<td>11/46</td>
<td>2/16</td>
<td>0.48</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>23/46</td>
<td>10/16</td>
<td>0.56</td>
</tr>
<tr>
<td>History of smoking, n</td>
<td>6/46</td>
<td>1/16</td>
<td>0.67</td>
</tr>
<tr>
<td>Medications, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEIs</td>
<td>15/46</td>
<td>8/16</td>
<td>0.24</td>
</tr>
<tr>
<td>β-blockers</td>
<td>25/46</td>
<td>9/16</td>
<td>0.99</td>
</tr>
<tr>
<td>Diuretics</td>
<td>19/46</td>
<td>11/16</td>
<td>0.08</td>
</tr>
<tr>
<td>ARBs</td>
<td>1/46</td>
<td>1/16</td>
<td>0.45</td>
</tr>
<tr>
<td>Digoxin</td>
<td>1/46</td>
<td>0/16</td>
<td>0.99</td>
</tr>
<tr>
<td>Statins</td>
<td>18/46</td>
<td>8/16</td>
<td>0.56</td>
</tr>
<tr>
<td>Insulin</td>
<td>0/46</td>
<td>3/16</td>
<td>0.015</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>74±2</td>
<td>74±4</td>
<td>0.96</td>
</tr>
<tr>
<td>LVPS, mm Hg</td>
<td>233±5</td>
<td>217±6</td>
<td>0.24</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>21.4±1.4</td>
<td>28.2±3.7</td>
<td>0.040</td>
</tr>
<tr>
<td>CI, L/min−1</td>
<td>2.31±0.11</td>
<td>2.08±0.20</td>
<td>0.39</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>64.6±1.5</td>
<td>62.1±2.1</td>
<td>0.35</td>
</tr>
<tr>
<td>LVSP, mm</td>
<td>11.1±0.3</td>
<td>11.0±0.4</td>
<td>0.29</td>
</tr>
<tr>
<td>LVWT, mm</td>
<td>13.38±0.3</td>
<td>13.60±0.5</td>
<td>0.82</td>
</tr>
<tr>
<td>Peak aortic valve velocity, m/s</td>
<td>4.65±0.17</td>
<td>4.29±0.21</td>
<td>0.26</td>
</tr>
<tr>
<td>Mean aortic transvalvular pressure gradient, mm Hg</td>
<td>60.1±2.6</td>
<td>57.6±1.9</td>
<td>0.56</td>
</tr>
<tr>
<td>AVA, cm²/m²</td>
<td>0.53±0.04</td>
<td>0.58±0.04</td>
<td>0.45</td>
</tr>
<tr>
<td>LVMI, g/m²</td>
<td>132±5</td>
<td>137±15</td>
<td>0.66</td>
</tr>
<tr>
<td>LVMI/LVEDV ratio</td>
<td>2.68±0.20</td>
<td>2.12±0.23</td>
<td>0.17</td>
</tr>
</tbody>
</table>

AS indicates aortic stenosis; DM, diabetes mellitus; ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers; LVPS, left ventricular (LV) peak-systolic pressure; LVEDP, LV end-diastolic pressure; CI, cardiac index; LVEF, LV end-systolic pressure; LVEF, LV end-diastolic pressure; LVSP, LV end-diastolic volume index; LVEF, LV ejection fraction; LVWT, LV posterior wall thickness; LVWT, interventricular-septum thickness; AVA, aortic valve area index; and LVMI, LV mass index. Values are given as mean±SEM when appropriate.

Myocardial Fibrosis and Advanced Glycation End Product Deposition

In Picrosirius Red–stained sections, CVF rose from 10.2±1.2% in AS (n=23) to 19.1±6.3% in AS-DM (n=9; P=0.013; Figure 2A). In hematoxylin and eosin–stained sections, CVF rose similarly from 12.9±1.1% in AS to 18.2±2.6% in AS-DM (P<0.001; Figure 2B). When AS and AS-DM data were pooled, CVF correlated with LVEDP (r=0.60, P<0.001; Figure 2C). In addition, CVF correlated with LVEDP (P=0.005) in a multiple regression analysis that included both AS status (AS versus AS-DM) and CVF. The CVF in AS-DM tended to be lower than the previously reported CVF in HFREF patients with DM (HFREF-DM) (22.4±2.2%; P=0.11).\(^{3}\) Deposition of AGEs

Downloaded from http://circ.ahajournals.org/ by guest on April 15, 2017
was inferred from CML immunostaining and occurred in endothelial and smooth muscle cells of intramyocardial arterioles, venules, and capillaries (Figure 3A and 3B). Deposition of AGEs was significantly higher in AS-DM (n=7) than in AS (n=7) patients (31.4±6.1 versus 14.4±2.1 score per 1 mm²; P=0.03; Figure 3C).

**Cardiomyocyte Dimensions and Cardiomyocyte Fpassive**

The MyD rose progressively from 13.1±0.3 μm in control myocardium to 22.9±0.3 μm in AS and to 26.4±0.3 μm in AS-DM (P<0.01 for trend). The MyD was higher in AS-DM (n=9) than in AS (n=24; P<0.001; Figure 4A) patients. The MyD in AS and AS-DM was larger than the MyD previously reported in HFNEF patients (19.8±1.7 μm; P<0.001) and HFNEF patients with DM (HFNEF-DM; 22.4±0.9 μm; P<0.001), respectively.3 Fpassive of isolated cardiomyocytes (Figure 4B) was comparable in AS (n=25) (3.7±0.4 kN/m²) and control (3.5±1.7 kN/m²) myocardium but higher in AS-DM (n=14; 5.1±0.7 kN/m²) than in AS (P=0.04; Figure 4C) or control (P=0.04). Fpassive in AS and AS-DM was lower than Fpassive previously reported respectively in HFNEF (5.1±0.7 kN/m²; P<0.001) and in HFNEF-DM (8.5±0.9 kN/m²; P=0.007).1 The passive length tension of individual cardiomyocytes was constructed by measuring Fpassive at various sarcomere lengths ranging from 1.6 to 2.2 μm (Figure 4D). Compared with AS, the passive length-tension relation of AS-DM was shifted upward over the entire range of sarcomere lengths.

**Titin Isoform Expression and Titin Isoform Phosphorylation**

There was no significant difference in the titin isoform ratio (N2BA/N2B) among control (0.39±0.05; n=8),29 AS (0.61±0.07; n=8), and AS-DM (0.50±0.07; n=6). Relative phosphorylation (P) of N2B and N2BA titin isoforms (Figure 5A) differed, however, as evident from the P-N2BA/P-N2B ratio, which was significantly higher in AS-DM compared with control (P<0.001; Figure 5B). The higher P-N2BA/P-N2B ratio resulted from both significant hypophosphorylation of the stiff N2B isoform (P=0.006) and hyperphosphorylation of the compliant N2BA isoform (P=0.005). Hypophosphorylation of the stiff N2B isoform was in agreement with the higher Fpassive in AS-DM because phosphorylation of titin lowers cardiomyocyte Fpassive in an isoform-specific way.3 In AS-DM, the P-N2BA/P-N2B ratio was also correlated with LVEDP (r=0.995, P=0.005; Figure 5C). Involvement of a titin phosphorylation deficit in the high Fpassive of AS-DM cardiomyocytes was further supported by administration of PKA to the isolated cardiomyocytes (Figure 4C), which reduced Fpassive to similarly low values in AS (1.9±0.2 kN/m²) and AS-DM (1.9±0.3 kN/m²).

Phosphorylation status of other myocardial proteins was comparable between AS and AS-DM (troponin I [AS, 0.56±0.16 versus AS-DM, 0.42±0.05]; troponin T [AS, 0.38±0.09 versus AS-DM, 0.34±0.04]; myosin light chain-2 [AS, 0.16±0.15 versus AS-DM, 0.14±0.08]; desmin [AS, 0.10±0.04 versus AS-DM, 0.20±0.05]; and myosin binding protein-C [AS, 0.15±0.02 versus AS-DM, 0.17±0.02]).
Discussion

Aortic Stenosis and Diabetes Mellitus as Comorbidities

Because of the rising prevalence of sclerocalcific aortic valve disease and DM type 2 in aging populations, more patients present with AS and DM as comorbidities. Most clinical studies investigating the association of AS and DM focused on the progression of sclerocalcific aortic valve disease to significant AS and observed faster progression in the presence of DM. Despite numerous studies reporting DM to attenuate eccentric LV remodeling after myocardial infarction and to raise diastolic LV stiffness in HFREF or HFNEF in the absence of coronary artery disease, myocardial effects of DM in AS have largely been overlooked, apart from...

Figure 3. Advanced glycation end product (AGE) deposition. A, Intense AGE deposition in an intramyocardial venule in aortic stenosis–diabetes mellitus (AS-DM). B, The AGE deposition in AS-DM was limited to endothelial and smooth muscle cells (arrow) in arterioles and venules and to endothelial cells in capillaries. C, The AGE deposition score was larger in AS-DM than AS patients. *P<0.05.

Figure 4. Cardiomyocyte dimension and $F_{\text{passive}}$. A, Cardiomyocyte diameter (MyD) was higher in aortic stenosis–diabetes mellitus (AS-DM) than in AS patients. B, Single cardiomyocyte mounted between a force transducer and a piezoelectric motor to measure $F_{\text{passive}}$. C, $F_{\text{passive}}$ was higher in cardiomyocytes of AS-DM than AS patients. Protein kinase A (PKA) decreased $F_{\text{passive}}$ in cardiomyocytes of AS and AS-DM patients. D, Cardiomyocyte passive length-tension relation was shifted upward in AS-DM compared with AS patients. SL indicates sarcomere length. *P<0.05 vs AS; ***P<0.001 vs AS; #P<0.05 vs before PKA (-PKA).
Diastolic LV dysfunction in AS has hitherto been attributed to interstitial fibrosis resulting from an imbalance between extracellular matrix production and degradation.33,34 The present study confirmed these findings in that it observed in AS a CVF twice as high as in control subjects and in AS-DM a CVF 3 times as high as in control subjects. Furthermore, for the pooled AS and AS-DM groups, a significant correlation was observed between CVF and LVEDP. In contrast to HFREF patients,25 \( F_{\text{passive}} \) of isolated cardiomyocytes of AS patients was comparable to that of control subjects and did not contribute significantly to diastolic LV dysfunction.

In the present study, the worse diastolic LV dysfunction of AS-DM patients could have resulted not only from more fibrosis but also from more AGE deposition and raised cardiomyocyte \( F_{\text{passive}} \). With the use of light microscopic immunohistochemical visualization of CML to measure AGEs deposition, more CML was detected in the endothelial and smooth muscle cells of intramyocardial arterioles, venules, and capillaries of the AS-DM patients. This increase in AGE deposition could be involved in the worse diastolic LV dysfunction of the AS-DM patients because vascular AGE deposition blunts endothelial nitric oxide release, as evident from improved flow-mediated dilation of the brachial artery in hypertensives treated with an AGE crosslink breaker35 and because blunted coronary endothelial nitric oxide release worsens diastolic LV function.36 When light microscopic visualization of CML was implemented with electron microscopy, vascular deposition of CML was recently shown to be accompanied by interstitial accumulation of CML, which can directly reduce myocardial diastolic distensibility because of collagen crosslinking.37

The findings of the present study differ from a previous study in HFREF and HFNEF patients free of coronary artery disease.3 In the present study, DM worsened diastolic LV dysfunction in AS through fibrosis, AGE deposition, and raised cardiomyocyte \( F_{\text{passive}} \). In HFREF, DM worsened diastolic LV dysfunction mainly through fibrosis, whereas in HFNEF, DM worsened diastolic LV dysfunction mainly through raised cardiomyocyte \( F_{\text{passive}} \). The extent of the DM-related increase in \( F_{\text{passive}} \), however, clearly differed between AS and HFNEF in that cardiomyocyte \( F_{\text{passive}} \) was significantly higher in HFNEF-DM than in AS-DM patients. A similar trend was observed for fibrosis with higher CVF in HFREF-DM than in AS-DM patients. The unequal extent of the DM-related myocardial effects in AS, HFREF, and HFNEF suggests that DM stimulates prevailing myocardial signal transduction pathways specifically activated by the underlying clinical condition.38

**Cardiomyocyte Hypertrophy and \( F_{\text{passive}} \)**

The MyD rose progressively from control to AS and to AS-DM. The larger MyD in AS-DM than in AS patients corresponded with a trend for higher LVMI. A larger MyD or higher LVMI was also observed recently in other situations combining LV pressure overload with deranged metabolism such as insulin-resistant mice subjected to transverse aortic constriction,21 streptozotocin-treated rats subjected to suprarenal aortic banding,22 and asymptomatic AS patients suffering from metabolic syndrome.23

An MyD larger than control was associated with higher \( F_{\text{passive}} \) in AS-DM patients but not in AS patients, whose cardiomyocyte \( F_{\text{passive}} \) was similar to values previously reported in control subjects.25 Furthermore, the MyD observed in AS patients was larger than that previously reported in a
group of HFNEF patients suffering from arterial hypertension and without DM.3 Despite this larger MyD, the cardiomyocytes of the AS patients had lower $F_{\text{passive}}$ than the cardiomyocytes of the HFNEF patients. The MyD observed in AS-DM patients was also larger than the MyD previously reported in HFNEF-DM patients.3 Again, despite this larger MyD, the cardiomyocytes of the AS-DM patients had lower $F_{\text{passive}}$ than the cardiomyocytes of the HFNEF-DM patients.

From these observations, it becomes apparent that cardiomyocyte hypertrophy is accompanied by reduced cytoskeletal distensibility in the setting of DM but not necessarily in the setting of mechanical overload because AS induces no change in $F_{\text{passive}}$, in contrast to arterial hypertension, which leads to a large increase in $F_{\text{passive}}$. The nature of the mechanical overload, which is more resistive in AS and more capacitive in arterial hypertension,39,40 could account for this divergence because distinct types of loading could trigger different signal transduction pathways for cardiomyocyte hypertrophy, some of which are harmless and others are harmful for cytoskeletal distensibility.

A comparison of $F_{\text{passive}}$ between AS and HFNEF and between AS-DM and HFNEF-DM challenges an obligatory cause-effect relationship between cardiomyocyte hypertrophy and impaired cardiomyocyte distensibility. Clinical observations reported a similar dissociation between myocardial hypertrophy and diastolic LV dysfunction. In the Valsartan in Diastolic Dysfunction (VALIDD) trial, only 3% of hypertensives had significant LV hypertrophy despite all having diastolic LV dysfunction, evident from a lower-than-normal lateral mitral annular age-specific relaxation velocity.41 Basic studies are also supportive of the idea that impaired cardiomyocyte distensibility does not result from but actually causes cardiomyocyte hypertrophy. The giant cytoskeletal protein titin, which determines cardiomyocyte distensibility, is indeed increasingly recognized as an important mechanosensor interacting at sarcomeric Z-disk, I-band, or M-band regions with prohypertrophic calcineurin–nuclear factor of activated T cells, mitogen-activated protein kinases, and extracellular signal-regulated kinase-2 signaling.42

**Cardiomyocyte $F_{\text{passive}}$ and Titin Isoform Phosphorylation**

Overexpression of the stiff N2B titin isoform, titin hypophosphorylation by PKA or protein kinase G, titin hyperphosphorylation by protein kinase C, and loop formation within the titin molecule caused by oxidative stress–induced disulfide bonds have all been identified as mechanisms that raise the intrinsic stiffness of titin and cardiomyocyte $F_{\text{passive}}$.42,43 Concerning the effects of titin on cardiomyocyte $F_{\text{passive}}$, the present study observed similar expression of titin isoforms in control subjects, AS patients, and AS-DM patients; hypophosphorylation of the stiff N2B titin isoform in AS-DM patients; and raised $F_{\text{passive}}$ in AS-DM patients that normalized after PKA administration. A recent experimental study showed insulin to enhance N2B titin isoform expression and titin phosphorylation in rat embryonic cardiomyocytes but failed to detect altered passive stiffness in skinned cardiac fibers of streptozotocin-treated rats.44 These findings are in agreement with the present study insofar as phosphorylation of the stiff N2B titin isoform was reduced in AS-DM patients. This reduction in phosphorylation, however, was accompanied by a significant rise in cardiomyocyte $F_{\text{passive}}$, which correlated with LVEDP and which was corrected by administration of PKA.

**Study Limitations**

Because LV tissue was procured peroperatively, its availability was limited. Hence, all histomorphometry, cardiomyocyte function, and proteomics data could not be acquired in a single patient, and comparison between AS and AS-DM patients was therefore frequently limited to subgroups of both populations. Furthermore, the statistical power of the study was hampered by the small size of the AS and AS-DM populations.

**Conclusions**

The present study observed an additional impairment of LV end-diastolic distensibility when severe, symptomatic AS was associated with DM. This additional impairment related to both structural and functional alterations of LV myocardium consisting of increased interstitial collagen deposition, augmented intramyocardial vascular AGE accumulation, and higher $F_{\text{passive}}$ of cardiomyocytes. Relative hypophosphorylation of the stiff N2B titin isoform in AS-DM myocardium accounted for the higher $F_{\text{passive}}$ of AS-DM cardiomyocytes because in vitro administration of PKA normalized $F_{\text{passive}}$.

**Sources of Funding**

This study was supported by a grant from the Dutch Heart Foundation, The Hague, The Netherlands (2006B035) (Dr Paulus); a Mecenaturá grant from the University of Debrecen Medical and Health Science Center, Debrecen, Hungary (Mec-4/2008; Dr Borbély); a János Bolayai Research Scholarship of the Hungarian Academy of Sciences (Dr Borbély); a basic research fellowship grant from the Heart Failure Association of the European Society of Cardiology, Nice, France (Dr Borbély); a grant from the Portuguese Foundation for Science and Technology (FCT, PIC/IC/82943/2007; Drs Leite-Moreira, Falca˜o-Pires, and Gavina); and a grant from the European Commission, Research Directorate General, Brussels, Belgium (FP7-Health-2010; MEDIA-261409; Dr Paulus and Leite-Moreira).

**References**


CLINICAL PERSPECTIVE

In aging populations, diabetes mellitus (DM) and aortic stenosis (AS) are becoming frequent comorbidities. Studies looking at the interaction between DM and AS investigated mainly the progression of sclerocalcific valvular dysfunction. In heart failure (HF), DM raises diastolic left ventricular (LV) stiffness, which adversely affects morbidity and mortality. The DM-related rise in diastolic LV stiffness was observed both in HF with reduced ejection fraction and in HF with normal ejection fraction. In HF with reduced ejection fraction, DM affected myocardial stiffness through excessive fibrosis and arteriolar or capillary deposition of advanced glycation end products, whereas in HF with normal ejection fraction, DM increased myocardial stiffness through elevation of cardiomyocyte resting tension ($F_{\text{passive}}$). The present clinical study extended these observations on DM-related worsening of diastolic LV stiffness to symptomatic AS and confirmed a similar increase in diastolic LV stiffness in patients suffering from both AS and DM. This increase was evident from higher LV end-diastolic pressure at comparable LV end-diastolic volume index. Furthermore, the increase in diastolic LV stiffness was shown to result from all 3 aforementioned mechanisms, namely excessive fibrosis, intramyocardial vascular advanced glycation end product deposition, and elevated cardiomyocyte $F_{\text{passive}}$. The latter could be attributed to hypophosphorylation of the stiff isoform of the cytoskeletal protein titin, which is largely responsible for cardiomyocyte $F_{\text{passive}}$. The observed increase in diastolic LV stiffness in patients suffering from both AS and DM could predispose them to earlier development of heart failure symptoms and an earlier need for aortic valve replacement.
Diabetes Mellitus Worsens Diastolic Left Ventricular Dysfunction in Aortic Stenosis Through Altered Myocardial Structure and Cardiomyocyte Stiffness


_Circulation._ published online August 15, 2011;

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2011/08/13/CIRCULATIONAHA.111.025270

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/