NOVEL THERAPEUTIC STRATEGIES IN DIABETES-ASSOCIATED CARDIOVASCULAR DISEASES

PhD Thesis

Adar Saeed

Rácz Károly Conservative Medicine Division Doctoral School
Semmelweis University

Supervisor: Dora Bianka Balogh, PhD

Official reviewers: Boglarka Laczy, MD, PhD
Alex Ali Sayour, MD, PhD

Head of the Complex Examination Committee:
Csaba Bodor, PhD, DSc

Members of the Complex Examination Committee:
Csaba Ambrus, MD, PhD
Tamas Kaucsar, MD, PhD

Budapest
2024

1 Introduction

Diabetes mellitus (DM) has emerged as one of the major global chronic diseases of our time. As per the latest data from the International Diabetes Federation (IDF), the prevalence of DM among adults was estimated to be 536.6 million in 2021, constituting 10.5% of the world's population. Projections indicate this number will increase to 783.2 million by 2045. In Hungary, over 600,000 individuals were reported to have DM, according to information from the IDF Europe website, which accounts for 9.1% of the country's population.

The complications of DM can be categorized into two primary types. The first is microvascular, which affects small blood vessels in the retina, peripheral nerves, and kidneys, leading to diseases, such as retinopathy, neuropathy, and nephropathy. The second is macrovascular, which impacts larger blood vessels and includes conditions like peripheral artery disease, coronary artery disease (CAD), and cerebrovascular disease. Patients with all forms of DM are at increased risk of developing serious acute and chronic complications.

Diabetic cardiomyopathy (DCM) is defined as structural heart defects and dysfunction without other cardiac risk factors, such as CAD, hypertension, and severe valvular diseases in patients with DM. In diabetic patients, the prevalence of DCM reached 6.1 million in 2020. Generally, the prevalence of cardiac dysfunction with type 1 DM (T1DM) and type 2 DM (T2DM) has been reported to be as high as 14.5% and 35.0%, respectively.

DCM is characterized by left ventricular (LV) stiffness and cardiac remodeling, with impaired early diastolic filling and elevated LV end-diastolic pressure. The later stage involves LV hypertrophy, advanced cardiac remodeling, and diastolic dysfunction, with symptoms of HF. As DCM progresses, it leads to systolic dysfunction and HF with reduced ejection fraction, prolonged pre-ejection performance, and increased filling pressures.

The underlying pathological factors are complex in both T1DM and T2DM. Hyperglycemia is the primary trigger of DCM and directly affects cardiomyocytes. Due to insulin deficiency in T1DM, glucose metabolism redirects towards alternative pathways, such as the polyol and hexosamines pathway, and the formation of advanced glycation end products (AGEs). Which further induces cardiac inflammation, oxidative stress, hypoxia, and the overactivation of the renin-angiotensin-aldosterone system (RAAS). Inflammatory cytokines and angiotensin II stimulate the production and secretion of TGF-β, which is the main regulator of myocardial fibrosis and cardiac fibroblast activation to produce extracellular matrix. All these glucose-induced pathological mechanisms lead to cardiac defect, remodeling, and stiffness.

Cardiac imaging is the gold standard diagnostic tool for detecting structural and functional cardiac changes in DCM. Moreover, several circulating biomarkers have been investigated for their role in predicting clinical outcomes in DCM such as brain natriuretic peptide (BNP). BNP are useful sensors for systolic/diastolic dysfunction in symptomatic DM patients. Moreover, cardiac troponin I (cTnI), a regulatory protein controls calcium-mediated interaction between actin and myosin and is a clinical indicator of myocardial cell damage. Additionally, Klotho forms a unique endocrine system that regulates multiple metabolic processes, such as the inactivation of oxidative stress, inflammation, and fibrotic pathways in the heart and kidney. Alterations in the levels of these biomarkers may indicate myocardial structural and functional dysfunction.

The most effective intervention strategy for DCM treatments to effectively manage DM is through lifestyle modifications, improved glycemic control, hypertension, and dyslipidemia if present. Regular physical exercise and a nutritious diet are crucial for managing DM.

RAAS inhibitors (RAASi) are the primary standard therapy when hypertension and albuminuria present or for diabetic patients with macrovascular complications plus hypertension, HF, or CKD. They have been shown to reduce all causes of CVD mortality in diabetic patients with HF and asymptomatic LV dysfunction. Furthermore, RAASi are not

recommended for diabetic normotensive patients without pre-existing cardiovascular complications, however, T1DM patients are younger at the onset of the disease, thus they lose more life-years due to CVD. Clinical investigations with low-dose RAASi in T1DM-related cardiovascular complications are lacking. Therefore, to address this gap, we designed a study to explore the potential benefits of RAASi in T1DM-related CVD.

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) are a new class of anti-diabetic drugs. Their mechanism of action involves inhibiting renal tubular glucose reabsorption, resulting in a reduction in blood glucose levels and inducing glucosuria in an insulin-independent action. The FDA has approved canagliflozin, dapagliflozin, and empagliflozin as SGLT2i for the treatment of DCM based on findings of large cardiovascular outcome trials (CVOTs) (EMPA-REG OUTCOME, CANVAS, and DECLARE-TIMI 58). These trials have demonstrated significant cardiorenal benefits, reducing the risk of cardiovascular complications and HF.

Following these promising results, clinical trials were initiated specifically for people with HF, including participants with or without T2DM. The first dedicated HF study with DAPA (DAPA-HF) showed that treatment significantly reduced the risk of a first HF event and death from cardiovascular causes in patients with reduced ejection fraction irrespective of the presence of T2DM. For these reasons, we aimed to investigate the efficacy of DAPA in mitigating the progression of CV complications in T1DM. Focusing on the molecular mechanism behind DAPA's anti-inflammatory and antifibrotic effects in cardiac remodeling,

2 Objective

Our studies aimed to explore novel therapeutic strategies to improve the management of DM-related cardiovascular complications. We hypothesized that low-dose RAASi could improve myocardial damage, cardiac fibrosis, and vascular function after five weeks of T1DM. We further hypothesized that SGLT2i offers cardioprotective benefits beyond glycemic control and prevents the progression of CVD in a T1DM rat model.

The objectives of the present studies were as follows:

1. To evaluate the cardioprotective effects of various low-dose RAASi treatments after five weeks of T1DM
2. To assess the antifibrotic effects of various low-dose RAASi in the left ventricle.
3. To characterize the preventive effect of DAPA on the progression of cardiovascular complications in T1DM.
4. To determine the possible anti-inflammatory effects of DAPA.
5. To assess the effect of DAPA on cardiac remodeling.

3 Methods

Study Approval

All animal experiments were conducted according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Semmelweis University Budapest, Hungary (PEI/001/380-4/2013 and PEI/001/1731-9-2015).

3.1 Animals and Experimental Design

The study was performed on eight-week-old male Wistar rats (Rattus norvegicus) housed in plastic cages under controlled light/dark conditions (12:12 hour light/dark cycle) and constant temperature of 24±2 °C. They had access to standard rodent chow and tap water. A qualified person monitors their health daily.

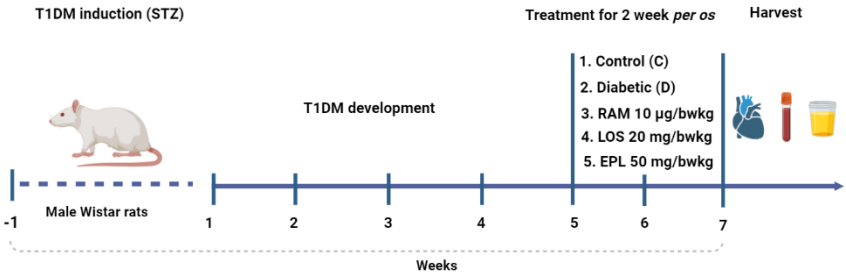
T1DM was chemically induced with a single intraperitoneal injection of 65 mg/bwkg streptozotocin (STZ) dissolved in 0.1 M citrate buffer. Blood glucose levels were measured three times from the tail vein 72 hours after the injection and following overnight fasting. Rats with a peripheral blood glucose value above 15 mmol/L were enrolled in the study. Control animals, non-diabetic, age- and body-weight-matched healthy animals, received citrate buffer without STZ once and isotonic saline (NaCl 154 mmol/L) daily. Two different experimental protocols were used in the study.

Protocol I

Following five weeks of T1DM induction, the rats were randomized into four groups (n = 6 animals/group). The dosages of RAASi ensure the effectiveness of RAAS blockade without affecting systemic blood pressure. Subsequently, a two-week oral treatment was administered, involving the following groups:

1. Isotonic saline as a non-diabetic control (C)
2. Isotonic saline as a diabetic vehicle (D)
3. Ramipril dissolved in isotonic saline (D + RAM, 10 μg/bwkg/day)

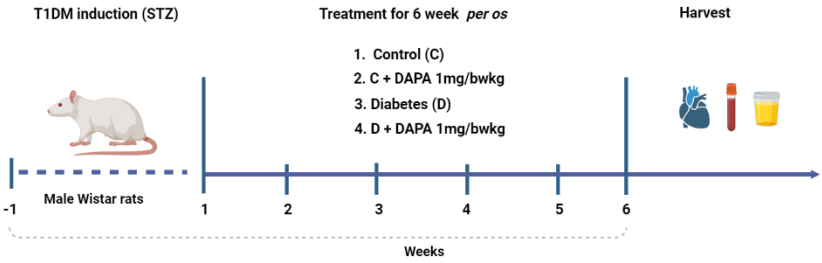
4. Losartan dissolved in isotonic saline (D + LOS, 20 mg/bwkg/day)
6. Eplerenone dissolved in isotonic saline (D + EPL, 50 mg/bwkg/day).



Protocol II

Diabetic (D) rats were randomly divided into four groups immediately after the onset of diabetes (n = 6/groups), six-week oral treatment was administered involving the following groups:

1. Isotonic saline as a non-diabetic control (C)
2. DAPA dissolved in isotonic saline in non-diabetic control (C + DAPA; 1 mg/bwkg/day)
3. Isotonic saline as a vehicle (D)
4. DAPA dissolved in isotonic saline (D + DAPA; 1 mg/bwkg/day)



At the end of the experiments, blood pressure was measured, and rats were anesthetized by a mixture of 75 mg/bwkg ketamine and 10 mg/bwkg xylazine. Subsequently, terminal blood samples were drawn from the abdominal aorta to euthanize the animals. Blood, urine, heart, and aorta samples were collected and stored for further investigations.

3.2 Metabolic, Molecular, and Histological Measurements

The measurement of arterial blood pressure through the tail vein was measured, and mean arterial pressure (MAP) was calculated in a clinically validated environment.

Measurement of metabolic and cardiac parameters, such as serum glucose, fructosamine, and lipid profile was determined with a photometric chemistry analyzer. Glucosuria was measured from 24-hour collected urine.

Measurement of the serum myocardial biomarkers such as serum levels of Klotho and cardiac troponin I were measured using the Rat ELISA Kit.

Relative mRNA expression was quantified with quantitative reverse transcription polymerase chain reaction (RT-qPCR). The following targets were assessed in rat LV samples: BNP (*Nppb*), TGF- β (*Tgfb1*), PDGF (*Pdgfb*), CTGF (*Ccn2*), fibronectin (*Fn*), IL-1β (*Il1b*), IL-6 (*Il6*), TNF-α (*Tnf*), MCP-1 (*Ccl2*), and 18S ribosomal RNA (*Rn18S*). Data were normalized to *Rn18S* as a housekeeping gene.

Histological analysis

Orcein-staining was performed to analyze the amount of elastic fiber in the aorta section of the rat heart. Histological examination was performed under 20x objective magnification. Intima-media thickness (IMT) was measured on cross sections of the aorta, and the mean value of ten measurements was calculated.

Picrosirius red staining was performed to analyze collagen accumulation and fibrosis of the rat LV samples. The red-stained area represents the intramyocardial collagen which is measured based on the red color intensity. An experienced cardiovascular histopathologist conducted multiple optimization measurements, achieving the best RGB pixel intensity parameters, and then performed red-stained area measurements on each histological slide.

3.3 Statistical Analysis

Data are expressed as means±standard deviations (SD). Statistical analysis was conducted using Prism software (version 10.1.0; GraphPad Software, San Diego, CA, USA). Multiple comparisons and interactions were evaluated by one-way ANOVA followed by the Holm-Sidak post hoc test. For non-parametrical data, the Kruskal-Wallis ANOVA on ranks was used, followed by Dunn's correction. A significance level P value <0.05 was considered statistically significant.

4 Results

4.1 RAASi treatment did not alter mean arterial pressure, heart rate, and heart-to-body weight ratio.

Systolic and diastolic pressures and heart rate were measured, and the MAP was calculated. After seven weeks of T1DM, MAP remained unaltered in all groups. This finding implies an effective inhibition of the RAAS without impacting systemic blood pressure. STZ-induced T1DM was associated with a decline in heart rate and an increased heart-to-body weight ratio. These parameters were unaffected by all RAASi.

4.2 RAASi mitigated aortic intima-media thickening.

Aortic IMT is an important early biomarker of atherosclerosis. The diabetic rats displayed intimal thickening and diffused elastic membranes. RAASi reduced enhancement and pathohistological changes in the aorta of diabetic rats; however, only eplerenone reached the level of significance.

4.3 RAASi normalized the levels of specific biomarkers of myocardial injury.

BNP has been an important diagnostic biomarker of LV dysfunction. BNP is mostly released in response to LV volume expansion and pressure overload. The mRNA expression of BNP (*Nppb*) levels was significantly increased in the diabetic group, and it was mitigated with all RAASi treatments.

In parallel, troponin I, a gold standard molecular marker of myocardial injury, was elevated in the serum of diabetic rats. RAASi treatment lowered this elevation to control levels. Furthermore, Klotho is a novel cardioprotective factor in the progression of diabetic cardiovascular complications. We observed decreased serum levels of Klotho in diabetic rats. This decline was reversed only by eplerenone treatment.

4.4 RAASis halted the progression of T1DM-induced myocardial fibrosis.

Profibrotic factors play a key role in the mechanisms of cardiac fibrosis. Here, we investigated the mRNA expressions of *Tgfb1*, *Pdgfb*, and *Ccn2* in the LV. *Tgfb1* and *Ccn2* were upregulated in diabetic rats. Low-dose RAASi treatment decreased the elevation of the investigated markers.

We further used picrosirius red staining to evaluate the collagen content in the myocardium of the LV. The treatment of RAASi significantly reduced collagen accumulation. In parallel, DM-induced higher fibronectin (*Fn*) expression was abolished by RAASi, suggesting a milder myocardial remodeling.

4.5 DAPA treatment prevented metabolic deterioration and cardiac hypertrophy in diabetic rats.

Typical metabolic characteristics of DM were manifested after six weeks of T1DM, including weight loss and elevated levels of serum glucose, fructosamine, and lipids profiles. Treatment with DAPA significantly improved all metabolic parameters in diabetic rats. The dysregulation of carbohydrate metabolism in diabetic rats was improved with DAPA treatment, as confirmed by the reduction in serum glucose and fructosamine levels. Simultaneously, DAPA increased the urinary glucose excretion in both the non-diabetic control and diabetic groups, confirming the mechanism of action of SGLT2i.

MAP remained unchanged in all experimental groups. In line with *Protocol I*, STZ-induced T1DM was associated with decreased heart rate, which was reversed by DAPA treatment. Diabetic rats showed cardiac hypertrophy, indicated by an increased heart-to-body weight ratio. DAPA treatment prevented cardiac hypertrophy.

4.6 DAPA treatment prevented intima-media thickening

In *Protocol II*, we also investigated the of aortic IMT. In diabetic rats, the aorta exhibited a thicker intimal appearance and diffused elastic membranes compared to the control group. All these histological changes were prevented by DAPA treatment demonstrating its efficacy in attenuating the progression of atherosclerosis in T1DM.

4.7 DAPA mitigated the upregulation of myocardial injury biomarkers.

Natriuretic peptides have a crucial role in the clinical assessment of myocardial injury. Therefore, mRNA expressions of *Nppb* (BNP) were assessed in the LV, revealing significant increases in diabetic rats. In the DAPA-treated group, the levels of *Nppb* were less elevated compared to the diabetic group.

We measured the serum levels of cardiac troponin I, and our findings showed that DAPA treatment decreased the DM-induced serum level of cardiac troponin I, ensuring the preventive effect of DAPA on cardiac injury. In parallel to *Protocol I*, we found that Klotho expression was decreased in the serum of T1DM rats. DAPA treatment halted this reduction.

4.8 DM-induced cardiac inflammation was diminished by DAPA treatment

The upregulation of cardiac cytokines and chemokines has been associated with myocardial injury, hypertrophy, impaired LV function, and cardiac fibrosis. Therefore, we measure mRNA expressions of *Il1b*, *Il6*, *Tnf*, and *Ccl2* (MCP-1) in the LV. All investigated inflammatory markers were increased in diabetic rats. Our results showed that the upregulated *Il1b*, *Il6*, and *Tnf* were abolished in the DAPA-treated group, suggesting reduced inflammation-mediated myocardial damage. These findings underscore the anti-inflammatory efficacy of DAPA.

4.9 DAPA mitigated the T1DM-induced cardiac remodeling progression.

We also investigated the role of DAPA in cardiac remodeling in this experiment. Therefore, we measured the mRNA expressions of profibrotic markers *Tgfb1*, *Pdgfb*, and *Ccn2* in the LV. Diabetic rats showed elevated mRNA expressions of these profibrotic markers. DAPA treatment significantly diminished the elevation of *Tgfb1* and *Ctgf* .

The histological analysis of the myocardial collagen content of the LV was examined with picrosirius red staining. DM led to myocardial collagen deposition in rats. The DAPA-treated group showed a substantial decrease in collagen accumulation, nearly matching the levels observed in the control group. These results confirm DAPA's protective role and effectiveness in reducing cardiac fibrosis. In parallel, DAPA halted DM-induced higher *Fn* expression, indicating milder myocardial remodeling.

5 Conclusion

1. Treatment with low-dose RAASi effectively counteracted T1DM-induced CV complications without affecting BP levels.
2. RAASi used as monotherapy impeded cardiac tissue remodeling.
3. Monotherapy with the MR antagonist eplerenone may provide equivalent or even superior efficacy compared to ACEi or ARB in halting the progression of arterial and myocardial injuries in T1DM.
4. Our study provides experimental evidence for DAPA's cardioprotective effect in a T1DM model. DAPA prevented metabolic decline, intimal thickening, cardiac inflammation, and fibrosis in T1DM.
5. These findings suggest that a complex system underlies the organ protective effects of SGLT2i and may offer a novel therapeutic opportunity to treat and improve outcomes for DM and related disorders simultaneously.

6 Bibliography

Publications related to the theme of the PhD thesis.

*Hodrea Judit, ***Adar Saeed**, Agnes Molnar, Attila Fintha, Adrienn Barczi, Laszlo J. Wagner, Attila J. Szabo, Andrea Fekete, and Dora B. Balogh; SGLT2 inhibitor dapagliflozin prevents atherosclerotic and cardiac complications in experimental type 1 diabetes. PLoS One 2022: **IF=3.7**

*** Shared first authorship**

Balogh, Dora B., Agnes Molnar, Arianna Degi, Akos Toth, Lilla Lenart, **Adar Saeed**, Adrienn Barczi, Attila J. Szabo, Laszlo J. Wagner, Gyorgy Reusz, and Andrea Fekete; Cardioprotective and Antifibrotic Effects of Low-Dose Renin–Angiotensin–Aldosterone System Inhibitors in Type 1 Diabetic Rat Model. International Journal of Molecular Sciences 2023: **IF=5.6**