Early Development of Calcific Aortic Valve Disease and Left Ventricular Hypertrophy in a Mouse Model of Combined Dyslipidemia and Type 2 Diabetes Mellitus

Khai Le Quang,* Rihab Bouchareb,* Dominic Lachance, Marc-André Laplante, Diala El Husseini, Marie-Chloé Boulanger, Dominique Fournier, Xiang Ping Fang, Rita Kohen Avramoglou, Philippe Pibarot, Yves Deshaies, Gary Sweeney, Patrick Mathieu, André Marette

Objective—This study aimed to determine the potential impact of type 2 diabetes mellitus on left ventricular dysfunction and the development of calcified aortic valve disease using a dyslipidemic mouse model prone to developing type 2 diabetes mellitus.

Approach and Results—When compared with nondiabetic LDLr−/−/ApoB100/100, diabetic LDLr−/−/ApoB100/100/IGF-II mice exhibited similar dyslipidemia and obesity but developed type 2 diabetes mellitus when fed a high-fat/sucrose/cholesterol diet for 6 months. LDLr−/−/ApoB100/100/IGF-II mice showed left ventricular hypertrophy versus C57BL6 but not LDLr−/−/ApoB100/100 mice. Transthoracic echocardiography revealed significant reductions in both left ventricular systolic fractional shortening and diastolic function in high-fat/sucrose/cholesterol fed LDLr−/−/ApoB100/100/IGF-II mice when compared with LDLr−/−/ApoB100/100. Importantly, we found that peak aortic jet velocity was significantly increased in LDLr−/−/ApoB100/100/IGF-II mice versus LDLr−/−/ApoB100/100 animals on the high-fat/sucrose/cholesterol diet. Microtomography scans and Alizarin red staining indicated calcification in the aortic valves, whereas electron microscopy and energy dispersive x-ray spectroscopy further revealed mineralization of the aortic leaflets and the presence of inflammatory infiltrates in diabetic mice. Gene expression studies showed upregulation of hypertrophic markers (anp, bnp, b-mhc) in myocardial tissues and of osteogenic markers (spp1, bmp, runx2) in aortic tissues of diabetic mice.

Conclusions—We have established the diabetes mellitus –prone LDLr−/−/ApoB100/100/IGF-II mouse as a new model of calcified aortic valve disease. Our results are consistent with the growing body of clinical evidence that the dysmetabolic state of type 2 diabetes mellitus contributes to early mineralization of the aortic valve and calcified aortic valve disease pathogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: aortic valve stenosis ■ diabetes mellitus, type 2 ■ inflammation ■ obesity

Calcific aortic valve disease is the most common heart valve disorder. The prevalence of aortic valve stenosis (AS) increases with age and has been associated with various risk factors such as type 2 diabetes mellitus (T2D), metabolic syndrome (MetS), hypertension, and dyslipidemia.1,2 Calcific aortic valve disease was previously viewed as a passive process related to aging, but studies in the past decade have uncovered some key molecular processes involved in the development of this disorder.3-5 In this regard, various pathogenic processes such as lipid infiltration/retention, inflammation, and osteogenesis are now considered as key drivers of aortic valve mineralization.6-8 A dysmetabolic state, characterized by insulin resistance/T2D, has been shown to play an important role in the development of aortic valve mineralization and the progression of AS.9,10 There is currently no pharmacological treatment known to slow the progression of the disease. Considering that the pathological processes leading to AS share some similarities with atherosclerosis, several studies explored the potential benefit of lipid-lowering therapy with statins on AS progression. However, although some studies reported that statins may limit aortic valve calcification and slow the hemodynamic progression of AS,11-13 several randomized clinical trials reported that statin treatment was ineffective in patients with AS.14,15 Accordingly, there is no primary indication for statin therapy in patients with AS.

One reason hindering the development of medical therapies for the treatment of AS is the lack of valid animal models that can reproduce the human disorder. AS can develop to a certain...
extent in rabbits fed cholesterol and supplemented with vitamin D.\textsuperscript{16,17} Mice lacking low-density lipoprotein receptor and expressing only apolipoprotein B100 (LRKOB100) can develop AS at a very old age (≈20 months), and only one third of these mice showed echocardiographic evidence of valve stenosis.\textsuperscript{18} Recently, another murine model of excess vitamin A intake (200 IU/g retinyl palmitate for 12 months) was also reported to develop heart valve calcification with a modest increase in transaortic velocity.\textsuperscript{19}

Using LDLr\textsuperscript{−/−}/ApoB100/100/IGF-II (LRKOB100/IGF) mice, which is a model combining atherosclerosis and several features of the MetS as well as increased susceptibility to T2D when fed a diabetogenic diet,\textsuperscript{20,21} we were able to closely recapitulate and characterize the pathophysiology of AS seen in humans. Noteworthy, although this model was previously characterized for atherosclerosis and calcification of atherosclerotic lesions, we are reporting for the first time that this model also develops early AS (6 versus 20 months) and at a much greater rate (80% versus 33%) than previous models when fed a diabetogenic diet. The significant correlation between AS severity and overnight fasting glycemia further points to diabetes mellitus as an important independent factor contributing to accelerated AS progression in our model. Such a preclinical model of AS should prove valuable and may help elucidate the mechanism whereby the development and progression of calcific aortic valve disease is accelerated in patients with MetS/T2D.\textsuperscript{9,22}

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Metabolic Phenotypes of LRKOB100 and LRKOB100/IGF Mice
It has been previously reported that LRKOB100/IGF mice are prone to diabetes mellitus even when fed a standard diet.\textsuperscript{21} We found that fasting glycemia was increased in LRKOB100/IGF mice compared to wild-type (WT) and LDLr\textsuperscript{−/−}/ApoB100/100 (LRKOB100) mice. This suggests that the addition of IGF-II to the diet further exacerbates the diabetic phenotype in these mice.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>aortic valve stenosis</td>
</tr>
<tr>
<td>HFSC</td>
<td>high-fat/sucrose/cholesterol</td>
</tr>
<tr>
<td>LRKOB100</td>
<td>LDLr\textsuperscript{−/−}/ApoB100/100</td>
</tr>
<tr>
<td>LRKOB100/IGF</td>
<td>LDLr\textsuperscript{−/−}/ApoB100/100/IGF-II</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>LVH</td>
<td>left ventricular hypertrophy</td>
</tr>
<tr>
<td>MetS</td>
<td>metabolic syndrome</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes mellitus</td>
</tr>
</tbody>
</table>

Figure 1. Plasma analysis for diabetic features and cardiac remodeling. Measurements were performed after an overnight fast. A, Glucose; B, Insulin; C, Cholesterol; and D, Triglycerides. E, Body weight (BW) of 3 genotypes fed chow diet and 6 months after high-fat/sucrose/cholesterol (HFSC) diet. F, The ratio of total ventricular weight (TVW) to BW in mice fed chow diet or HFSC diet. G, The ratio of TVW corrected for tibia length (TL). H, Left ventricular (LV) mass as assessed by transthoracic echocardiography (M-mode) corrected for BW in mice fed chow diet or HFSC diet (chow diet, n=8, 7, 8 and HFSC diet, n=10, 12, 15 for wild-type [WT], LDLr\textsuperscript{−/−}/ApoB100/100 [LRKOB100], and LDLr\textsuperscript{−/−}/ApoB100/100/IGF-II [LRKOB100/IGF] mice, respectively). *P<0.05, **P<0.01, ***P<0.001; 2-way ANOVA was applied.
versus C57BL/6 wild-type (WT) mice but not versus LRKOB100 kept on standard chow diet. The metabolic phenotypes of LRKOB100 and LRKOB100/IGF animals diverged further on feeding a diabetogenic high-fat/sucrose/cholesterol (HFSC) diet for 6 months. Indeed, only the latter genotype developed diabetes mellitus based on fasting hyperglycemia (Figure 1A). This was explained by a lack of compensatory increase in insulin secretion (Figure 1B). WT mice also developed hyperglycemia when fed the HFSC diet, but these animals gained more weight as compared with both LRKOB100 models (Figure 1E), thus explaining the relative protection of LRKOB100 mice (Figure 1A) against hyperglycemia, especially given the compensatory insulin response in these animals. Resistance against diet-induced obesity has also been reported for the low-density lipoprotein receptor knockout mouse model. Both cholesterolemia and triglyceridemia were similarly increased in LRKOB100 and LRKOB100/IGF mice as compared with WT on either diet (Figure 1C and 1D). These data thus indicate that LRKOB100/IGF mice are prone to T2D, after only 6 months of feeding a diabetogenic diet.

**Left Ventricular Hypertrophy and Dysfunction in HFSC-Fed Diabetic LRKOB100/IGF Mice**

Echocardiographic parameters for each group are shown in the Table. The total ventricular weight corrected for body weight was significantly elevated in LRKOB100 and LRKOB100/IGF mice fed the HFSC diet (Figure 1F). However, when corrected for tibia length, the total ventricular weight was increased only in LRKOB100/IGF mice consuming either the chow or HFSC diets (Figure 1G). Calculation of left ventricular (LV) mass by echocardiography also confirmed the augmentation of LV mass/body weight ratio in diabetic LRKOB100/IGF mice compared with WT mice (Figure 1H). Representative M-mode echocardiographic images of short-axis LV systolic function are shown in Figure 2E (top tracings) for the 3 genotypes fed HFSC diet. Echocardiography also showed a significant reduction in systolic fractional shortening in LRKOB100 and LRKOB100/IGF mice compared with WT mice on the same diet (Table, Figure 2A). Moreover, this decrease was more severe in LRKOB100/IGF mice on HFSC diet as compared with LRKOB100. However, stroke volume and cardiac output were not significantly different between LRKOB100 and LRKOB100/IGF mice on HFSC diet (Table, Figure 2B).

LV diastolic function was also impaired in diabetic animals. The E/E’ ratio was significantly increased in LRKOB100/IGF mice compared with WT mice on HFSC diet (Figure 2C). The value of isovolumic relaxation time, corrected for heart rate, was decreased in both LRKOB100 models as compared

---

**Table. Measures of Echocardiographic Parameters Obtained From WT, LRKOB100, and LRKOB100/IGF Mice Fed Chow Diet or HFSC Diet for 6 Months**

<table>
<thead>
<tr>
<th>Echocardiography</th>
<th>Chow Diet</th>
<th>HFSC Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=8)</td>
<td>LRKOB100 (n=7)</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>227.0±4.3</td>
<td>233.0±4.9</td>
</tr>
<tr>
<td>LVId, mm</td>
<td>4.01±0.10</td>
<td>4.10±0.09</td>
</tr>
<tr>
<td>FS, %</td>
<td>39.8±1.3</td>
<td>34.9±1.3*</td>
</tr>
<tr>
<td>EF, %</td>
<td>76.6±1.5</td>
<td>70.7±1.7*</td>
</tr>
<tr>
<td>SV, mL</td>
<td>0.09±0.004</td>
<td>0.10±0.005</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>430±10</td>
<td>456±22</td>
</tr>
<tr>
<td>CO, L/min</td>
<td>0.04±0.002</td>
<td>0.04±0.003</td>
</tr>
<tr>
<td>LVOT, mm</td>
<td>1.51±0.02</td>
<td>1.53±0.03</td>
</tr>
<tr>
<td>Mitral E velocity, cm/s</td>
<td>93.9±4.6</td>
<td>96.4±3.6</td>
</tr>
<tr>
<td>Mitral A velocity, cm/s</td>
<td>77.0±3.2</td>
<td>82.2±4.6</td>
</tr>
<tr>
<td>Mitral E/A ratio</td>
<td>1.22±0.05</td>
<td>1.19±0.05</td>
</tr>
<tr>
<td>Mitral E/E’ ratio</td>
<td>25.7±1.2</td>
<td>27.6±1.9</td>
</tr>
<tr>
<td>IVRTc</td>
<td>1.64±0.06</td>
<td>1.57±0.10</td>
</tr>
<tr>
<td>Aortic jet velocity, cm/s</td>
<td>121.8±3.7</td>
<td>132.9±5.7</td>
</tr>
<tr>
<td>AVA, mm²</td>
<td>1.69±0.05</td>
<td>1.67±0.07</td>
</tr>
<tr>
<td>LV max PG, mm Hg</td>
<td>5.38±0.29</td>
<td>6.78±0.54*</td>
</tr>
<tr>
<td>FSVR</td>
<td>0.68±0.04</td>
<td>0.50±0.04§</td>
</tr>
</tbody>
</table>

AVA indicates aortic valve area; CO, cardiac output; E/E’, mitral inflow measured by pulsed-wave over tissue Doppler; EF, ejection fraction; FS, fractional shortening; FSVR, fractional shortening–velocity ratio; HFSC, high-fat/sucrose/cholesterol; IVRTc, rate-corrected isovolumic relaxation time; LRKOB100, LDLr −/−/ApoB100/100; LRKOB100/IGF, LDLr −/−/ApoB100/100/IGF-II; LV, left ventricular; LV max PG, LV maximum pressure gradient; LVId, LV interior diameter in end-diastole; LVOT, LV outflow tract; MV, mitral valve; SV, stroke volume; and WT, wild type.

*P<0.05 vs WT.
†P<0.05 vs LRKOB100 (on the same diet).
‡P<0.01 vs WT.
||P<0.01 vs WT.
||P<0.01 vs LRKOB100 (on the same diet).
¶P<0.001 vs WT.
with WT mice on HFSC diet (*P<0.001, Table). In summary, echocardiographic analyses showed an impairment of LV function in both LRKOB100 and LRKOB100/IGF mouse models on chow diet, but feeding a diabetogenic diet was found to worsen cardiac dysfunction to a greater degree in the LRKOB100/IGF model.

Development of Aortic Stenosis in HFSC-Fed Diabetic LRKOB100/IGF Mice

Aortic valve hemodynamics, as assessed by echocardiography, (representative images of peak aortic jet velocity for HFSC-fed mice, Figure 2E, bottom tracings) revealed that aortic stenosis developed in both LRKOB100 mice and to
a greater extent in LRKOB100/IGF mice after 6 months of HFSC feeding. Indeed, the peak aortic jet velocity, the peak transvalvular pressure gradient, and the mean transvalvular pressure gradient were all markedly increased in LRKOB100/IGF and LRKOB100 versus WT mice but to a greater magnitude in the former group (Figure 3A–3C). Accordingly, although no WT mice developed AS (based on a threshold value of 150 cm/s for peak transvalvular pressure gradient, see Materials and Methods in the online-only Data Supplement), we found that ≈80% of diabetic LRKOB100/IGF and ≈40% of nondiabetic LRKOB100 mice did develop AS after 6 months of HFSC feeding (Figure 3D). Of note, the proportion of nondiabetic LRKOB100 mice having developed AS in the present study (≈40%) was similar to that previously observed (≈33%) by the group of Weiss et al.\(^{18}\) However, AS was induced in a much shorter period in our study (6 versus 20 months) which may be explained by our use of a diabetogenic diet (high-fat and -sucrose diet) enriched in cholesterol versus a standard diet used by Weiss et al.\(^{18}\) A significant correlation between peak aortic jet velocity and fasting glucose and cholesterol (Table I in the online-only Data Supplement) suggests that both diabetes mellitus and hypercholesterolemia\(^{24}\) contributed to accelerated aortic stenosis development. Furthermore, the aortic valve area was significantly decreased in the HFSC-fed LRKOB100/IGF mice in comparison with both WT and LRKOB100 mice (P≤0.001, Figure 2D), further supporting more severe AS in LRKOB100/IGF versus LRKOB100 mice.

**Aortic Valve Calciﬁcation in HFSC-Fed LRKOB100/IGF Mice**

A microtomography (microcomputed tomography) scanner was used to document the presence of calcification in vivo. In mice on HFSC diet, we observed the presence of minerals in the aortic valves of LRKOB100/IGF mice but not in WT mice fed the same diet (red arrow, Figure 4A). By using alizarin red staining, we next conﬁrmed in histological sections that calcification was present at the base of leaflets and aortic root in LRKOB100/IGF mice (black arrows, Figure 4B) compared with WT and LRKOB100 mice. Scanning electron microscopy studies of aortic valves from LRKOB100/IGF mice fed the HFSC diet showed the presence of electron-dense areas on the aortic surface of leaflets (Figure 5A). Microanalyses of electron-dense areas with energy dispersive x-ray spectroscopy revealed the presence of calcium and phosphate (Ca/P ratio, 1.87) in LRKOB100/IGF mice (Figure 5E) versus WT mice (Figure 5D). Further scanning electron microscopy analyses of HFSC-fed LRKOB100/IGF valve sections showed that some areas of the aortic surface leaflet (facing the aorta) were denuded of endothelial cells and revealed the presence of aggregates of inﬂammatory cells, platelets, and ﬁbrin covering the extracellular matrix (Figure 5B). Most inﬂammatory cells were the size of macrophages (10–15 μmol/L) and were activated as indicated by the presence of membrane rufﬂes (Figure 5C).

**Upregulation of Osteogenesis and Fibrosis in the Aortic Root of Diabetic LRKOB100/IGF Mice**

The expression of osteoblast-related genes in aortic tissues was next examined. We found that the {\textit{sp}1} gene (encoding osteopontin) was increased in the aortic root of both LRKOB100 models fed the HFSC diet versus WT mice, but more signiﬁcantly so (P<0.001) in LRKOB100/IGF mice (Figure 5F). The expression of {\textit{bglap}} (encoding osteocalcin) was also increased in aortic tissue from both LRKOB100 models, even in chow-fed animals, but again the expression of the gene was further enhanced (P<0.001) in aortas from HFSC-fed diabetic LRKOB100/IGF mice as compared with LRKOB100 mice on the same diet (Figure 5G). Furthermore, the expression of runx2, a key transcription factor and early marker of osteoblast differentiation,\(^{25}\) was signiﬁcantly increased in aortic tissues of both LRKOB100 genotypes on HFSC diet (Figure 5H). Interestingly, runx2 was inversely modulated in chow-fed mice, showing lower levels in aortic tissues from LRKOB100 mice, but elevated levels in aortic tissues from LRKOB100/IGF mice as compared with the WT controls on the same diet. Hence, both LRKOB100 genotypes

![Figure 3](https://example.com/figure3.png)

*Figure 3. Hemodynamic perturbation caused by aortic valve stenosis (AS) in LDLr\(^{-/-}\)/ApoB100/100/IGF-II (LRKOB100/IGF) mice after 6 months on high-fat/sucrose/cholesterol (HFSC) diet. Peak aortic jet velocity (A), peak transvalvular pressure gradient (peak TA; B), mean transvalvular pressure gradient (C), and percentage (D) of animals developing aortic stenosis in the 3 genotypes fed chow or HFSC diet (chow diet, n=8, 7, and HFSC diet, n=10, 12, 15 for wild-type [WT], LDLr\(^{-/-}\)/ApoB100/100 [LRKOB100], and LRKOB100/IGF mice, respectively). *P<0.05, **P<0.01, ***P<0.001; 2-way ANOVA was applied.*
exhibit higher expression of osteogenic genes in the aortic root, especially after consuming the HFSC diet, but this osteogenic phenotype appeared to be substantially greater in diabetic LRKOB100/IGF animals. As illustrated in Figure 6A (upper), \textit{spp1} immunolabeling performed on aortic valve tissue was clearly increased in LRKOB100/IGF versus WT mice fed the HFSC diet, which further supports increased valvular osteogenesis in the former group.

In parallel, Masson trichrome staining also revealed substantial aortic valve fibrosis in LRKOB100/IGF versus WT (Figure 6A, lower). Of note, there was a significant correlation between aortic valve fibrosis and aortic root \textit{spp1} gene expression (Figure 6B), suggesting that aortic valve fibrosis and calcification occur jointly in the progression of AS. Interestingly, the inverse correlations between the aortic valve fibrosis and the LV ejection fraction (%) further indicate that aortic valve alteration not only underlie AS progression but also contribute to altered cardiac systolic function (Figure 6C).

**Increased Expression of Hypertrophic Cardiac Markers in Diabetic LRKOB100/IGF Mice**

Several genetic markers of cardiac hypertrophy were significantly increased in LRKOB100/IGF mice fed the HFSC diet compared with WT (Figure 7A–7F). Indeed, genes encoding atrial natriuretic peptide (\textit{anp}; 2.86±0.65-fold), brain natriuretic peptide (\textit{bnp}; 2.34±0.48-fold), \(\beta\)-myosin heavy chain (\(\beta\)-mhc; 1.83±0.26-fold) and type 1a collagen (\textit{colla1}; 1.53±0.15-fold) were significantly increased. In contrast, \(\alpha\)-mhc mRNA levels were decreased in cardiac tissue of LRKOB100/IGF mice under HFSC-fed versus LRKOB100 mice (\(P<0.05\)). Furthermore, expression of \textit{cdh13}, the gene encoding T-cadherin, was significantly decreased (0.42±0.06-fold) in the diabetic mice.

**Discussion**

There is growing evidence that AS prevalence is associated with the MetS and T2D, but this remains poorly investigated because of the lack of an appropriate rodent model that shares the multiple features of human MetS, such as dyslipidemia, obesity, insulin resistance, and T2D. The LRKOB100/IGF mouse was generated to duplicate the development of T2D through initial overproduction of insulin followed by pancreatic fatigue and loss of \(\beta\)-cell response to a glucose challenge.\(^{20}\)

We have also recently shown that this model develops marked insulin resistance in both liver and skeletal muscle and, on feeding a high-fat sucrose diet, is prone to develop T2D.\(^{24}\) These animals do not show increased aortic plaque size compared with a control LRKOB100 mouse, but they have been reported to accumulate more calcium within their aortic lesion.\(^{21}\) In this study, we demonstrate that the LRKOB100/
IGF mice develop AS after only 6 months of HFSC diet as opposed to ≈20 months for previously reported models. As the lifetime expectancy of a mouse is ≈24 months, the earlier development of AS in our model will allow (1) a better characterization of the disease and (2) the investigation of treatments potentially delaying or even regressing its evolution.

Noteworthy, when compared with nondiabetic LRKOB100 mice, the proportion of diabetic LRKOB100/IGF having developed AS was doubled (80% versus 40%) indicating that AS progression is accelerated by T2D. Accordingly, we found a significant correlation between peak aortic jet velocity and overnight fasting glycemia. Concurring previous studies,24,27 hypercholesterolemia also contributed to AS progression in our model as reflected by the significant correlation between plasma cholesterol level and peak aortic jet velocity. Of note, the increased AS rate found in LRKOB100/IGF versus LRKOB100 mice despite similar plasma cholesterol levels further supports the notion that diabetes mellitus has an additive effect on AS progression.

Microcomputed tomographic analysis revealed the mineralization of the aortic valve in LRKOB100/IGF mice, which occurred after only 6 months of feeding the diabetogenic diet. This was further documented using scanning electron microscopy and energy dispersive x-ray spectroscopy, confirming that aortic valve cusps were covered with minerals composed of calcium hydroxyapatite. Of interest, these findings in the diabetic LRKOB100/IGF mice are consistent with the finding that calcific lesions observed in patients with AS...
are characterized by the presence of calcium deposits containing calcium hydroxyapatite. Mineralization of the aortic valve in patients with AS is also accompanied by local inflammation, which is characterized by the presence of an inflammatory infiltrate, mostly composed of macrophages. Scanning electron microscopy analysis confirmed the presence of macrophages, which were adherent to the surface of the aortic valve, where endothelial denudation was also observed. Most of the macrophages observed on the aortic side of cusps appeared activated, as evidenced by membrane ruffling. In this regard, it has been shown that after exposure to low-density lipoprotein, macrophages exhibit morphological changes such as membrane ruffles. It should also be pointed out that inflammation of the aortic valve in humans is closely associated with the expression of osteoblast markers. Consistent with previous observations made in calcific aortic valve disease, we found increased expression of osteoblast gene markers such as runx2 (runt-related transcription factor 2, osteoblastic differentiation), spp1 (osteopontin), and bglap (osteocalcin) in the aortic tissue of LRKOB100/IGF mice. Immunohistochemistry analysis further revealed that spp1 expression was significantly increased in aortic valve leaflets of LRKOB100/IGF mice. To further substantiate that LRKOB100/IGF mice closely recapitulate the calcific AS progression seen in humans, we also observed important fibrosis in aortic valve leaflets of those animals.

The early development of AS in the diabetic LRKOB100/IGF mouse model was accompanied by LV hypertrophy (LVH) and systolic/diastolic dysfunction. The LVH in LRKOB100/IGF mice, as evidenced by morphological and echocardiographic measurements, was also confirmed by determination of cardiac gene expression markers. Indeed, quantitative polymerase chain reaction analysis showed that ANP and BNP were significantly upregulated in LRKOB100/IGF mice compared with LRKOB100 and WT mice fed the HFSC diet. There is emerging evidence of cross-talk between the myocardium and systemic metabolic pathways. Specifically, there is interest in potential distant metabolic effects of ANP and BNP, produced in the myocardial tissue in response to ventricular stretch and cardiac overload. At the molecular level, pathological stresses have been reported to induce multiple changes, including genetic reprogramming, as revealed by the re-expression of fetal genes (β-mhc) and the downregulation of multiple adult genes (α-mhc). In the present study, we found that expression of α-mhc in LRKOB100/IGF mice tended to be downregulated, whereas myocardial expression of β-mhc was upregulated. This is consistent with the increased expression of the mRNA encoding for col1a1 in the myocardium of LRKOB100/IGF mice.

Echocardiographic analyses further demonstrated that when compared with both WT and LRKOB100 mice, the LRKOB100/IGF mice had a reduced fractional shortening (%), suggesting that LV systolic function is depressed in diabetic mice. Thus, our results showed a significant reduction in systolic function in LRKOB100/IGF mice versus LRKOB100 mice after 6 months of feeding the HFSC diet. Noteworthy, we observed significant inverse correlations between LV ejection fraction (%) and the aortic valve fibrosis, supporting the idea that alteration of aortic valve leaflets underlying AS contributes to impair cardiac function.
Although no significant difference in LV systolic dysfunction between LRKOB100 and LRKOB100/IGF mice was reported in a previous study, the latter used much older mice (18 months). It is therefore possible that with aging, even the non-diabetic LRKOB100 mouse model also develops systolic dysfunction, catching up with cardiac dysfunction that occurs much earlier in diabetic LRKOB100/IGF mice. Moreover, along with the decreased fractional shortening (%), we found that LV diastolic dysfunction was also present in the LRKOB100/IGF mice. This finding supports the concept that LV diastolic dysfunction represents an important feature of diabetic cardiomyopathy and that it develops early in the course of the disease.

It is worthy of note that we have observed cardiac abnormalities such as LVH even in LRKOB100/IGF mice that were fed a standard chow diet. We have previously shown, using hyperinsulinemic-euglycemic clamp studies, that insulin resistance is already present in chow-fed LRKOB100/IGF mice, whereas glucose intolerance and T2D develop only on feeding these animals a high-fat sucrose diet. This suggests that the combined dyslipidemic and insulin resistance states of chow-fed LRKOB100/IGF mice are sufficient to promote LVH in this model. This is in accordance with a previous report that insulin resistance is a powerful and independent predictor of the progression of LVH in patients with AS.

Our finding of marked downregulation of myocardial cdh13 levels in HFSC-fed diabetic LRKOB100/IGF mice is also of significant interest. Indeed, Denzel et al showed that T-cadherin (encoded by cdh13) protects against increased myocardial pressure through its association with adiponectin in mice. Their results revealed an extensive colocalization of T-cadherin and adiponectin in cardiomyocytes in vivo. Pressure overload stress in T-cadherin–deficient mice resulted in exacerbated LVH. This suggests that reduced adiponectin signaling through T-cadherin may contribute to LVH development in this mouse model of cardiometabolic syndrome and T2D.

Conclusions

This study presents the HFSC-fed LRKOB100/IGF mouse as a novel and unique model of diabetes mellitus–linked AS. The early development of AS in this model correlates with several metabolic alterations related to insulin resistance and T2D and is concomitant with LVH and LV dysfunction. Several characteristics of this model reiterate the pathogenic features of AS in humans and thus may help understand the pathophysiological and molecular mechanisms underlying the development of AS in obese patients with T2D. This unique animal model of T2D-linked AS may also prove to be useful for the evaluation of novel pharmacological strategies for AS prevention or treatment.

Acknowledgments

We thank Christine Dion, Kim Denault, and Alcione Lescano for help with animal procedures and cardiomyocyte experiments.

Sources of Funding

This work was supported by grants (No. 161971 to A. Marette and 245048 to P. Mathieu) from the Canadian Institutes of Health Research (CIHR) and a CIHR/Pfizer Research Chair in the pathogenesis of insulin resistance and cardiovascular diseases to A. Marette. M.-A. Laplante was supported by the Heart and Stroke Foundation of Canada. P. Mathieu is a research scholar from the Fonds de Recherche du Québec–Santé. P. Pibarot holds the Canada Research Chair in Valvular Heart Disease.

Disclosures

None.

References

In this study, we have characterized aortic valve stenosis (AS) development in a new mouse model of AS combining atherosclerosis and several features of the metabolic syndrome seen in humans. This model is also genetically prone to the development of type 2 diabetes mellitus when fed a high-fat diet which we find greatly accelerates the development of AS. Such a preclinical model of accelerated AS will help elucidate the mechanism whereby the development and progression of calcific aortic valve disease is increased in patients with diabetes mellitus and also prove valuable for testing the therapeutic efficacy of drugs targeting AS.
Early Development of Calcific Aortic Valve Disease and Left Ventricular Hypertrophy in a Mouse Model of Combined Dyslipidemia and Type 2 Diabetes Mellitus

Khai Le Quang, Rihab Bouchareb, Dominic Lachance, Marc-André Laplante, Diala El Husseini, Marie-Chloé Boulander, Dominique Fournier, Xiang Ping Fang, Rita Kohen Avramoglu, Philippe Pibarot, Yves Deshaies, Gary Sweeney, Patrick Mathieu and André Marette

Arterioscler Thromb Vasc Biol. published online August 14, 2014;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2014/08/14/ATVBAHA.114.304205

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/08/14/ATVBAHA.114.304205.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

**Animals** - All animal protocols were conducted according to guidelines set out by the Laval University Animal Care and Handling Committee. Male mice were housed in a pathogen-free, temperature-controlled environment under a 12:12 hour light-dark cycle and fed *ad libitum* either a standard rodent diet (Harlan Teklad T-2018,) or a diet high in fat, sucrose and cholesterol (55% calories from fat, 28% from sucrose, 0.2% cholesterol) for 6 months starting at 12 weeks of age. LRKOB100 and LRKOB100/IGFmice (both on C57Bl/6J background) were generated from an established colony at the Heart and Lung Institute of Laval University from original founders kindly provided by Dr. Seppo Ylä-Herttuala. Age-matched C57B1/6J mice were obtained from Jackson Laboratories (Bar Harbour, ME).

**Plasma measurements** - Mice were fasted for 12 hours and blood samples taken under anaesthesia from the saphenous vein and immediately analyzed for glucose using a Precision PCx glucometer (MediSense, Abbott Laboratories, MA). Plasma insulin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO). Plasma triglyceride and cholesterol were measured enzymatically as specified by the manufacturer (Randox Laboratories, Kearneysville, WV).

**Echocardiography** - Transthoracic echocardiography was performed under 2.5%-isoflurane anaesthesia, with L15-7io (5-12 Megaherz) and S12-4 (4-12 Megaherz) probes connected to a Philips HD11XE ultrasound system (Philips Healthcare Ultrasound, The Netherlands). Left ventricular (LV) M-mode imaging was obtained in parasternal short-axis view at the level of the papillary muscles. LV dimensions were measured at end-diastole (LVDd) and end-systole (LVDs), and LV fractional shortening (LVFS) was calculated as (LVDd – LVDs)/LVDd × 100%. LV volumes and ejection fraction (EF) were calculated using the Teichholz formula and the Quinones formula, respectively. The diameter of the LV outflow-tract (DLVOT) was measured in a zoomed parasternal long-axis view. LVOT cross sectional area (CSCLVOT) was calculated as π(D/2)2. LVOT flow velocity was obtained by pulsed-wave Doppler (PW) in the apical 5-chamber view. The LV stroke volume (SV) was calculated as VTILVOT × CSCLVOT, where VTILVOT is the velocity-time integral measured in the LVOT. Cardiac output (CO) was measured as: HR × SV. Pulsed-wave Doppler was used to record transmural flow in the apical 4-chamber view and from this signal we measured peak velocity of E- and A-waves and time interval from mitral valve closing to opening (MVco) was measured. Mitral annulus motion velocity was recorded by Doppler tissue imaging, velocity during early filling E’ was measured, and E/E’ ratio was calculated. LV isovolumetric relaxation time (IVRT) was measured using continuous wave Doppler at the conjunction of LV inflow and outflow in apical 5-chamber view and was corrected (IVRTC) by the square root of the R-R interval (msec). Continuous-wave Doppler was also used to record aortic jet flow velocity in the apical 5-chamber view and peak aortic jet velocity and velocity-time integral were measured. Aortic valve area (AVA) was calculated by the continuity equation method using the maximum LVOT and peak aortic jet velocities: 0.785 × ((CSCLVOT)2 × VSUIT)/VPeak. The average of 3 consecutive cardiac cycles was used for each measurement. The function-corrected fractional shortening-velocity ratio was calculated with the following formula (FSVR= (FS%/x1000)/4[Vpeak]. To evaluate the proportion (%) of mice having developed AS in each group, we fixed a threshold value for the peak TA gradient at 150 cm/sec, where mice with a value greater than the threshold were considered to have AS. Although there is no echocardiographic guidelines in mice to discriminate between the presence or absence or AS, we used 150 cm/sec as a threshold value based on our results and those of Weiss et al showing that
WT (without any tissue and molecular valve alteration) never reaches a peak TA gradient above 150 cm/sec.

**Micro-CT scan** - CT scan images of mice were acquired through the eXplore CT 120 system from Gammamedica (Northridge, CA, USA). A protocol, designed especially for our needs, was named « low noise cardiac scan ». It has the following characteristics: 102 M Gy, 80 kV, 32 mA, « binning » = 4X4, exposure = 16 msec, voxel = 100 uM, 440 views, format « step & shoot ». ECG gating was performed with the use of a module (model 1025T) from Small Animal Instruments (Stony Brook, NY, USA) connected to the system. Calcium deposits in the heart and aorta were quantified using the Agatston method with the use of a dedicated software (eXplore Microview v.2.2).

**Energy dispersive X-ray microanalysis** - Energy dispersive X-ray (EDX) was used to compare the calcific deposits on cell surface. The operating program (FEI) identified peaks by position in the spectrum and shape of counts distribution, matching these to a stored reference library of elemental peak spectra and their derivatives. Accurate quantifications depended on the identification of every peak present in the spectrum: counts data were collected for all detected peaks, and calcium and phosphorus were quantified from proportions present in the region of interest relative to the other elemental species present.

**Tissue staining and immunohistochemistry** – Tissue staining and immunohistochemical analysis were performed on transversal sections of aortic valve leaflets (5 micron slices) from embedded OCT tissues or in paraffin (fixed in a solution of 4% paraformaldehyde) as specified.

**Alizarin red staining**: Sections were deparaffinized and stained with an alizarin red solution (2% alizarin red in water, pH 4.2 adjusted by using 10% ammonium hydroxide in water). Sections were incubated in acetone for 30 seconds and then rinsed with a 1:1 solution of acetone and xylene. Finally, sections were rinsed in xylene and mounted in cytoseal. Pictures were acquired using ImageProPlus7.0 software (MediaCybernetics, Rockville, MD, USA) with a 10X objective (insets were enlarged fourfold) and images were processed using Photoshop CS4 (Adobe).

**Masson Trichrome staining**: Staining was done with a Masson Trichrome kit (Sigma-Aldrich, ON, Canada) on aortic valve sections embedded in OCT. Sections were fixed with acetone-methanol (60:40) and then rinsed in water followed by 5 minutes in Weigert’s iron hematoxylin staining. Slides were once again rinsed in running tap water for 5 minutes.Sections were then stained with Scarlet-Acid fuchsin for 5 minutes and rinsed with deionized water. They were then incubated in phosphotungstic/phosphomolybdic acid solution for 5 minutes. Slides were then placed in aniline blue solution for 5 minutes and in 1% acetic acid for 2 minutes. Finally slides were mounted in cytoseal. Images were acquired using a Zeiss Axios Observer microscope using the Zen software (Zeiss, ON, Canada), with a LD A-Plan 20x/0.25 Ph1 objective (Zeiss) with mosaic mode in bright field light. Images were processed and quantifications were performed using Image J1.47g (NIH, USA).

**Osteopontin immunostaining**: Immunostaining analyses were performed from OCT embedded tissue using osteopontin primary antibody (AB10910, Millipore, CAN). Slides were then incubated with EnVision Dual Link System-HRP, followed by AEC substrate (Dako, Carpinteria, CA, USA). Nonimmunized rabbit serum was used as a negative control in all immunohistology experiments. Slides were analysed in bright field microscopy using the BX51 microscope from Olympus (Objective 10X, NA 0.4). Images
were acquired with a camera evolution QEi from MediaCybernetics driven by the ImagePro Plus 7.0 software (MediaCybernetics, MD, USA).

**Scanning electron microscopy** - Samples were fixed 2 h in 2.5% glutaraldehyde at 4°C and washed in 0.1M cacodylate before being post-fixed with 1% osmium tetroxide for 1h at 4°C. Dehydration was then performed with increasing ethanol concentrations up to the critical point of drying with hexamethyldisilazane overnight. Dried samples were sputtered with palladium (Nanotech, USA) and observed by scanning electron microscopy (SEM) in 30 kv (Quanta FEG 3D, FEI, USA).

**Q-PCR analysis** - Mouse LV or aortic root samples were homogenized, total RNA was isolated using Trizol, purified and further quantified via Nanodrop. DNAse I (Invitrogen) treatment was performed. The integrity of total RNA was assessed with an Agilent bioanlyser. RNA with a RIN number above 7.5 was used for gene expression analysis. cDNA was synthesized from 220 ng total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Burlington, ON) with random hexamer primers. Quantitative real-time PCR (q-PCR) was performed with Quantitec SYBR Green PCR kit from Qiagen on the Rotor-Gene 6000 system (Corbett Robotics Inc, San Francisco, CA, USA). Primers for the following transcripts were obtained from Qiagen (Mississauga, ON, Canada): *spp1*, *bglap*, *runx2*, *anp*, *bnp*, *α-mhc*, *β-mhc*, *col1a* and *cdh13*. The expression of hypoxanthine guanine phosphoribosyltransferase (*hprt1*) was used as a reference gene to normalize the results, and the \( \Delta C_t \) method was applied to determine the mRNA expression level.

**Statistical analysis** - Data are presented as mean ± SEM. Where only chow diet was described, a one way ANOVA was used followed by Bonferroni’s post-hoc test. For analysis of multiple genotypes and diets, two-way ANOVA followed by Bonferroni’s post-hoc test was performed. Pearson’s correlation coefficients were used to assess relationship between the aortic jet velocity or left ventricle ejection % (LVEF%) with various systemic parameters (i.e. O/N fasting level of glycemia, TG, cholesterol) or levels of aortic valve fibrosis and aortic root *spp1* gene expression. Statistical analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software Inc. LaJolla, CA). A \( P < 0.05 \) was considered significant.

**Reference:**
**Supplemental Table I:** Correlation between the peak aortic jet velocity and left ventricle ejection fraction (LVEF) with systemic metabolic parameters.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Peak aortic jet velocity (n=8)</th>
<th>LVEF (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>R²</td>
</tr>
<tr>
<td>Glycemia</td>
<td>0.022*</td>
<td>0.609</td>
</tr>
<tr>
<td>AUC OGTT</td>
<td>0.092</td>
<td>0.401</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.002**</td>
<td>0.771</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.641</td>
<td>0.033*</td>
</tr>
</tbody>
</table>

Plasma glucose, triglyceride and cholesterol levels were measured in the fasting state. AUC OGTT: Area under curve of the oral glucose tolerance test. *: P<0.05 and **: P<0.01.