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Original article

# Reduction of cardiac and renal dysfunction by new inhibitor of DPP4 in diabetic rats

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ABSTRACT

*Background:* Increased mortality due to type 2 diabetes mellitus (T2DM) has been associated with renal and/or cardiovascular dysfunction. Dipeptidyl dipeptidase-4 inhibitors (iDPP-4s) may exert cardioprotective effects through their pleiotropic actions *via* glucagon-like peptide 1–dependent mechanisms. In this study, the pharmacological profile of a new iDPP-4 (LASSBio-2124) was investigated in rats with cardiac and renal dysfunction induced by T2DM.

*Methods:* T2DM was induced in rats by 2 weeks of a high-fat diet followed by intravenous injection of streptozotocin. Metabolic disturbance and cardiac, vascular, and renal dysfunction were analyzed in the experimental groups.

*Results:* Sitagliptin and LASSBio-2124 administration after T2DM induction reduced elevated glucose levels to $319.8 \pm 13.2$ and $279.7 \pm 17.8$ mg/dL, respectively ($p < 0.05$). LASSBio-2124 also lowered the cholesterol and triglyceride levels from $76.8 \pm 8.0$ to $42.7 \pm 3.2$ mg/dL and from $229.7 \pm 25.4$ to $100.7 \pm 17.1$ mg/dL, in diabetic rats. Sitagliptin and LASSBio-2124 reversed the reduction of the plasma insulin level. LASSBio-2124 recovered the increased urinary flow in diabetic animals and reduced 24-h proteinuria from $23.7 \pm 1.5$ to $13.3 \pm 2.8$ mg ($p < 0.05$). It also reduced systolic and diastolic left-ventricular dysfunction in hearts from diabetic rats.

*Conclusion:* The effects of LASSBio-2124 were superior to those of sitagliptin in the cardiovascular systems of T2DM rats. This new prototype showed promise for the avoidance of comorbidities in a T2DM experimental model, and thus may constitute an innovative therapeutic agent for the treatment of these conditions in the clinical field in future.



## Introduction

Diabetic cardiomyopathy (DCM), a significant contributor to high morbidity and mortality in diabetic patients, is characterized by the presence of diastolic and late-onset systolic dysfunction, hypertension, and vascular heart disease. DCM occurs due to reduced insulin-induced metabolic actions in the heart, which lead to increased left ventricle (LV) end-diastolic pressure (EDP), decreased lusitropism, and a reduced LV ejection fraction independent of other risk factors, including coronary heart disease [1]. Type 2 diabetes mellitus (T2DM)-induced cardiac dysfunction initiates with LV fibrosis and diastolic dysfunction, eventually progressing to systolic dysfunction and culminating in global heart failure [2,3]. Additionally, increased mortality is associated with diabetic nephropathy (DN), detected in 25% of patients with T2DM and referred to as cardiorenal metabolic syndrome [4]. Evidence suggests that inflammation is an important pathogenic factor in the development of DCM and DN [5,6]. Furthermore, many preclinical and clinical studies have explored the inhibition of the dipeptidyl peptidase-4 (DPP-4) enzyme, with subsequent elevation of the plasma insulinotropic hormone glucagon-like peptide 1, which represents a promising

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strategy for the treatment of T2DM [7–10] and its comorbidities, such as cardiorenal dysfunction [11–13]. The identification of a new therapeutic strategy to address the inflammatory and oxidative stress components of this metabolic disorder remains of great importance. Accordingly, the design and synthesis of new drug prototypes are important steps to expand available treatments.

In the present work, the new compound LASSBio-2124 was synthesized by molecular hybridization of sitagliptin, a dipeptidyl peptidase-4 inhibitor (iDPP-4) [14], and an anti-inflammatory agent (LASSBio-1772) [15]. LASSBio-2124 [16] (Fig. 1) promoted 50% inhibition of DPP-4 enzyme activity at a concentration of 10.6 μM (data not shown). The pharmacological profile of LASSBio-2124 was investigated in a rat model of advanced-stage T2DM with comorbidities such as DCM and DN.

## Materials and methods

### *Induction of T2DM*

All experiments were approved by the Ethics and Animal Care and Use Committee of Universidade Federal do Rio de Janeiro. Male Wistar rats (240–300 g, 8 weeks old) were randomly divided into two groups: a control (non-diabetic) group that received standard chow ($n = 6$); and a diabetic group fed a hypercaloric chow diet (Pragsoluções biociências, Jaú, SP, Brazil) composed of 20 kcal% protein, 25 kcal% carbohydrates, and 45 kcal% fat ($n = 18$). After 14 days of the experimental protocol, streptozotocin (STZ; Sigma-Aldrich Co., St Louis, MO, USA) was dissolved in a citrate buffer (pH 4.5) and immediately injected into the caudal veins (35 mg/kg body weight) of animals fed the hypercaloric chow to induce T2DM [17,18]. Non-diabetic rats received injection of citrate buffer and then, oral administration of the compound vehicle, which was a mixture of benzyl alcohol, polysorbate 80, disodium EDTA, hydroxyethylcellulose and water. Disease onset was confirmed 8 weeks after STZ administration; animals with fasting blood glucose levels ≥250 mg/dL were considered to be diabetic. Then, rats with T2DM were divided randomly into three groups of 6 rats each: T2DM + vehicle, T2DM + sitagliptin (22.6 μmol/kg), and T2DM + LASSBio-2124 (22.6 μmol/kg). Since sitaglipitin at the dose of 10 mg/kg produces antidiabetic effect in rats [19–22], it was used the corresponding dose of 22.6 μmol/kg to compare the efficacy of sitaglipitin and LASSBio-2124 when administered by oral gavage once a day for 2 weeks. Fig. 2 shows the experimental timeline used to characterize the evolution of T2DM. At baseline and at 2, 10, and 12 weeks during the protocol, the rat's metabolic status, blood pressure, and renal function were evaluated.

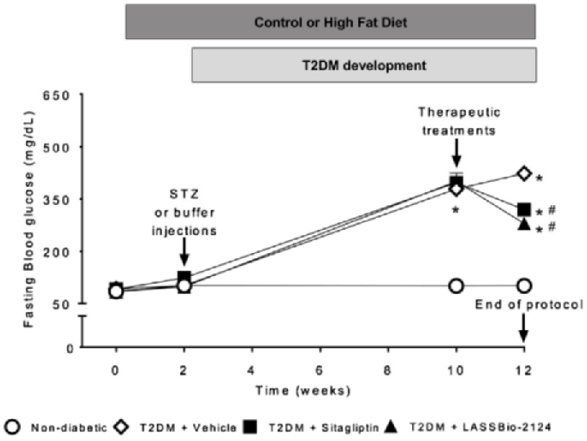


**Fig. 2.** Overview of the experimental protocol. Changes in glucose levels during 12 weeks of protocol in rats fed with a hypercaloric chow diet and low dose of streptozotocin (type 2 Diabetes Mellitus group; T2DM) or fed with a standard chow and citrate buffer (non-diabetic group). Note that T2DM was confirmed at week 10 of protocol through a significant increase of serum glucose levels. At this moment, sitagliptin or LASSBio-2124 started being administrated by oral gavage for 2 weeks totaling 12 weeks of experimental protocol. Data represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group; $\#p < 0.05$ compared with T2DM group treated with vehicle. Ordinary one-way ANOVA with multiple comparisons. STZ, streptozotocin.

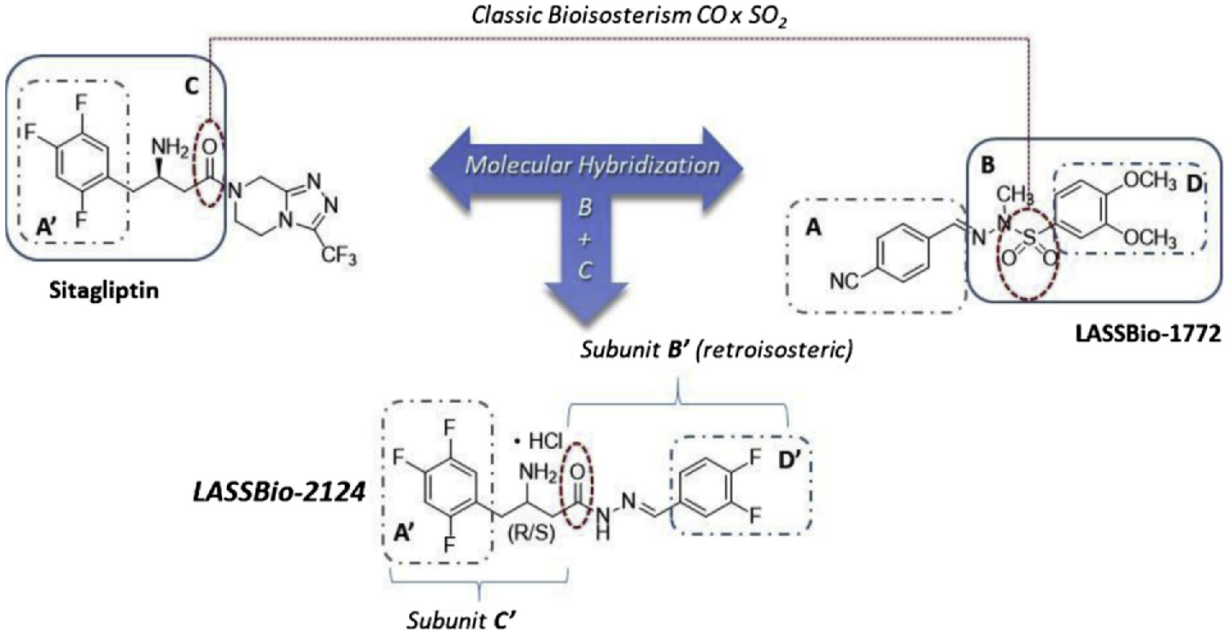


**Fig. 1.** Genesis concept of compound LASSBio-2124 (chemical name: (*E*)-4-(2-(3,4-difluorobenzylidene)hydrazinyl)-4-*oxo*-1-(2,4,5-trifluorophenyl)butan-2-aminium chloride) by molecular hybridization between the prototypes sitagliptin and LASSBio-1772 (chemical name: (*E*)-*N*′-(4-cyanobenzylidene)-*N*-methylbenzenesulfonylhydrazide). As addressed in the text, LASSBio-2124 promoted inhibition of 50% in the DPP-4 enzyme activity with a concentration of 10.6 μM.

*Biochemical analysis*

To measure the serum levels of glucose, insulin, total cholesterol, and triglycerides, animals were fasted for 12 h overnight and tail blood samples were taken on the following morning. Glycemia and insulin levels were determined using an Accu-Chek® monitoring system (ROCHE, Germany) and a commercial kit (Insulin Ultrasensitive ELISA, Germany), respectively. Lipid profiles were assessed using a commercial diagnostic kit (Bioclin, Belo Horizonte, MG, Brazil).

The oral glucose tolerance test (OGTT) was performed *via* determination of the plasma glucose level in response to the oral administration (by gavage) of glucose (2 g/kg body weight) using a commercial diagnostic kit (Bioclin, Belo Horizonte, MG, Brazil). Tail blood samples were taken before and 30, 60, and 120 min after glucose administration.

*Non-invasive blood pressure measurements*

Non-invasive blood pressure measurements were performed in the experimental groups using tail-cuff plethysmography (Letica model LE 5001; Cornella, Barcelona, Spain). Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were measured at 2, 10, and 12 weeks of the protocol.

*Evaluation of renal function*

Non-diabetic and diabetic rats were placed in metabolic cages (Tecniplast, Buguggiate, VA, Italy) with free access to water and food for 24 h. After this period, urinary flow was measured using the following equation: V = urine volume (mL)/24 h (1440 min). Urine samples (1 mL) were collected to assess proteinuria (mg/mL) using a commercial kit (Gold Analisa, Rio de Janeiro, RJ, Brazil).

*Invasive hemodynamic measurement*

At the end of the experimental period, the rats were anesthetized with ketamine (80 mg/kg, *ip*) and xylazine (15 mg/kg, *ip*). Anesthesia depth was verified by pinching the animal's paw with forceps. Subsequently, a catheter (PE-50) was introduced into the right carotid artery, connected to a pressure transducer (MLT884; ADInstruments, Inc.; Sydney, NSW, Australia), and introduced into the LV for the recording of intracavitary left ventricular systolic pressure (LVSP) and left ventricle end diastolic pressure (LVEDP) on a polygraph (Powerlab; ADInstruments, Inc., Sydney, NSW, Australia) using the LabChart software (version 7.0; ADInstruments, Inc.). The left ventricle (LV) contraction and relaxation rates were assessed by maximal positive and negative dP/dt, respectively. Immediately after the hemodynamic measurements, the animals were killed *via* exsanguination by cardiac puncture, and tissues were collected for the evaluation of hypertrophy, histology, and molecular pathways.

*Evaluation of endothelial function in the aorta*

After euthanasia, the thoracic aorta was removed from each rat, cleaned of connective tissue, and prepared for isometric tension recording, as described previously [23]. Briefly, aortic rings 3–4 mm in length were placed in chambers filled with physiological solution (123 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $KH_2PO_4$, 11.5 mM glucose, 15.5 mM $NaHCO_3$, 1.2 mM $CaCl_2$), oxygenated with 95% $O_2$/5% $CO_2$, and maintained at 37 °C. After 2 h, the preparations were exposed to increasing concentrations of phenylephrine (1–10 μM), followed by the addition of increasing concentrations of acetylcholine (ACh; 1–10 μM), to determine endothelial integrity.

*Histomorphometric analysis*

For histological analysis, the right kidney and heart apex were fixed in Gendre's fluid and zinc formalin, respectively, and embedded in paraffin. Tissue sections (5 μm) were stained and examined under a microscope (Axiostar; Zeiss, Germany). The collagen content was detected in 15–20 fields (400×) each of picro-Sirius red–stained sections of LV and renal tissues, and analyzed using Fiji software [24]. Kidney sections were also stained with periodic acid–Schiff to evaluate tubular and glomerular damage. The mesangial area was measured in 20 cortical glomeruli (1000×), and tubulointerstitial damage was characterized using a semi-quantitative score (0, absent; 1, minimal; 2, mild; 3, moderate; 4, severe), with consideration of five parameters: interstitial edema, luminal casts, tubular dilation, intracellular glycogen accumulation, and epithelial injury [25–29].

*Cardiac p38-α mitogen-activated protein kinase activation*

Immunohistochemical analysis of heart paraffin sections (5 μm) was performed using a standard technique [30], in which sections were blocked with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) for 30 min, followed by incubation overnight at 4 °C with primary antibody (anti–p38-α, ab7952; Abcam). After blockade of endogenous peroxidase activity using 3% hydrogen peroxide, the samples were incubated for 2 h with secondary antibody (Nichirei, 414191F, diluted 1:3 in PBS). Chromogenic detection was performed with 3,3′-diaminobenzidine, and counterstaining was performed with Harris' hematoxylin. The activation of p38-α mitogen-activated protein kinase (MAPK) in cardiac tissue was analyzed by determining the percentages of positive (brown) cardiomyocyte nuclei found in eight fields (1000×) of the LV.

*Membrane preparation and western blot analysis*

Heart subcellular fractions were prepared for western blot analysis and protein expression as described previously [31].

*Data analysis*

Data were expressed as means ± standard errors of the mean, and were analyzed using the GraphPrism software (version 6.0). The experimental groups were compared using one-way analysis of variance, with a significance level of $p < 0.05$. Pearson's correlation was used to test for relationships between vasodilation in the aorta and MAP, and between the collagen fraction and RAGE protein expression in LV tissues.

## Results

The serum glucose level in diabetic rats treated with LASSBio-2124 was reduced from 400.5 ± 24.0 to 270.7 ± 17.8 mg/dL (Fig. 2; $p < 0.05$). Similar results were obtained with the orally administered equimolar dose of sitagliptin in diabetic rats (blood glucose was reduced from 387.0 ± 13.8 to 319.8 ± 13.2 mg/dL; $p < 0.05$; Fig. 2). During the experimental period, the non-diabetic group gained weight properly, but animals that received the hypercaloric diet + STZ injection (diabetic group) did not follow the pattern of increasing body weight (Fig. 3A). Additionally, the T2DM + vehicle group showed impaired fasting glucose tolerance in the OGTT, whereas this intolerance was reduced in diabetic animals treated with sitagliptin or LASSBio-2124 (Fig. 3B; $p < 0.05$). Increased levels of total cholesterol and triglycerides were observed in diabetic animals, and this metabolic disturbance was recovered at

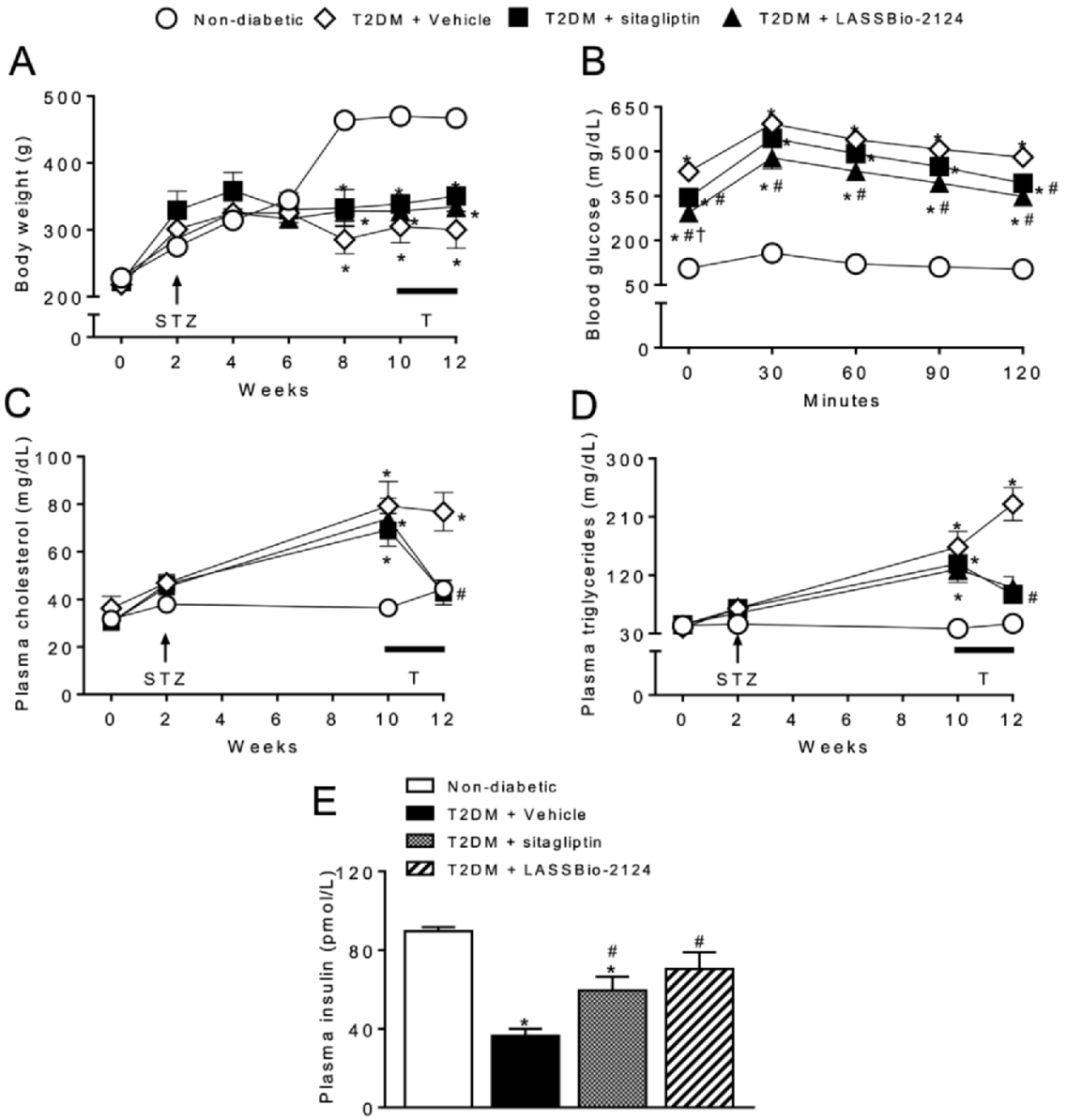


**Fig. 3.** Effects of sitagliptin, LASSBio-2124 or vehicle administration for 2 weeks on **A**, body weight change during protocol development. **B**, serum glucose levels measured in rats fasted overnight for 12 h by the oral glucose tolerance test (OGTT) at the end of protocol. **C**, serum total cholesterol, and **D**, triglycerides levels measured during protocol development. **E**, plasma insulin concentration at the end of experimental approaches. Data represent the mean ± SEM (n = 6 rats per group); *$p < 0.05$ compared with non-diabetic group, #$p < 0.05$ compared with T2DM group treated with vehicle, †$p < 0.05$ compared with T2DM + sitagliptin rats. Ordinary one-way ANOVA with multiple comparisons. STZ, streptozotocin; T, treatments; T2DM, type 2 Diabetes Mellitus.

the end of treatment period by sitagliptin and LASSBio-2124 (Fig. 3C, D; $p < 0.05$). At the end of the experimental period, the serum insulin level was significantly lower in the T2DM + vehicle group than in the non-diabetic group ($p < 0.05$; Fig. 3E). Therefore, LASSBio-2124, but not sitagliptin, normalized the insulin level in diabetic animals ($p < 0.05$). Sitagliptin only partially reversed the insulin elevation detected in diabetic animals (Fig. 3E).

*Non-invasive blood pressure parameters and endothelial function in the aorta*

Before the initiation of treatment with sitagliptin or LASSBio-2124, the diabetic animals exhibited high SBP, DBP, and MAP in comparison with the non-diabetic group ($p < 0.05$). After 2 weeks of treatment of T2DM rats with LASSBio-2124, those values had been reduced from 179.8 ± 6.1 to 143.8 ± 12.3 (mmHg; $p < 0.05$), from 142.3 ± 4.9 to 111.5 ± 9.0 (mmHg; $p < 0.05$), and from 159.2 ± 8.9 to 123.3 ± 10.4 (mmHg; $p < 0.05$), respectively (Fig. 4A–C). The treatment of diabetic rats with sitagliptin also promoted significant reductions in the SBP, DBP, and MAP from 180.8 ± 8.7 to 127.0 ± 11.1 (mmHg; $p < 0.05$), from 147.8 ± 8.9 to 103.2 ± 11.5 (mmHg; $p < 0.05$), and from 154.3 ± 4.2 to 114.2 ± 13.9 (mmHg; $p < 0.05$), respectively. T2DM promoted the impairment of vascular reactivity; ACh-induced relaxation in the aorta was significantly lesser in diabetic than in non-diabetic rats (63.1% ± 4.8% *vs.* 99.4% ± 0.5%; $p < 0.05$; Fig. 4D). This impairment was totally reversed after treatment with LASSBio-2124 (91.3% ± 3.4%, $p < 0.05$), indicating the improvement of endothelial function, but only partially after treatment with sitagliptin (Fig. 4D). MAP correlated with the ACh-induced vascular response (Fig. 4E; $p < 0.05$).

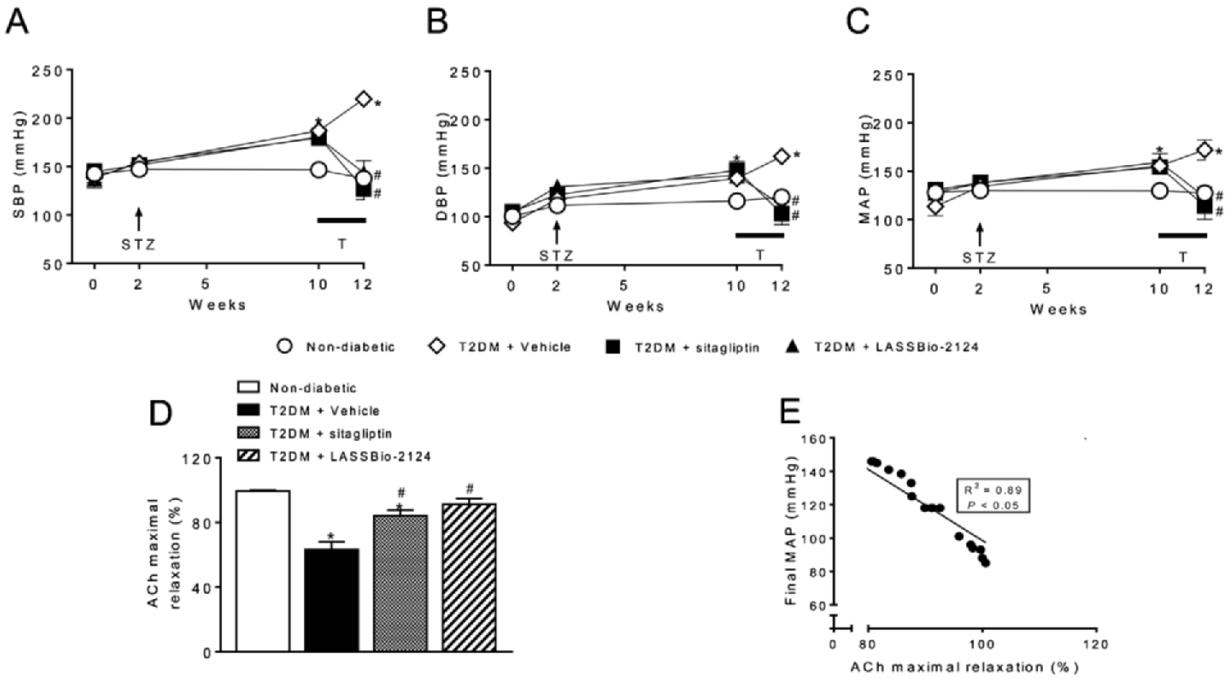


**Fig. 4.** Effects of sitagliptin, LASSBio-2124 or vehicle administration for 2 weeks on **A**, systolic pressure; **B**, diastolic pressure. **C**, mean arterial pressure acquired during protocol development. **D**, acetylcholine-induced maximal relaxation in aortas from T2DM or non-diabetic rats at the end of protocol. **E**, correlation between MAP measure at the end of protocol and acetylcholine-induced maximal relaxation in aortas. Data represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group, $\#p < 0.05$ compared with T2DM group treated with vehicle. Ordinary one-way ANOVA with multiple comparisons. STZ, streptozotocin; T, treatments; T2DM, type 2 Diabetes Mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

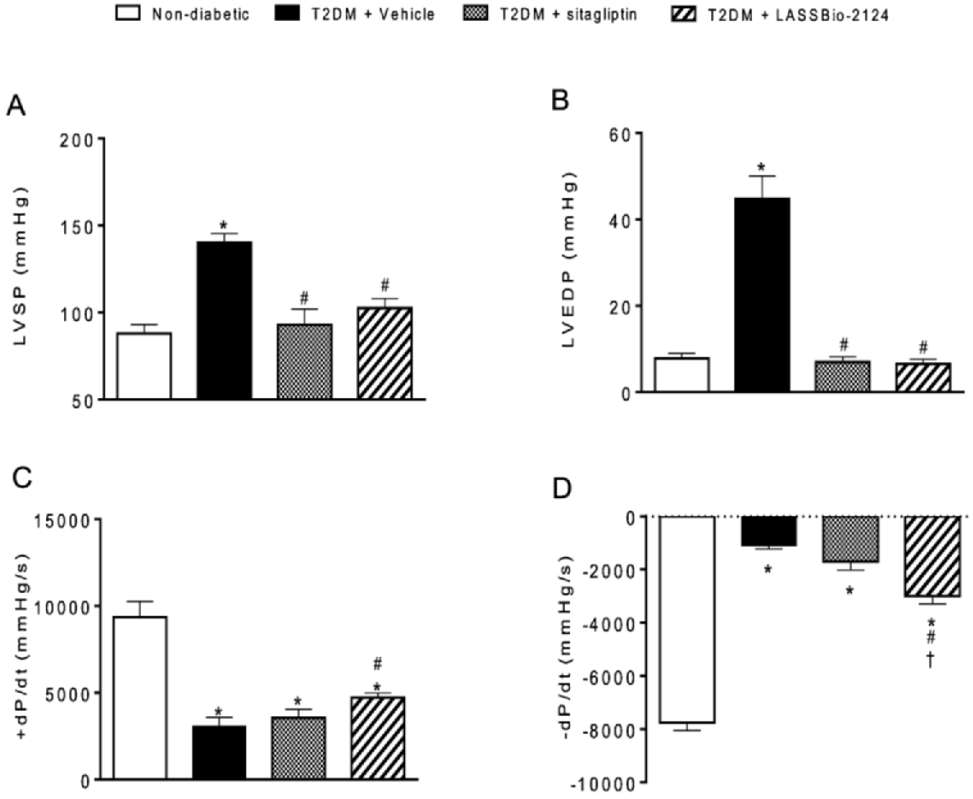


**Fig. 5.** Effects of sitagliptin, LASSBio-2124 or vehicle administration for 2 weeks on **A**, LVSP. **B**, LVEDP. **C**, resting maximum rates of rise (positive dP/dt) and **D**, fall (negative dP/dt) in LV pressures. Data represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group, $\#p < 0.05$ compared with T2DM group treated with vehicle, $\dagger p < 0.05$ compared with T2DM + sitagliptin rats. Ordinary one-way ANOVA with multiple comparisons. T2DM, type 2 Diabetes Mellitus; LVSP, left ventricular systolic pressures; LVEDP, left ventricular end-diastolic pressures.

*Hemodynamic parameters*

A significant increase in the LVSP was observed in T2DM rats treated with vehicle when compared with non-diabetic animals (Fig. 5A; $p < 0.05$). In contrast, normal pressure was detected in diabetic animals treated with sitagliptin or LASSBio-2124 ($p < 0.05$). The LVEDP was significantly higher in the vehicle-treated T2DM group than in non-diabetic rats ($p < 0.05$), and sitagliptin and LASSBio-2124 reduced this hemodynamic parameter (Fig. 5B; $p < 0.05$). Positive and negative dP/dt were altered by T2DM compared to non-diabetic counterparts (Fig. 5C, D), with reductions from $9359.0 \pm 914.2$ to $3079 \pm 497.0$ mmHg/s and from $-7751.0 \pm 299.0$ to $-1094.0 \pm 138.0$ mmHg/s ($p < 0.05$, respectively).

*Heart histopathological properties*

Representative images of LV tissue, showing the collagen content and nuclear p38-α MAPK expression, are shown in Fig. 6. The ratio between heart weight and tibial length was increased significantly in animals with T2DM treated with vehicle (Fig. 6B; $p < 0.05$). LASSBio-2124 and sitagliptin similarly prevented heart hypertrophy. Collagen deposition and nuclear p38-α MAPK expression in the LV were increased significantly in T2DM rats (Fig. 6D, E; $p < 0.05$). Only fibrosis was reversed by sitagliptin and LASSBio-2124 ($p < 0.05$). The expression of RAGE, which is involved in extracellular matrix deposition in the hearts of diabetic rodents [32], was increased in T2DM rats, but normalized after treatment with sitagliptin and LASSBio-2124 (Fig. 6F; $p < 0.05$). RAGE

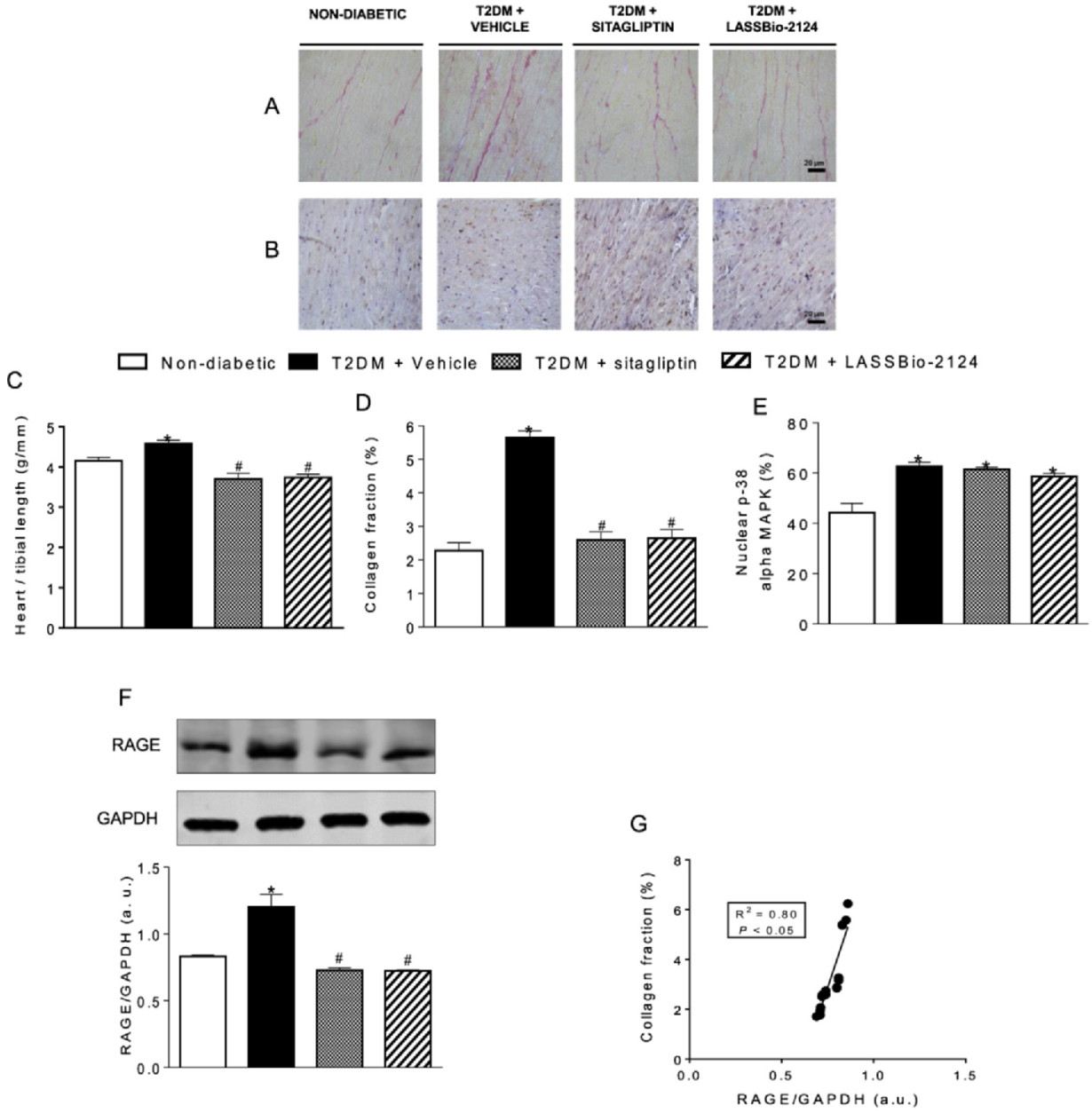


**Fig. 6.** Effects of sitagliptin, LASSBio-2124 or vehicle administration for 2 weeks on extracellular matrix deposition in the heart tissue. **A**, picrosirius red staining. **B**, representative immunostaining for nuclear p-38 alpha MAPK protein in left ventricular tissue. **C**, ratio between heart weight and tibial length. **D**, collagen fraction in left ventricle tissues. **E**, western blot analyses and quantification of RAGE protein expression in left ventricles from all animal groups. GAPDH was used for normalization. G, linear regression between collagen content in left ventricle tissues and RAGE protein expression. Each column and bar represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group, $\#p < 0.05$ compared with T2DM group treated with vehicle. Ordinary one-way ANOVA with multiple comparisons. T2DM, type 2 Diabetes Mellitus; MAPK, mitogen-activated protein kinase; RAGE, receptor for advanced glycation end products.

expression was also correlated significantly with the collagen fraction (Fig. 6G; $p < 0.05$).

*Calcium handling, apoptosis, and inflammation markers in the heart*

Fig. 7 shows representative western blot images demonstrating the expression of SERCA2a, p-PLB, PLB, TNF-α, active caspase 3, and Bcl-2 in LVs from all groups. T2DM did not alter SERCA2a expression in the LV (Fig. 7A). However, reductions in the p-PLB/PLB ratio and Bcl-2 level, with concomitant increases in TNF-α and active caspase 3 expression, were observed in LV tissues from T2DM rats (Fig. 7B-E; $p < 0.05$). Sitagliptin and LASSBio-2124 promoted returns to control levels of PLB, p-PLB, TNF-α, and Bcl-2 protein expression ($p < 0.05$), but only treatment with LASSBio-2124 normalized the expression of active caspase 3, a pro-apoptotic protein, in LV tissues from T2DM rats (Fig. 7D; $p < 0.05$).

*Renal function*

Optical microscopy images of kidney sections are shown in Fig. 8. Non-diabetic rats showed normal glomerular and tubular structure, but T2DM induced evident expansion of the glomerular mesangial matrix and increased tubular injury and tubulointerstitial collagen deposition (Fig. 8D–F; $p < 0.05$). Treatment with sitagliptin or LASSBio-2124 significantly reduced the glomerular matrix expansion, but not the tubular injury (Fig. 8E, F). Persistent polyuria was noted in T2DM rats compared with the non-diabetic group (Fig. 8G; $p < 0.05$). Importantly, proteinuria detected in diabetic animals was significantly reduced by LASSBio-2124 ($p < 0.05$), but not by sitagliptin (Fig. 8H).

## Discussion

The *in vivo* and *in vitro* experiments conducted in this work closely recapitulate the main characteristics of clinical T2DM-induced cardiomyopathy and nephropathy. Orally administered LASSBio-2124 in rats with T2DM induced by a hypercaloric diet and low-dose STZ rescued the evolution of cardiovascular and kidney dysfunction, sometimes showing a better pharmacological profile than that promoted by sitagliptin, thereby suggesting a possible future therapeutic application of this chemical prototype in subjects with DCM and DN comorbidities. Sitagliptin and LASSBio-2124 are both iDPP-4s; we propose that the greater efficacy of the latter for some parameters in this study reflects additional actions on injured myocardial and kidney tissues in T2DM rats.

The rat model used in this work replicated the metabolic features and complications of chronic T2DM, as indicated by overt hyperglycemia, dyslipidemia, hypoinsulinism, heart and blood vessel disease, and nephropathy [33–38]. The expected significant gain in body weight in T2DM animals by the end of the study period was not observed, but may be justified by the weight-lowering effect of STZ in rats [39]. Additionally, we observed that sitagliptin and LASSBio-2124 administered at equimolar doses of 22.6 μmol/kg were weight-neutral agents.

In our experimental protocol, the hypercaloric diet together with STZ injection induced pancreatic β-cell dysfunction, as the serum insulin levels were reduced 12 weeks after study initiation compared with rats fed with a standard diet and given citrate buffer injections. This reduction might have caused the overt hyperglycemia observed during the study period. Interestingly, the treatment of T2DM animals with sitagliptin for 2 weeks resulted in

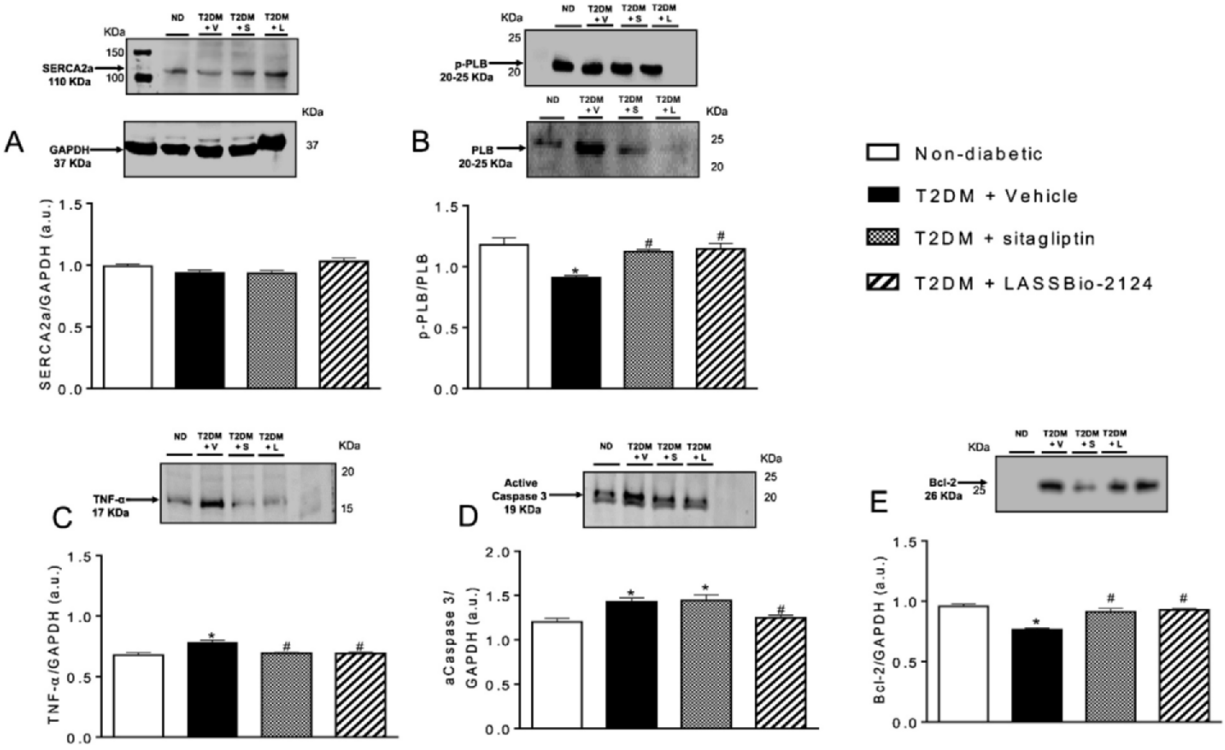


**Fig. 7.** Effects of T2DM model on LV protein expression over 12 weeks of protocol and oral treatment with vehicle, sitagliptin or LASSBio-2124 at week 12 of protocol. Figure **A** shows quantification of SERCA 2a expression. **B**, relative expression ratio of p-PLB to PLB. **C**, quantification of TNF-α. **D**, active caspase 3 expression. **E**, quantification of Bcl-2. Data represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group, $\#p < 0.05$ compared with T2DM group treated with vehicle, $\dagger p < 0.05$ compared with T2DM + sitagliptin rats. Ordinary one-way ANOVA with multiple comparisons. T2DM, type 2 Diabetes Mellitus; ND, non-diabetic. V, vehicle. S, sitagliptin. L, LASSBio-2124. SERCA2a, sarco-endoplasmic reticulum $Ca^{2+}$-ATPase 2a; p-PLB, phosphorylated phospholamban. PLB, total phospholamban. TNF-α, tumor necrosis factor alpha. aCaspase 3, active caspase 3. Bcl-2, B-cell lymphoma 2.

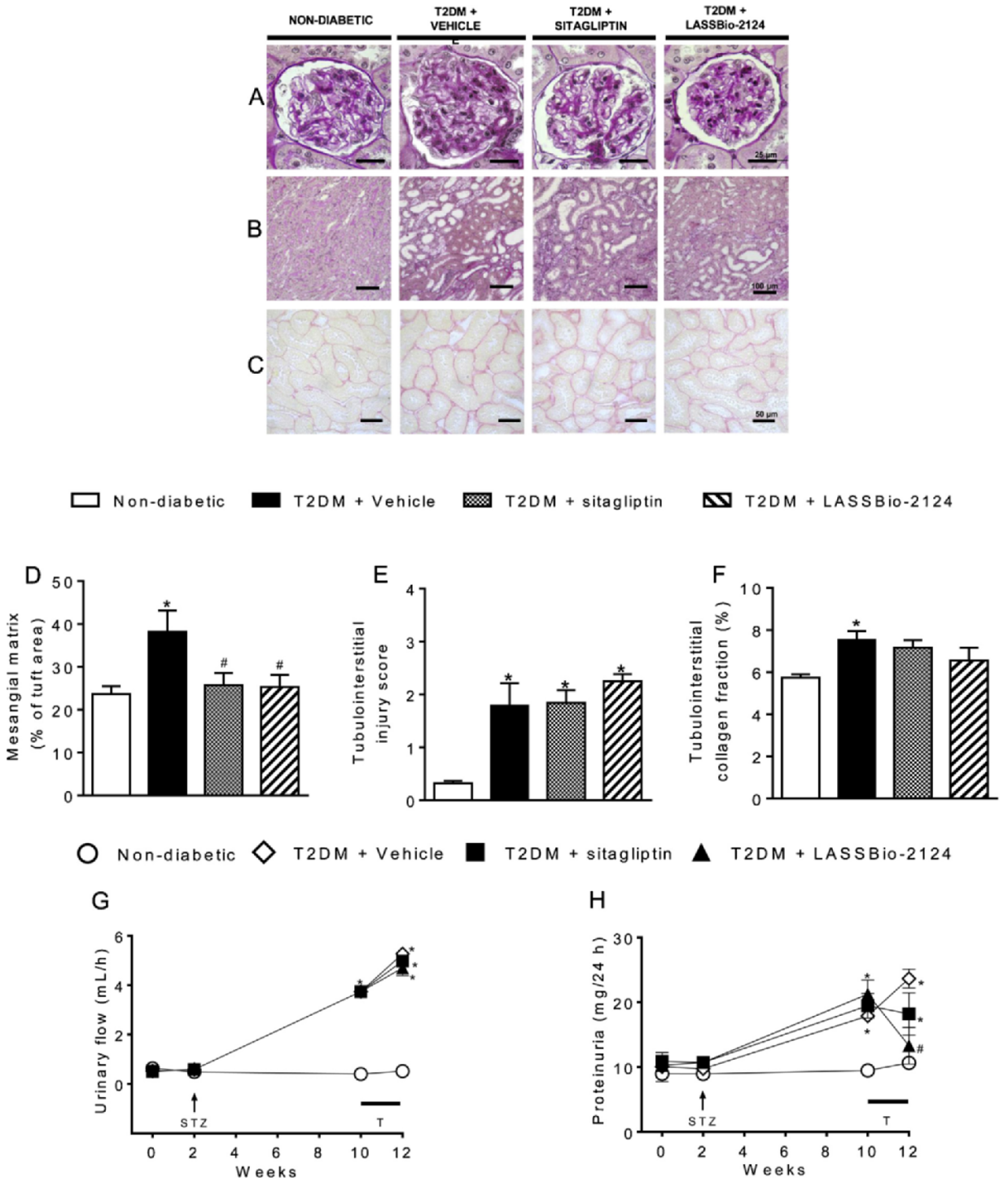


**Fig. 8.** Renal function and histological analysis. **A–B**, representative micrographs of PAS and. **C**, stained picrosirius red kidney sections. Figure **D** shows mesangial matrix area quantification. **E**, kidney injury score based on pathological findings in renal medulla. **F**, collagen deposition in peritubular matrix. **G**, urinary flow. **H**, proteinuria. Data represent the mean ± SEM (n = 6 rats per group); $^{*}p < 0.05$ compared with non-diabetic group, $\#p < 0.05$ compared with T2DM group treated with vehicle. Ordinary one-way ANOVA with multiple comparisons. STZ, streptozotocin; T, treatments; T2DM, type 2 Diabetes Mellitus. PAS, periodic acid-Schiff stain.

only partial normalization of diabetes-induced hypoinsulinemia, whereas the administration of LASSBio-2124 completely normalized insulin production, probably through the beneficial sensibilization of pancreatic $\beta$-cells to the high blood glucose levels. We assume this effect based on the observation of better hypoglycemic action of LASSBio-2124 than of sitagliptin in the OGTT performed in fasted T2DM rats.

Preclinical and clinical studies have provided conclusive evidence that lipids play an important role in the pathogenesis of progressive cardiovascular and kidney damage [40–42]. In the present study, the T2DM rats had significantly elevated plasma total cholesterol and triglyceride levels, which were normalized and reduced, respectively, by 2 weeks of treatment with sitagliptin or LASSBio-2124.

Endothelial dysfunction is an imbalance in the production of vasodilator factors that predisposes the vasculature toward prothrombotic and pro-atherogenic effects. The delicate balance of the production of vasoactive substances by the endothelial cells is disrupted in diabetic subjects, resulting in vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, impaired coagulation and nitric oxide (NO) production, vascular inflammation, atherosclerosis, and thrombosis [43]. Insulin increases the endothelial isoform of nitric oxide synthase (NOS) expression [44]. Thus, ACh and insulin cause endothelium-dependent NO-mediated vasodilation. High glucose concentrations cause a reduction in NOS expression in endothelial cells [45,46]. High blood levels of free fatty acids (FFAs) in hypertriglyceridemia also contribute to abnormal vascular reactivity because FFAs induce an inflammatory state in addition to reducing NO release by the endothelium. FFAs also reduce the secretion of vascular prostacyclin, a vasodilatory mediator, from the endothelium [47–49]. Accordingly, the context of diabetes associated with hyperglycemia, increased FFAs, and lack of insulin is a pro-constrictor state that would support evolution to a hypertensive phenotype in subjects with T2DM [50]. In this study, endothelial dysfunction due to reduced ACh-induced maximal relaxation was confirmed in aortas from T2DM animals. We did not assess correlations of the serum insulin, glucose, and lipid levels with ACh-induced aorta relaxation, but we propose that hypoinsulinemia, hyperglycemia, and hyperlipidemia were all involved in the endothelial dysfunction in our T2DM animals. Importantly, the T2DM rats also developed hypertension, with significantly increased SBP, DBP, and MAP during the experimental period. Final MAP data were also correlated with ACh-induced maximal relaxation. Two weeks of treatment of T2DM rats with LASSBio-2124 and sitagliptin promoted anti-hypertensive effects, but sitagliptin only partially reversed endothelial dysfunction, whereas LASSBio-2124 at the same dose and treatment duration completely normalized the impairment of ACh-induced relaxation; these findings validate the innovative pharmacological importance of LASSBio-2124 compared with sitagliptin.

The LV SBP and LV EDP were higher in the T2DM group than in non-diabetic rats. Resting maximum rates of rise (positive dP/dt) and fall (negative dP/dt) in LV pressures were also impaired at the end of the study period in T2DM rats, indicating significantly impaired LV systolic and diastolic function. The hemodynamic disturbances reflected by the altered LV SBP and LV EDP were attenuated after treatment of T2DM animals with sitagliptin or LASSBio-2124. However, only LASSBio-2124 treatment had a beneficial effect on the reduced positive and negative dP/dt. Importantly, diastolic function was better in LASSBio-2124-treated than in sitagliptin-treated T2DM rats, as reflected by the higher negative dP/dt. We should comment that the development of heart dysfunction in our T2DM rats was related to increased myocardial collagen deposition, which resulted in LV adaption (fibrosis/remodeling) and hypertrophy, as reflected by the greater heart hypertrophy index (ratio of heart weight to tibial length). Additionally, RAGE activation in the myocardium in diabetic mice may trigger diabetes-like cardiac dysfunction through several intra- and extracellular mechanisms, including cross-linking between RAGE and collagen-induced myocardial fibrosis [32]. Our data corroborate these findings, as RAGE expression was correlated with the collagen fraction in LV tissues from our experimental groups, and the RAGE level in the myocardium was increased in T2DM rats, followed by the enhancement of collagen deposition in the LV. The sitagliptin and LASSBio-2124 treatments dampened heart hypertrophy and reduced heart remodeling, due to the evident normalization of LV collagen content by the end of the study period and normalized RAGE expression, in T2DM rats compared with the vehicle-treated T2DM group. These findings might in part explain the improvement of LV function in diabetic rats treated for 2 weeks with sitagliptin or LASSBio-2124.

Innumerable mechanisms have been proposed to explain how the interaction between RAGE activation and the function of calcium cycling proteins, such as SERCA2a, contributes to the development of DCM [51]. Arai [51] showed that RAGE signaling generates oxidative stress, which is related to decreased intracellular calcium uptake by SERCA with no change in the expression of this ATPase located in the sarcoplasmic reticulum membrane. Our results reproduced these findings, as LV tissues from our T2DM rats showed no change in SERCA2a expression. However, we observed that the levels of total PLB protein and the p-PLB to PLB ratio were significantly altered in LVs from T2DM rats compared with those from the non-diabetic group, indicating that less SERCA2a was inhibited by PLB in the diabetic than in the non-diabetic group. Thus, the LV dysfunction in T2DM animals evidenced by our hemodynamic data might be also related to abnormal uptake of calcium by SERCA2a, followed by a subsequent reduction in calcium storage for normal heart contraction and an increase in the cytosolic calcium concentration by the moment that hearts from diseased animals needed to relax. Thus, our molecular data corroborates the impaired systolic and diastolic function observed in T2DM + vehicle compared with non-diabetic rats. Importantly, LVs from diabetic rats treated with sitagliptin or LASSBio-2124 showed the normalization of PLB levels, which may explain the improvement of heart function in these animals compared with T2DM + vehicle rats. However, these findings raise an intriguing question: why do our molecular data on calcium-handling proteins show similar capacities of sitagliptin and LASSBio-2124 to normalize PLB levels, given the better diastolic function in T2DM rats treated with LASSBio-2124 relative to sitagliptin-treated diabetic rats? To address this question, we consider that the expression of active caspase 3, a protein involved in cardiomyocyte apoptosis in the diabetic heart [52], was greater in LVs from diabetic than from non-diabetic rats, and that LASSBio-2124 is probably more effective than sitagliptin in reversing LV diastolic dysfunction in T2DM animals (given its normalization of cardiac active caspase 3 expression), which may help to improve the lusitropic property of the LV.

Increased apoptosis in hearts with DCM occurs as a consequence of sustained oxidative stress and inflammation [52,53]. The proinflammatory cytokine TNF-α promotes cell death by increasing the levels of active caspase 3 [53], and its proapoptotic action is involved with the activation of nuclear factor-κB [54]. In this study, we observed increased TNF-α expression in LVs from T2DM rats compared with those from the non-diabetic group, which was normalized by treatment with sitagliptin or LASSBio-2124. These observations corroborate the previous finding that sitagliptin has an anti-inflammatory effect [55], and, interestingly, demonstrate the same for LASSBio-2124. The antiapoptotic protein Bcl-2 was recently found to protect against cardiomyocyte dysfunction in diabetic hearts by participating in several molecular pathways [56]. In this study, we found a lower Bcl-2 content in LVs from T2DM rats than in those from non-diabetic rats, which was normalized by treatment with sitagliptin and LASSBio-2124 compared with vehicle. However, whereas sitagliptin and LASSBio-2124 treatments had important cardiac effects in T2DM rats, *via* the normalization of several *in vivo* and molecular alterations, they did not normalize the increased nuclear p38-α MAPK level in the heart.

In conclusion, our findings suggest that LASSBio-2124, a new iDPP-4, reduces metabolic disturbances and cardiorenal dysfunction consequent to T2DM, and may be promising for the treatment of T2DM comorbidities.

## Author contributions

BEOA, AKNA, LERG, JSS, JSCA, TLM, LVPM, PMP-C, VMNC, and GMMO carried out experimental analysis, acquisition, analysis and interpretation of the data and drafted the manuscript. RM-O, LML, EJB, RTS and GZ-S contributed to the conception, design and supervision of the study, funding acquisition and interpretation of data. All authors read and approved the final version of the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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