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NOVEL THERAPEUTIC STRATEGIES IN DIABETES-ASSOCIATED CARDIOVASCULAR DISEASES

PhD Thesis

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LIST OF ABBREVIATIONS

ACC	American College of Cardiology
ACEi	Angiotensin-Converting Enzyme Inhibitor
ADA	American Diabetes Association
AGEs	Advanced Glycation End products
AHA	American Heart Association
Ang	Angiotensin
ARB	Angiotensin II type 1 Receptor Blockers
AT1R	Angiotensin II type 1 receptor
BNP	Brain Natriuretic Peptide
BP	Blood Pressure
CAD	Coronary Artery Disease
CANVAS	Canagliflozin Cardiovascular Assessment Study
CKD	Chronic Kidney Disease
CTGF	Connective Tissue Growth Factor
CV	Cardiovascular
CVD	Cardiovascular Disease
CVOTs	Cardiovascular Outcome Trials
DAPA	Dapagliflozin
DCM	Diabetic Cardiomyopathy
DECLARE-TIMI 58	Dapagliflozin Effect on Cardiovascular Events Thrombolysis in Myocardial Infarction 58
DM	Diabetes Mellitus
DPP-4i	Dipeptidyl-Peptidase-4 inhibitors
ECM	Extracellular Matrix
EMPA-REG	Empagliflozin Cardiovascular Outcome Event Trial in Type 2
OUTCOMES	Diabetes Mellitus Patients-Removal of Excess Glucose
FDA	Food and Drug Administration
GLP-1RA	Glucagon-like Peptide-1 Receptor Agonists
HbA1c	Glycosylated hemoglobin
HF	Heart Failure
HFmrEF	Heart Failure Mildly Reduced Ejection Fraction

HFpEF	Heart Failure Preserved Ejection Fraction
HFrEF	Heart Failure with reduced Ejection Fraction
IDF	International Diabetes Federation
IL-1β	Interleukin-1β
IL-6	Interleukin-6
MACE	Major Adverse Cardiovascular Events
MCP-1	Monocyte Chemoattractant Protein 1
NF-κB	Nuclear Factor kappa B
NYHA	New York Heart Association
PCT	Proximal Convoluted Tubule
PDGF	Platelet Derived Growth Factor
RAAS	Renin Angiotensin-Aldosterone System
RAASi	Renin-Angiotensin-Aldosterone System Inhibitors
RAGE	Receptor for Advanced Glycation End products
RGB	Red Green Blue
ROS	Reactive Oxygen Species
SGLT1	Sodium-Glucose Cotransporter-1
SGLT2i	Sodium-Glucose Cotransporter-2 inhibitors
STZ	Streptozotocin
T1DM	Type 1 Diabetes
T2DM	Type 2 Diabetes
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
VERTIS CV	Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial
WHO	World Health Organization

1 INTRODUCTION

1.1 Diabetes Mellitus

1.1.1 Prevalence

Diabetes mellitus (DM) has emerged as one of the significant 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 (1). 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 (2).

People with DM face an increased likelihood of experiencing severe health complications, resulting in reduced quality of life, increased mortality, and escalating healthcare costs. Global spending on DM-related healthcare is estimated to reach USD 966 billion annually (3).

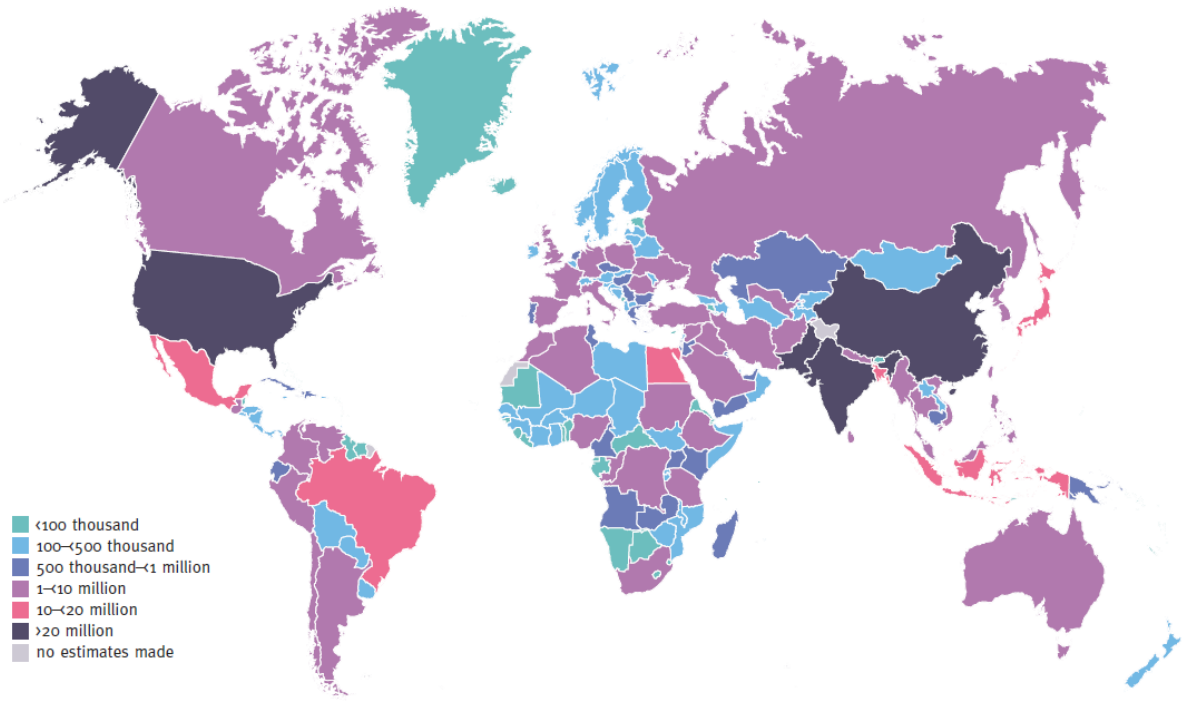

Figure 1 The estimated total number of diabetic adults (20-79 years) in 2021. *(IDF DIABETES ATLAS tenth edition, 2021(3)).*

1.1.2 Classification

The origins of DM date back to ancient times, as evidenced by a description of polyuria in an Egyptian papyrus around 1550 BC. In the 2nd century BC, Aretaeus of Cappadocia offered a detailed account of type 1 DM (T1DM), coining the term "diabetes" to depict the condition characterized by constant urination, insatiable thirst, and short survival (4). "Diabetes" refers to a metabolic disorder associated with carbohydrate, lipid, and protein metabolism disruptions. It is defined by persistent hyperglycemia resulting from either lack of insulin secretion, insulin resistance in peripheral tissues, or a combination of both. The consequences of DM include complications such as cardiovascular (CV) complications, neuropathy, retinopathy, and nephropathy (5). DM can be categorized into the following classes according to the "Standards of Care in Diabetes" by the American Diabetes Association (ADA) and IDF.

1. T1DM: It results from the autoimmune destruction of insulin-producing β-cells in the pancreas. It is usually diagnosed in children and young adults.
2. Type 2 DM (T2DM): It is characterized by insulin resistance, where the body's cells do not respond effectively to insulin, and progressive β-cell dysfunction. It is the most common form of DM and is often associated with lifestyle factors and genetics. It can develop at any age but is more common in adults.
3. Gestational DM: This condition occurs during pregnancy and is characterized by glucose intolerance. It increases the risk of developing T2DM for both the mother and child later in life.
4. Other Types of DM: This category includes specific types of DM with known causes, such as monogenic diabetes syndromes (resulting from a single gene mutation) and DM associated with certain medical conditions or medications (3,6).

T1DM represents approximately 5-10% of DM cases in adults. It is a complex condition characterized by a deficiency in insulin due to the loss of pancreatic β-cells, resulting in elevated blood glucose levels. Various factors contribute to its pathogenesis, including genetics, environmental factors, microbiome, metabolism, and immune systems (7). While symptoms typically manifest during childhood or adolescence, they can also

emerge later. The typical symptoms of T1DM are polydipsia, polyuria, sudden weight loss, fatigue, constant hunger, and blurred vision (3). There is currently no cure for the condition, necessitating lifelong insulin injections for patients. Management of T1DM involves a combination of insulin therapy, blood glucose monitoring, and lifestyle modifications (8).

T2DM is the most common type of DM. Its development is primarily caused by a combination of two main factors: defective insulin secretion by pancreatic β-cells and the inability of insulin-sensitive tissues to respond to insulin (9). T2DM is often associated with risk factors such as obesity, a sedentary lifestyle, family history of DM, age, and certain ethnicities (10). While T2DM primarily manifests in adults, its prevalence in children and adolescents has become a concern due to rising rates of obesity and metabolic syndrome in this population. The treatment of T2DM requires personalized approaches. Lifestyle changes, such as a healthy diet, regular physical activity, effective weight management, and medications (e.g., antidiabetic drugs and insulin), are critical in glucose control (11).

1.1.3 Diagnosis

The diagnosis of DM is based on a combination of clinical symptoms, laboratory tests, and the monitoring of blood glucose levels (6). Following the present recommendations from both the ADA and the World Health Organization (WHO), a diagnosis can be confirmed if any of the following criteria are met:

- The fasting plasma glucose ≥ 7.0 mmol/L. Fasting is defined as no calorie intake for at least 8 hours before the test.
- Two-hour plasma glucose ≥ 11.1 mmol/L during oral glucose tolerance test. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75g of anhydrous glucose dissolved in water.
- Glycosylated hemoglobin (HbA1c) ≥ 6.5% (48 mmol/mol).
- In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, random plasma glucose ≥ 11.1 mmol/L (12).

1.1.4 Complications

Hyperglycemia or uncontrolled blood glucose levels can lead to various DM complications. 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 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 (13). Patients with all forms of DM are at increased risk of developing serious acute and chronic complications. Diabetic ketoacidosis and severe hypoglycemia are the major life-threatening acute complications of diabetic patients. Poor glycemic control, misdiagnosis, lack of access to insulin, or low socioeconomic status result in acute complications leading to permanent neurological consequences or death (14). Adults with DM have 2-4 times increased CV risk compared with adults without DM, and this is a significant risk factor that can lead to the development of heart failure (HF) (19). The primary focus of this thesis is diabetic cardiomyopathy (DCM), which will be extensively discussed in the following chapter.

1.2 Diabetic cardiomyopathy

1.2.1 Background and prevalence

Rullber *et al*. introduced the definition of DCM in 1972 (15). 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 (16). In diabetic patients, the prevalence of DCM reached 6.1 million in 2020 (17). Generally, the prevalence of cardiac dysfunction with T1DM and T2DM has been reported to be as high as 14.5% and 35.0%, respectively (18). The Framingham Heart Study showed that DM is a significant independent risk factor for developing HF. The risk of HF is 2.4-fold and 5-fold higher in diabetic men and women than in non-diabetic people (19).

1.2.2 Classification

The early stage of DCM is clinically asymptomatic, yet it is structurally defined by left ventricular (LV) stiffness and cardiac remodeling. Functionally, it is characterized by

impaired early diastolic filling, heightened atrial filling, and elevated LV end-diastolic pressure. The later stage of DCM is characterized by induction of LV hypertrophy, advanced cardiac remodeling, and diastolic dysfunction with apparent symptoms of heart failure with preserved ejection fraction (HFpEF). In addition, the progression of DCM leads to systolic dysfunction and heart failure with reduced ejection fraction (HFrEF), dilated left ventricle, shortened ejection period, prolonged pre-ejection performance, and increased filling pressures (20,21). Table 1 shows the comparative clinical classification between DCM and HF stages proposed by Maisch *et al*. (23), the New York Heart Association (NYHA), and the American College of Cardiology (ACC)/American Heart Association (AHA).

Table 1 Classification of DCM stages.

DCM: diabetic cardiomyopathy, HF: heart failure, NYHA: New York Heart Association, ACC: American College of Cardiology, AHA: American Heart Association, HFrEF: heart failure reduced ejection fraction.

Classification	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
DCM Stages	Early	Middle	Middle/late	Late
Characteristics	HF with normal EF, hypertrophy	HF with normal EF, hypertrophy	Including hypertension, microangiopathy	HFrEF including coronary artery disease
Structural changes	Hypertrophy	Hypertrophy Dilatation Fibrosis	Dilatation Fibrosis Microangiopathy (small vessel disease)	Dilatation Fibrosis Microangiopathy Macroangiopathy
Functional changes	Diastolic dysfunction	Diastolic and systolic dysfunction	Diastolic and systolic dysfunction	Diastolic and systolic dysfunction
NYHA classes	**Class 1**	**Class 2**	**Class 3**	**Class 4**
Characteristics	Asymptomatic, no limitation of physical activity	Slight limitation during ordinary physical activity, with fatigue, palpitation, dyspnoea, or angina	Marked limitation, with symptoms occurring during minimal physical activity	Symptoms present at rest Unable to carry out any physical activity without discomfort
ACC/AHA HF stages	**Stage A**	**Stage B**	**Stage C**	**Stage D**
characteristics	At risk of HF, but no structural heart disease or symptoms	Asymptomatic structural heart disease	Symptomatic HF with structural heart disease	Refractory HF requiring specialist interventions

1.2.3 Diagnosis

Cardiac imaging is the gold-standard diagnostic tool for detecting structural and functional cardiac changes in DCM. It includes both noninvasive techniques (echocardiography, magnetic resonance imaging, doppler imaging, pulsed wave Doppler, and electrocardiography) and invasive methods (endomyocardial biopsy) (21,22).

Furthermore, various circulating biomarkers have been investigated for their role in predicting clinical outcomes in DCM. Brain natriuretic peptide (BNP) is predominantly released in response to LV volume expansion and pressure overload (23). BNP are useful sensors for systolic/diastolic dysfunction in symptomatic DM patients which promote diuresis, natriuresis, hypotension, and smooth muscle relaxant activities (24). Cardiac troponin I, a regulatory protein controls calcium-mediated interaction between actin and myosin and is a clinical indicator of myocardial cell damage (25). Troponins have also been associated with myocardial contractility in various cardiomyopathies. Increased serum troponin levels are generally assumed to reflect severe cardiomyocyte injury associated with LV hypertrophy and HF (26). Klotho has received great attention in the past few years as a possible sensitive and specific marker for chronic kidney disease (CKD) and cardiovascular disease (CVD). Klotho is released from the kidney and plays a main role in the progression of hypertension, DM, CVD, and kidney diseases. Many studies indicate that a lack of Klotho contributes to aging and CV damage. In contrast, increased expression impedes vascular calcification, LV hypertrophy, and fibrosis in the myocardium, and reduces atrial stiffness (27). Klotho proteins form 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 (28,29). Alterations in the levels of these biomarkers may indicate myocardial structural and functional dysfunction.

1.2.4 Pathogenesis

DCM develops independently of hypertension or coronary heart disease. The underlying pathological factors are complex and include metabolic disturbances, systemic and cardiac inflammation, oxidative stress, hypoxia, and the overactivation of the renin-angiotensin-aldosterone system (RAAS) in both T1DM and T2DM (22). These factors

collectively contribute to the development and progression of DCM, underscoring the complex interplay between metabolic dysregulation and cardiac pathology in diabetic patients.

1.2.4.1 Hyperglycemia

Hyperglycemia is the main trigger of DCM and induces several metabolic, molecular, and functional changes in cardiomyocytes (30). Under euglycemic conditions, glycolysis contributes to the cardiomyocyte energy formation to support systolic and diastolic functions (31). In T1DM, due to insulin deficiency, glucose metabolism redirects away from the glycolytic pathway towards alternative pathways, such as the polyol and hexosamines pathway, and the formation of advanced glycation end products (AGEs). The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol and converted to fructose (32). The hexosamine pathway in cardiomyocyte proteins undergoes enzymatic *O*-GlcNAcylation (33). Besides, hyperglycemia also promotes the non-enzymatic reaction between glucose, proteins, and lipids, leading to the formation of AGEs through a process known as glycation (34).

AGEs exert their effects by binding to the receptor for AGEs (RAGE) and activating intracellular signaling pathways, leading to increased oxidative stress through the formation of reactive oxygen species (ROS), triggering inflammation by activating the nuclear factor kappa B (NF-κB) pathway which in turn upregulates the expression of pro-inflammatory cytokines and chemokines (35). It also causes tissue damage by altering protein structure and function, impairing cardiac elasticity and function (35). Hyperglycemia itself, along with the byproducts of the alternative pathway, can trigger complex molecular pathways in the heart, including increased levels of ROS, accumulation of AGEs, altered calcium regulation by mitochondria, overactivation of the RAAS, transforming growth factor-β (TGF-β) production, and cardiac remodeling by accumulation of extracellular matrix (ECM). These processes ultimately result in cardiomyocyte apoptosis, LV hypertrophy, and impaired systolic and diastolic function (36).

1.2.4.2 Inflammation

Inflammation plays a significant role in the progression of DCM. Systemic and local inflammation (cardiomyocytes, fibroblasts, coronary smooth muscle, and endothelial cells) is associated with abnormal cardiac function and structure (37). Dysregulated glucose metabolism and dyslipidemia directly lead to the activation of white blood cells and the infiltration of inflammatory neutrophils, monocytes, and macrophages in the heart of DCM patients. Hyperglycemia and insulin deficiency trigger the upregulation of pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), which are optimal clinical markers used for monitoring and progression of HF patients (38), and chemokines, such as monocyte chemoattractant protein 1 (MCP-1), activating a common signaling pathway involving NF-κB transcription factor (39). Activation of NF-κB results in exacerbated downstream proinflammatory cytokines release (40).

Besides hyperglycemia, various mechanisms lead to its activation during DM. The interplay between oxidative stress, AGEs, inflammation, and RAAS activation contributes to NF-κB activation in the myocardium during T1DM. NF-κB activation has been demonstrated to promote adverse cardiac remodeling, hypertrophy, apoptosis, and fibrosis, leading to the development and progression of DCM (39,41).

1.2.4.3 RAAS

The RAAS is a crucial regulator of blood volume, electrolyte balance, and systemic vascular resistance. It is a hormonal cascade activated by the release of renin from juxtaglomerular cells in the kidneys. Renin cleaves the hepatic angiotensinogen into angiotensin (Ang) I, which is further transformed into Ang II by pulmonary angiotensin-converting enzyme. Ang II is the main RAAS mediator, binding to angiotensin type 1 receptor (AT1R) in different tissues, including the heart (42). Ang II is a potent vasoconstrictor and acts directly to increase vascular smooth muscle tone (43). Ang II contributes to blood pressure (BP) regulation through several mechanisms, including vasoconstriction, renal sodium and water retention, activation of the sympathetic nervous system, and aldosterone release from the adrenal glands (44). Hyperglycemia induces both systemic and cardiac RAAS activation. This activation stimulates an increase in the

levels of Ang II and aldosterone (45). Patients with DM exhibit a 3.4-fold higher concentration of Ang II compared to non-diabetic individuals (46). Increased production of Ang II and aldosterone enhances stimulation of the AT1R and of mineralocorticoid receptor signaling in heart muscle, triggering an adaptive pro-inflammatory response. This contributes to oxidative stress, increased cytokine expression, and the adherence and infiltration of leukocytes and macrophages. Together, these processes further stimulate cardiac fibrosis, leading to impaired contractile function, diastolic dysfunction, and potentially HF (47,48).

Moreover, Ang II and aldosterone are implicated in inhibiting insulin metabolic signaling in insulin-sensitive tissues, which plays a role in impaired endothelial-mediated vascular relaxation and hypertension development. Over time, this process promotes insulin resistance through non-genomic mechanisms. Prolonged abnormal activation of RAAS due to hyperglycemia has been associated with increased arterial pressure and vascular resistance (43).

1.2.4.4 Fibrosis

Myocardial fibrosis is one of the main histological manifestations of DCM. It causes myocardial damage, including increased myocardium stiffness and cardiac diastolic and systolic dysfunction (49). Experimental evidence suggests that many different mediators could promote myocardial fibrosis in DM. As mentioned earlier, hyperglycemia is the main trigger in DCM, which increases oxidative stress and the inflammatory response and indirectly activates neurohumoral factors, growth factors, and adipokines (50). Inflammatory cytokines and Ang II stimulate the production and secretion of TGF-β, the main regulator of myocardial fibrosis. TGF-β is produced as an inactive precursor, known as latent TGF-β. Once activated through proteolytic cleaves by different mediators (such as plasmin, matrix metalloproteinase 2 and 9) (51). TGF-β is present in both cardiomyocytes and myocardial fibroblasts and binds to TGF-β type 1 receptors (ALK5) to initiate intracellular signaling cascades. ALK5 activation initiates canonical TGF-β signaling through the Smad protein pathway, which translocates to the nucleus and regulates gene expression involved in ECM synthesis (e.g., collagen I, collagen III, and fibronectin) (51). TGF-β also promotes the expression of profibrotic markers like platelet-

derived growth factor (PDGF) and connective tissue growth factor (CTGF) (52,53). In addition, TGF-β regulates fibroblast activation. TGF-β released by cardiomyocytes can activate cardiac fibroblasts, promoting their transition into myofibroblasts. Increased production of ECM proteins leads to fibrotic remodeling of the myocardium, characterized by the accumulation of collagen fibers and stiffening of the cardiac tissue (54).

1.2.5 Treatment

The most effective intervention strategy for DCM is to effectively manage lifestyle modifications, improve glycemic control, hypertension, and dyslipidemia if present, and reduce cardiac adverse effects. Regular physical exercise and a nutritious diet are crucial for managing DM, especially considering global obesity prevalence (55). The new classes of antidiabetic drugs with the most effective and novel mechanisms of action are sodium-glucose cotransporter-2 inhibitors (SGLT2i), glucagon-like peptide-1 receptor agonists (GLP-1RA), and dipeptidyl-peptidase-4 inhibitors (DPP- 4i). GLP-1RA and DPP- 4i regulate blood glucose levels by targeting the incretin system. GLP-1 and gastric inhibitory peptides are produced by the gastrointestinal system and increase insulin production. DPP- 4 facilitates the degradation of GLP-1 by inhibiting DPP- 4, leading to an elevation in GLP-1 and gastric inhibitory peptide levels, which stimulates insulin release, resulting in a decrease in blood glucose levels (56). GLP-1RA enhances myocardial insulin sensitivity, increases glucose uptake, and prevents cardiomyocyte apoptosis, contributing to DM management (57). However, preliminary studies of DPP-4i are controversial regarding cardioprotection (58).

SGLT2i are novel oral glucose-lowering drugs. Several large clinical trials have shown the CV safety of SGLT2i in T2DM (59–62). Each of these studies was associated with a reduction in the relative risk of HF, suggesting a class effect of SGLT2i. In addition, dapagliflozin (DAPA) was more effective than placebo in preventing CV death and HF, regardless of the presence or absence of DM. The latest AHA/ACC/ Heart Failure Society of America guidelines recommend their use in stage A HF (63). The role of SGLT2i is discussed in a later section of the thesis. RAAS inhibitors (RAASi) are the primary standard therapy when hypertension and albuminuria present (64) or for diabetic patients

with macrovascular complications plus hypertension, HF, or CKD (65,66). They have been shown to reduce all causes of CVD mortality in diabetic patients with HF and asymptomatic LV dysfunction (67). Furthermore, RAASi reduce myocardial fibrosis and LV stiffness in DCM. RAASi are not recommended for diabetic normotensive patients without pre-existing CV complications. T1DM patients are younger at the onset of the disease and lose more life-years due to CVD. Clinical investigations with low-dose RAASi in T1DM-related CV complications are lacking. Therefore, to address this gap, we designed a study to explore the potential benefits of RAASi in T1DM-related CVD.

1.3 Novel antidiabetic drugs: SGLT2 inhibitors

1.3.1 The role of SGLTs in glucose reabsorption

The kidneys play a crucial role in regulating glucose levels through three well-known pathways: glucose utilization for energy needs, gluconeogenesis, and glucose reabsorption (68). Healthy kidneys can filter 140-160 g of glucose daily, with almost 99% reabsorbed in the proximal convoluted tubule (PCT) (69). SGLT2s are characterized by a high-capacity and low-affinity glucose transporter that utilizes one sodium ion per glucose molecule to transport glucose into the intracellular space. They are specifically located in the brush border of the PCT (S1/S2 segment) of kidney nephrons, accounting for the reabsorption of the majority of glucose (> 90%) under normoglycemic conditions (Figure 2) (70,71). On the other hand, the low-capacity and high-affinity SGLT1, which exhibits a similar structure to SGLT2 and transports two sodium ions per glucose molecule into the intracellular space, is abundant in the brush border of the distal part (S2/S3 segment) of the PCT, and accounts for the reabsorption of remnant glucose (< 10%) (72). Studies indicate that patients with T2DM show higher glucosuria threshold and improved glucose reabsorption, possibly due to increased SGLT2 expression in the kidneys (73).

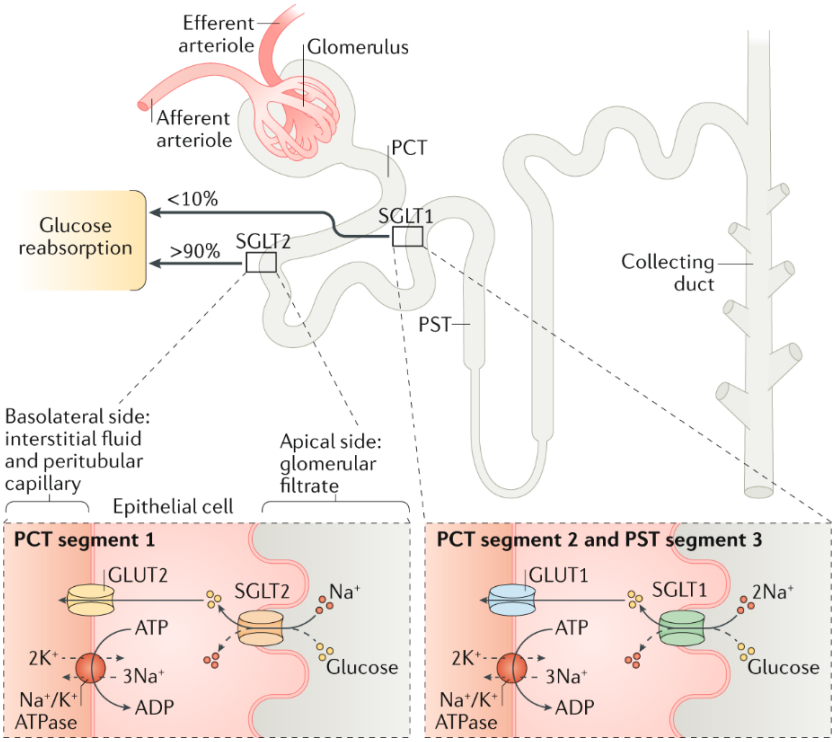

Figure 2 Renal glucose reabsorption is mediated by SGLT2 and SGLT1. In segment S1 of the PCT, glucose transport is facilitated by SGLT2, which actively transports glucose into cells by coupling it with the inward diffusion of sodium ions. This sodium ion influx is sustained by a Na^+/K^+ ATPase pump on the basolateral membrane. Glucose is then transported from cells into the interstitial compartment via GLUT2. SGLT1 and GLUT1 employ a similar transport mechanism in segment S3. Based on *Cowie MR* and *Fisher M* (74).

1.3.2 SGLT2i

Phlorizin was discovered by French chemists in 1835 from the root bark of apple trees. It was initially indicated as a treatment for malaria (75). It is a potent glucosuric agent that effectively inhibits both SGLT1 and SGLT2 but has a low bioavailability. Since the inhibition of SGLT1 leads to glucose-galactose malabsorption associated with diarrhea, dehydration, and other adverse reactions, phlorizin was not considered a suitable drug candidate (76). As a result, scientists have made chemical modifications to the structure of phlorizin, resulting in the development of novel derivatives such as O-glucoside, C-glucoside, and N-glucoside. C-glucosides are widely utilized in medical settings due to their notable SGLT2 selectivity and safety profile (77). DAPA was the frontrunner of C-glucoside-based SGLT2i development, demonstrating strong inhibitory potential with a

1200-fold selectivity for SGLT2 over SGLT1 compared to phlorizin's 10-fold selectivity. SGLT2i are used as second-line treatments for T2DM and are off-label for T1DM (78,79). Forxiga™ (DAPA) was the first agent approved by the European Medicines Agency and then by the Food and Drug Administration (FDA) in the United States for the treatment of T2DM, followed by canagliflozin (Invokana), empagliflozin(Jardiance®), ertugliflozin (Steglatro®) and bexagliflozin in 2023. Sotagliflozin, a dual SGLT1 and SGLT2 inhibitor, was approved for use by T1DM patients in Europe, although it has not yet been launched for clinical use. The use of SGLT2i has increased significantly, primarily due to their unique mechanism of action and favorable CV and renal outcomes. This new class of oral drugs has similar pharmacokinetic characteristics: rapid oral absorption, an extended elimination half-life, extensive hepatic metabolism, minimal renal elimination of the parent drug, and the lack of clinically relevant drug-drug interactions (80,81). Notably, all of the SGLT2i induce sustained glucosuria associated with reduced blood glucose levels in T2DM, and their efficacy remains unchanged despite progressive β-cell dysfunction and/or insulin resistance. Additionally, they can be prescribed as add-on therapy to other antidiabetic agents.

DAPA was first FDA-approved in 2020 to reduce the risk of CV death and hospitalization for HF in patients with reduced ejection fraction (HFrEF), regardless of diabetes status (82). In May 2023, the FDA expanded the indication of DAPA to include HF across the entire spectrum of LV ejection fraction. This includes HF with mildly reduced ejection fraction (HFmrEF) and HFpEF (83).

1.3.3 The protective effects of SGLT2i

CV complications are prevalent and have severe outcomes in diabetic patients. As per FDA regulations, all anti-diabetic drugs must provide evidence of their safety through CV outcome trials (CVOTs). Four significant CVOTs are Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients-Removal of Excess Glucose (EMPA-REG OUTCOME), Canagliflozin Cardiovascular Assessment Study (CANVAS), Dapagliflozin Effect on Cardiovascular Events Thrombolysis in Myocardial Infarction 58 (DECLARE-TIMI 58) and Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial (VERTIS CV) investigated the cardiovascular benefits

and safety of SGLT2i in T2DM patients (59–62). The purpose of these trials was to examine the CV safety of SGLT2i. These studies have shown a decrement in major adverse CV events (MACE), which include CV death, non-fatal cerebral infarction, and non-fatal myocardial infarction (84). Reductions in the risk of hospitalizations for HF of 30%, 33%, 27%, and 35% were reported in these trials (59–62). Furthermore, a meta-analysis of CVOTs involving 34,000 patients revealed that SGLT2i resulted in an 11% reduction in MACE (85). Following these promising results, clinical trials were explicitly initiated for people with HF, including participants with or without T2DM. The first dedicated HF study with DAPA (Dapagliflozin in Patients With Heart Failure and Reduced Ejection Fraction, DAPA-HF) showed that treatment significantly reduced the risk of a first HF event and death from CV causes in patients with reduced EF irrespective of the presence of T2DM.

CV and Renal Outcomes with Empagliflozin in Heart Failure Reduced Ejection Fraction (EMPEROR-R) (86), Empagliflozin in Heart Failure with a Preserved Ejection Fraction (EMPEROR-P) (87), and Dapagliflozin in Heart Failure with Mildly Reduced or Preserved Ejection Fraction (DELIVER) (83) treatment given either DAPA or empagliflozin was associated with 26% and 23% reduced the risk of the CV death or hospitalization for HF (88). The expanded indication for DAPA in with HF, including those with HFmrEF and HFpEF, is based on data from the Phase 3 DELIVER trial. In this study, DAPA demonstrated a statistically significant and clinically meaningful early reduction in the primary composite endpoint of CV death or worsening HF in patients with HFmrEF or HFpEF regardless of DM (83). Large randomized CV outcome trials in patients with DM and HF carried out with SGLT2i are presented in Table 2.

The explanations for the reduction in HF hospitalizations and the exact mechanisms associated with these CV benefits still need to be completed, but several hypotheses have been proposed. Firstly, Studies have confirmed that SGLT2i can reduce HbA1c and reduce the risk of hypoglycemia, resulting in CV benefits. SGLT2i causes glycosuria and impaired energy balance, leading to weight loss (89). SGLT2i can exert antihypertensive effects in many ways such as decreased uric acid levels, metabolic fuel switching (ketogenic) activity, reduced body weight, hemodynamic mechanisms secondary to

volume depletion caused by diuresis and natriuresis, and thus play a role in CV protection (90).

Table 2 An Overview of randomized double-blinded large-scale clinical trials investigating CV advantages of SGLT2i in patients with DM and HF.

HF: heart failure, CV: cardiovascular, MACE: major adverse cardiovascular events, HFmrEF: HF with mildly reduced ejection fraction, HFpEF: HF with preserved ejection fraction, HFrEF: HF with reduced ejection fraction; HR: hazard ratio, SGLT2: sodium-glucose cotransporter 2, T2DM: type 2 diabetes mellitus.

Trial name	Patient Population	Patient Numbers	SGLT2 inhibitor (*vs.* placebo)	Primary Outcome	HR
EMPA-REG (2015)	T2DM and CVD risk	7020	Empagliflozin	MACE	0.86
CANVAS (2017)	T2DM and high CVD risk	10,142	Canagliflozin	MACE	0.86
DECLARE-TIMI 58 (2019)	T2DM and high CVD risk	17,160	Dapagliflozin	MACE	0.93
DAPA-HF (2019)	HFrEF ± T2DM	4744	Dapagliflozin	worsening HF or CV death	0.74
VERTIS-CV (2020)	T2DM and high CVD risk	8246	Ertugliflozin	MACE 12%	0.99
EMPEROR-R (2020)	HFrEF ±T2DM	3730	Empagliflozin	hospitalization for worsening HF or CV death	0.75
SOLOIST–WHF (2021)	hospitalization for HF + T2DM	1222	Sotagliflozin	hospitalizations and urgent visits for HF, or CV death	0.67
EMPEROR-P 2021	HFmrEF or HFpEF ±T2DM	5988	Empagliflozin	hospitalization for HF or CV death	0.79
DELIVER (2022)	HFmrEF or HFpEF ± T2DM	6263	Dapagliflozin	worsening HF or CV death	0.82

The direct cardioprotective effects of SGLT2i are associated with reducing preload and afterload by enhancing urinary glucose excretion and lowering blood volume, thereby lowering cardiac preload and BP. They improve cardiac metabolism and bioenergetics by shifting myocardial metabolism from glucose to ketone bodies. Besides, they also enhance CV function by reducing oxidative stress and endothelial cell inflammation, thereby promoting favorable effects on cardiac remodeling (91). Moreover, SGLT2i could decrease cardiac fibrosis by alleviating TGF-β production, regulating macrophage polarization (92), and altering the production of adipokines, cytokines, chemokines, and the amount of epicardial adipose tissue (93).

Overall, this study highlights DCM, a condition characterized by structural and functional cardiac changes linked to molecular mechanisms that lead to HF. The analysis of a randomized clinical trial involving patients with HF revealed that the addition of DAPA to therapy significantly reduced the risk of worsening HF or CV death, independently of diabetes status. For these reasons, we aimed to investigate the efficacy of DAPA in mitigating the progression of CV complications in T1DM. There are likely to be multiple mechanisms underpinning these benefits, from improvements in cardiometabolic factors to cardiac remodeling.

2 OBJECTIVES

Our studies aimed to explore novel therapeutic strategies to improve the management of DM-related CV 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 LV.
3. To characterize the preventive impact of DAPA on the progression of CV 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).

Materials

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), and all standard plastic laboratory equipment was purchased from Sarstedt (Numbrecht, Germany) unless specified otherwise.

3.1 Animals and experimental design

Experiments were performed on eight-week-old male Wistar rats (*Rattus norvegicus*) purchased from Toxi-Coop Ltd. (Budapest, Hungary). Rats were housed in groups of three in plastic cages under controlled light/dark (12:12 hour light/dark cycle) with a constant temperature of 24±2 °C. They had *ad libitum* access to standard rodent chow and tap water. A qualified person monitors the health of the animals daily, focusing on housing, husbandry, enrichment, and socialization to prevent distress.

T1DM was chemically induced with a single intraperitoneal injection of 65 mg/bwkg streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 4.5). 72 hours after the STZ injection and following overnight fasting, blood glucose levels were measured three times from the tail vein using a Dcont IDEAL device (77 Elektronika, Budapest, Hungary). Rats with a peripheral blood glucose value above 15 mmol/L were enrolled in the study. As controls, non-diabetic, age- and body-weight-matched healthy animals (control: $n = 6$ animals/group) received an equivalent volume of citrate buffer without STZ once and the same amount of isotonic saline (NaCl 154 mmol/L) through oral gavage daily. They followed the same treatment duration as the diabetic animals in both protocols in this study. Two different experimental protocols were used:

Protocol I

Following five weeks of T1DM induction, the rats were randomized into four groups (*n* = 6 animals/group). The dosages of RAASi were based on prior experiments conducted by our research group, ensuring the effectiveness of RAAS blockade without affecting systemic BP (94,95). 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)
5. Eplerenone dissolved in isotonic saline (D + EPL, 50 mg/bwkg/day)

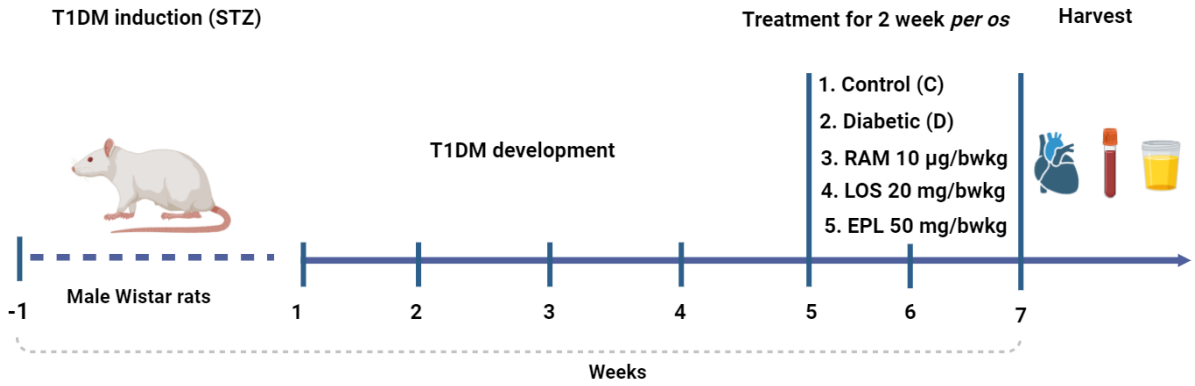

Figure 3 Experimental Design of Protocol I. After five weeks of DM, rats were randomized into five groups (*n* = 6/group) and treated daily by oral gavage for two weeks with isotonic saline as C: control or vehicle (D: diabetic), or D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone.

Protocol II

Diabetic (D) rats were randomly divided into four groups immediately after the onset of diabetes (*n* = 6/groups). The 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)

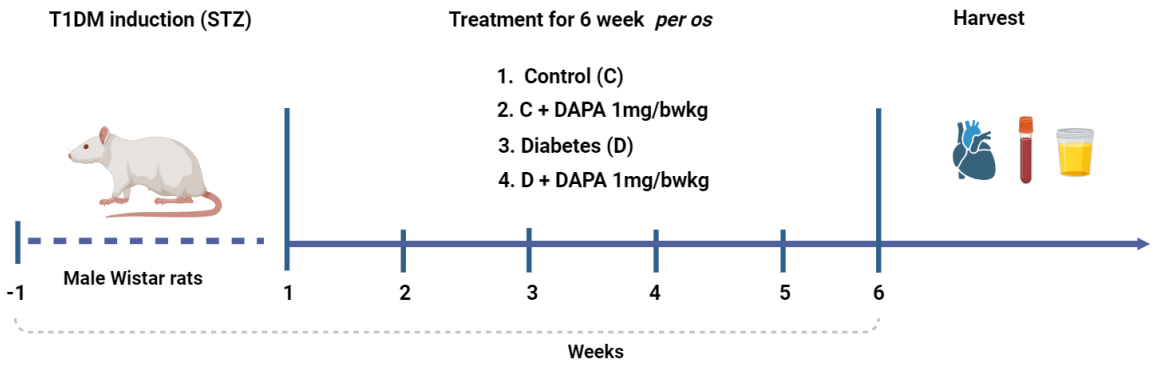

Figure 4 Experimental Design of Protocol II. Rats were randomly divided into four groups immediately after the onset of diabetes (n = 6/groups) and treated daily by oral gavage for six weeks with isotonic saline as C: control or vehicle (D: diabetic), or C + DAPA: dapagliflozin-treated control non-diabetic group, D + DAPA: dapagliflozin treated diabetic group.

In both protocols, rats were placed into metabolic cages for a 24-hour period to collect urine before euthanasia. At the end of the experiments, BP was measured, and rats were anesthetized by a mixture of 75 mg/bwkg ketamine (Richter Gedeon, Budapest, Hungary) and 10 mg/bwkg xylazine (Medicus Partner, Biatorbagy, Hungary). 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 Measurement of arterial blood pressure

Systolic and diastolic pressures were measured on the tail vein using a non-invasive CODA Standard monitor system (EMKA Technologies, Paris, France), which uses proprietary volume pressure recording that is clinically validated. Mean arterial pressure (MAP) was calculated. The recording was conducted in an appropriate environment devoid of any distractions.

3.3 Measurement of metabolic and cardiac parameters

Blood samples were collected and centrifuged at 3600 rpm for 6 min. The serum and urine samples were photometrically determined with commercially available kits on a

Hitachi 912 photometric chemistry analyzer (Roche Hitachi, Basel, Switzerland). Glucosuria was measured from 24-hour collected urine.

3.4 Measurement of Klotho and Cardiac Troponin I

Serum levels of Klotho and cardiac troponin I were measured using the Rat Klotho ELISA Kit (ABclonal, Woburn, MA, USA) and the Rat Cardiac Troponin I ELISA Kit (Abcam, Cambridge, UK), respectively, according to the manufacturer's protocols. The concentration was determined by measuring the absorbance at 450 nm with wavelength correction at 650 nm using 96-well microplates with a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany).

3.5 Cardiac histology

Orcein-staining

The amount of elastic fiber in the aorta section was determined using the orcein staining. The aorta was dissected under a light microscope, immediately fixed in 8% paraformaldehyde, and embedded in paraffin for immunohistochemistry. 5 μm sections were deparaffinized in xylene and rehydrated (100%, 90%, 70% ethanol, and distilled water). Sections were immersed in 1% orcein at 60 °C for 30 min, followed by differentiation in acid-alcohol (1:99 hydrochloric acid and 70% ethanol) for 10 seconds to remove the dye excess, and then into distilled water. Histological examination was performed under 20x objective magnification using Case Viewer 2.4. (3DHISTECH, Budapest, Hungary). Intima-media thickness (IMT) was measured on cross sections of the aorta, and the mean value of ten measurements was calculated.

Picrosirius red staining

Picrosirius red staining was used to evaluate collagen accumulation and fibrosis of the left ventricle. Fresh frozen heart tissues were sectioned (4 μm thickness) and stained, and slides were digitalized with a Pannoramic1000 slide scanner (3DHistech, Budapest, Hungary). The slides were visualized with SlideViewer2.5 (3DHistech, Budapest, Hungary), and intramyocardial collagen represented a red-colored area was measured with the Quant Center HistoQuant 2.5 module (3DHistech, Budapest, Hungary). The myocardial field of measurement was manually selected with the caution of avoiding the

endocardium and perivascular connective tissue. The surface measurement was 1,6 mm^2 in each heart. The red-stained area was measured based on the red color intensity. First, multiple optimization measurements were performed with the supervision of an experienced CV histopathologist, reaching the best RGB pixel intensity parameters: red channel 84-221, Green channel 8-142, and blue channel 10-137. Then, the red-stained area measurements were performed on each histological slide.

3.6 Lyophilization of the tissue samples

Heart tissue samples from protocol I were frozen at -80°C after collection, then lyophilized using a ScanVac CoolSafe Touch Superior device (LaboGene A/S, Allerod, Denmark). The samples were arranged in 2 ml tubes to maximize surface exposure, and the tubes were left open during the freeze-drying process. Pre-freezing was done at -40°C for 1 hour, followed by primary drying in six 2-hour steps at 0.22 hPa with a gradual increase in temperature up to 30°C. Secondary drying was conducted at 0.1 hPa and 40°C for 3 hours. After drying, the tissue products were manually smashed with 20 Gauge needles and further pulverized using a TissueLyser LT. The powdered tissue samples were stored at 4°C until further analysis.

3.7 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using the Total RNA Mini Kit (Geneaid Biotech, New Taipei City, Taiwan). Measurement of quality and quantity of isolated RNA was performed by NanoDrop ND-1000 spectrophotometer (Baylor College of Medicine, Houston, TX, USA). First-strand cDNA was reverse-transcribed using the Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA). 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*) were determined by using LightCycler 480 SYBR Green I Master enzyme mix (Roche Diagnostics, Indianapolis, IN, USA) and specific primers listed in Table 2. Data were analyzed by LightCycler® 480 software (version 1.5.0.39, Roche Diagnostics). The above mentioned gene expressions were normalized to *Rn18S* as a housekeeping gene.

Table 3 Sequences of primer pairs for quantitative RT-PCR.

Gene	NCBI reference	Primer pairs	Product length (bp)
Nppb	NM_031545.1	Forward: 5’ TGACGGGCTGAGGTTGTTTT 3’ Reverse: 5’ ACACTGTGGCAAGTTTGTGC 3’	198
Il1b	NM_031512.2	Forward: 5’ GACTTCACCATGGAACCCGT 3’ Reverse: 5’ GGAGACTGCCCATTCTCGAC 3’	104
Il6	NM_012589.2	Forward: 5’ AGCGATGATGCACTGTCAGA 3’ Reverse: 5’ TAGCACACTAGGTTTGCCGA 3’	409
Tnf	NM_012675.3	Forward: 5’ ACTGAACTTCGGGGTGATCG 3’ Reverse: 5’ GCTTGGTGGTTTGCTACGAC 3’	153
Ccl2	NM_031530.1	Forward: 5’ GATCCCAATGAGTCGGCTGG 3’ Reverse: 5’ ACAGAAGTGCTTGAGGTGGTT 3’	294
Tgfb1	NM_021578.2	Forward: 5' GCACCGGAGAGCCCTGGATACC 3' Reverse: 5' CCCGGGTTGTGTTGGTTGTAGAGG 3'	222
Pdgfb	NM_031524.1	Forward: 5' TCGATCGCACCAATGCCAACTTCC 3' Reverse: 5' CACGGGCCGAGGGGTCACTACTGT 3'	236
Ccn2	NM_022266.2	Forward: 5' TCCACCCGGGTTACCAATGACAATAC 3' Reverse: 5' CTTAGCCCGGTAGGTCTTCACACTGG 3'	195
Fn	NM_019143.2	Forward: 5’ GGATCCCCTCCCAGAGAAGT 3’ Reverse: 5’ GGGTGTGGAAGGGTAACCAG 3’	188
Rn18S	NR_046237.1	Forward: 5' GCGGTCGGCGTCCCCCAACTTCTT 3' Reverse: 5' GCGCGTGCAGCCCCGGACATCTA 3'	105

3.8 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. The Kruskal-Wallis ANOVA on ranks was used for non-parametrical data, followed by Dunn’s correction. A significance level P value <0.05 was considered statistically significant.

4 RESULTS

4.1 Metabolic parameters remained unchanged with RAASi treatment.

After seven weeks of T1DM induction, metabolic parameters were measured. The rats showed significant weight loss and dysregulation of carbohydrate metabolism, such as elevated levels of blood glucose and fructosamine. Besides, the disruption of the lipid profile was observed. These changes confirm the development of T1DM. Treatment with low-dose RAASi did not affect these parameters, except for cholesterol levels, which were improved by losartan and eplerenone. Liver enzyme levels remained unchanged in all experimental groups (Table 4).

Table 4 RAASi treatment did not alter metabolic parameters. D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. HDL-C: high-density lipoprotein-cholesterol, GOT: glutamate-oxaloacetate transaminase, GPT: glutamate-pyruvate transaminase. Values indicate means±SDs. *p < 0.05 *vs*. control, **p < 0.01 *vs*. control, ***p < 0.001 *vs*. control, §p < 0.05 *vs*. diabetic, §§§p < 0.001 *vs*. diabetic (*n* = 6/group).

	Control	**Diabetic (D)**	**D + RAM**	**D + LOS**	**D + EPL**
Body weight (g)	338±14.7	283±46.7*	266±21.6	246±29.4	278±34.3
Non-fasting serum glucose (mmol/L)	12.8±1.68	37.4±7.36***	40.8±3.35***	41.8±6.44***	33.2±2.45***
Fructosamine (µmol/L)	153 ±8.28	243 ±16.3***	249 ±10.6***	256 ± 17.6***	239 ± 16.5***
Total cholesterol (mmol/L)	1.86±0.10	2.35±0.17**	2.08±0.16	1.98±0.21§	1.80±0.28§§§
Triglycerides (mmol/L)	0.58±0.14	1.28±0.69*	0.98±0.34	1.74±1.15	1.10±0.56
GOT (U/L)	134±10.5	239±95.4*	149±25.0	169±85.2	142±17.4
GPT (U/L)	29.6±2.42	128±78.7**	91.6±38.0	137±84.5	69.8±17.0

4.2 RAASi 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 BP. In line with the literature and our previous studies, 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 (96,97) (Table 5).

Table 5 Blood pressure was not affected by RAASi. Mean arterial pressure, heart rate, and the heart-to-body weight ratio of control, D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. Values indicate means ± SDs. *p < 0.05 *vs.* control, ***p < 0.001 *vs*. control (*n* = 6/group).

	Control	**Diabetic (D)**	**D + RAM**	**D + LOS**	**D + EPL**
Mean arterial pressure (mmHg)	77.4±11.9	76.2±6.86	76.5±11.8	74.5±17.2	77.7±17.1
Heart rate (bpm)	479±58.6	371±23.9***	375±22.9***	375±12.9***	329±22.0***
Heart-to-body weight ratio (%)	0.34±0.03	0.40±0.02*	0.43±0.04*	0.39±0.05	0.41±0.05*

4.3 RAASi mitigated aortic intima-media thickening.

Aortic IMT is an essential early biomarker of atherosclerosis, showing progressive changes with disease duration, suggesting its potential as an evolving biomarker (98). The histological examination of aortic IMT in the control, diabetic, and RAASi-treated rats showed distinct histological features. The control rats showed a wavy internal elastic lamina, while the diabetic rats displayed intimal thickening, irregularities, and diffused elastic membranes. RAASi reduced enhancement and pathohistological changes in the aorta of diabetic rats; however, only eplerenone reached the level of significance (Figure 5).

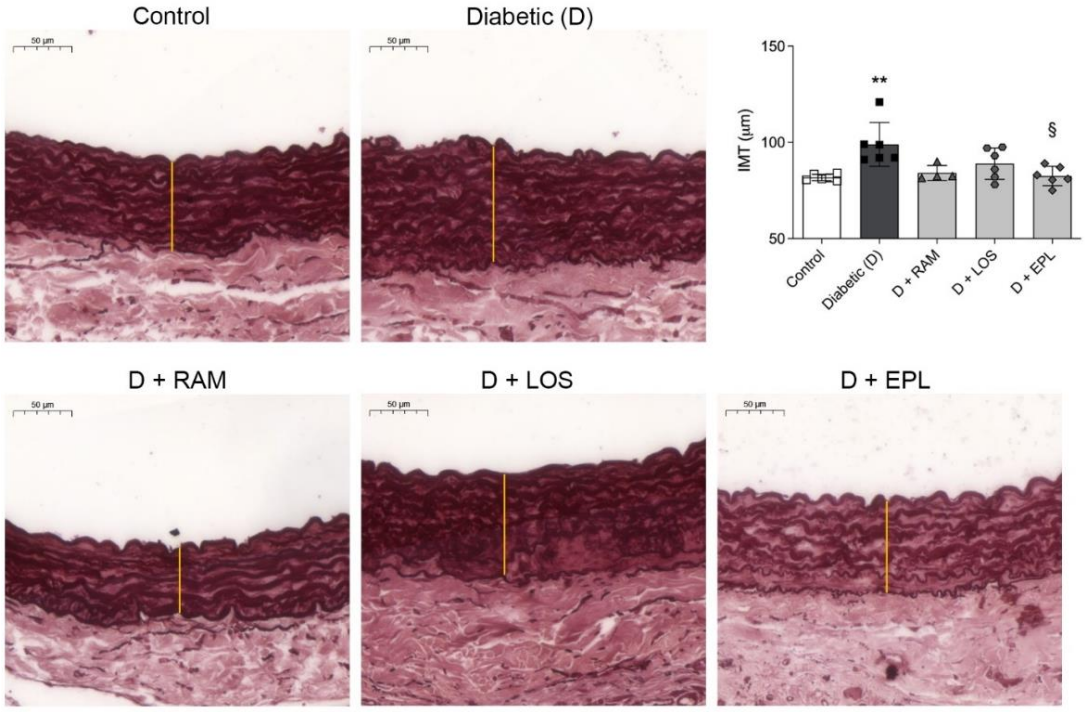

Figure 5 RAASi reduced aortic intima-media thickening (IMT) increment. Representative Orcein-stained aorta sections of control, D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. Elastic fibers are stained brown after Orcein staining. They are visualized as either thin fibers or elastic lamella. Original magnification, ×40. Scale bar: 50 µm. Bars indicate means ± SDs. **$p < 0.01$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic (n = 6/group).

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

BNP has been suggested as an important diagnostic biomarker of LV dysfunction and is widely used in clinical applications for risk stratification and management of patients with HF. BNP is mainly released in response to LV volume expansion and pressure overload (99). The mRNA expression of BNP (*Nppb*) levels significantly increased in the diabetic group and were mitigated with all RAASi treatments (Figure 6A).

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 (Figure 6B). Klotho is a novel cardioprotective factor (29), and its deficiency is one of the hallmarks in the progression of diabetic CV complications (100). We observed decreased serum levels of Klotho in diabetic rats. This decline was reversed only by eplerenone treatment (Figure 6C).

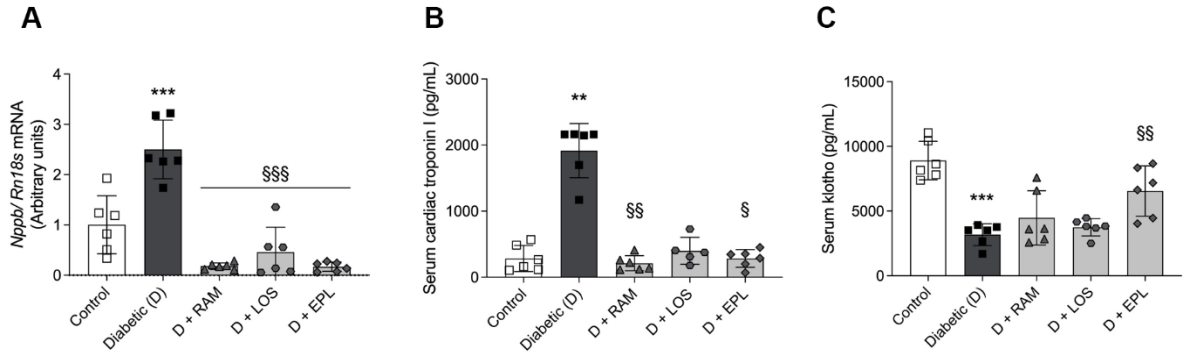

Figure 6 RAASi decreased specific biomarkers of myocardial injury. Control, D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. (A) mRNA expressions of B-type natriuretic peptide (*Nppb*). mRNA expressions were normalized to *Rn18S* mRNA expression; (B) the quantitative evaluation of serum cardiac troponin I level; (C) the quantitative evaluation of serum Klotho levels. Bars indicate means ± SDs. **p < 0.01 *vs.* control, ***p < 0.001 *vs*. control, §p < 0.05 *vs*. diabetic, §§p < 0.01 *vs*. diabetic, §§§p < 0.001 *vs*. diabetic (*n* = 6/group).

4.5 RAASi halted the progression of T1DM-induced myocardial fibrosis.

4.5.1 RAASi mitigated profibrotic growth factor levels.

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 (Figure 7A-C).

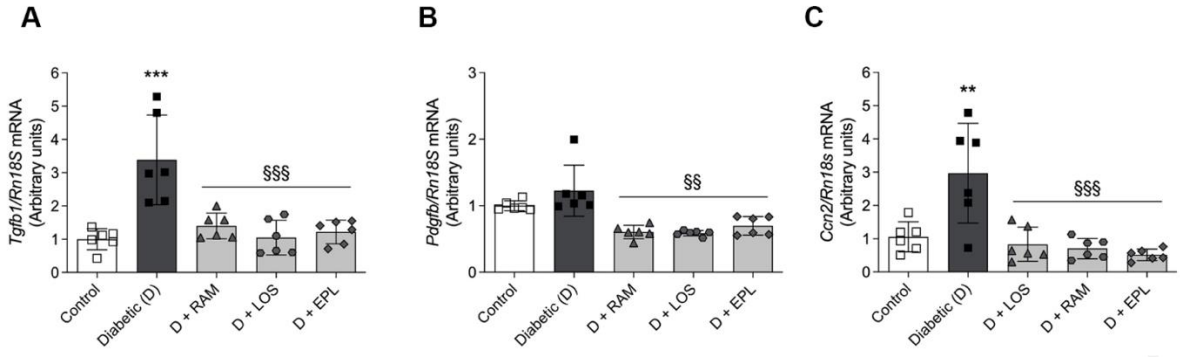

Figure 7 RAASi decreased the levels of profibrotic factors in the LV. Control, D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. (A-C) mRNA expression of transforming growth factor β (*Tgfb1*), platelet-derived growth factor (*Pdgfb*), and connective tissue growth factor (*Ccn2*). mRNA expressions were normalized to Rn18S mRNA expression. Bars indicate means ± SDs. **p < 0.01 *vs*. control, ***p < 0.001 *vs*. control, §§p < 0.01 *vs*. diabetic, §§§p < 0.001 *vs*. diabetic (*n* = 6/group).

4.5.2 RAASi attenuated collagen accumulation in the left ventricle of diabetic rats.

We used picrosirius red staining to evaluate the collagen content in the LV myocardium. The analysis showed increased myocardial collagen deposition in the diabetic group. The treatment of RAASi significantly reduced collagen accumulation (Figure 8A). In parallel, DM-induced higher fibronectin (*Fn*) expression was abolished by RAASi, suggesting a milder myocardial remodeling (Figure 8B).

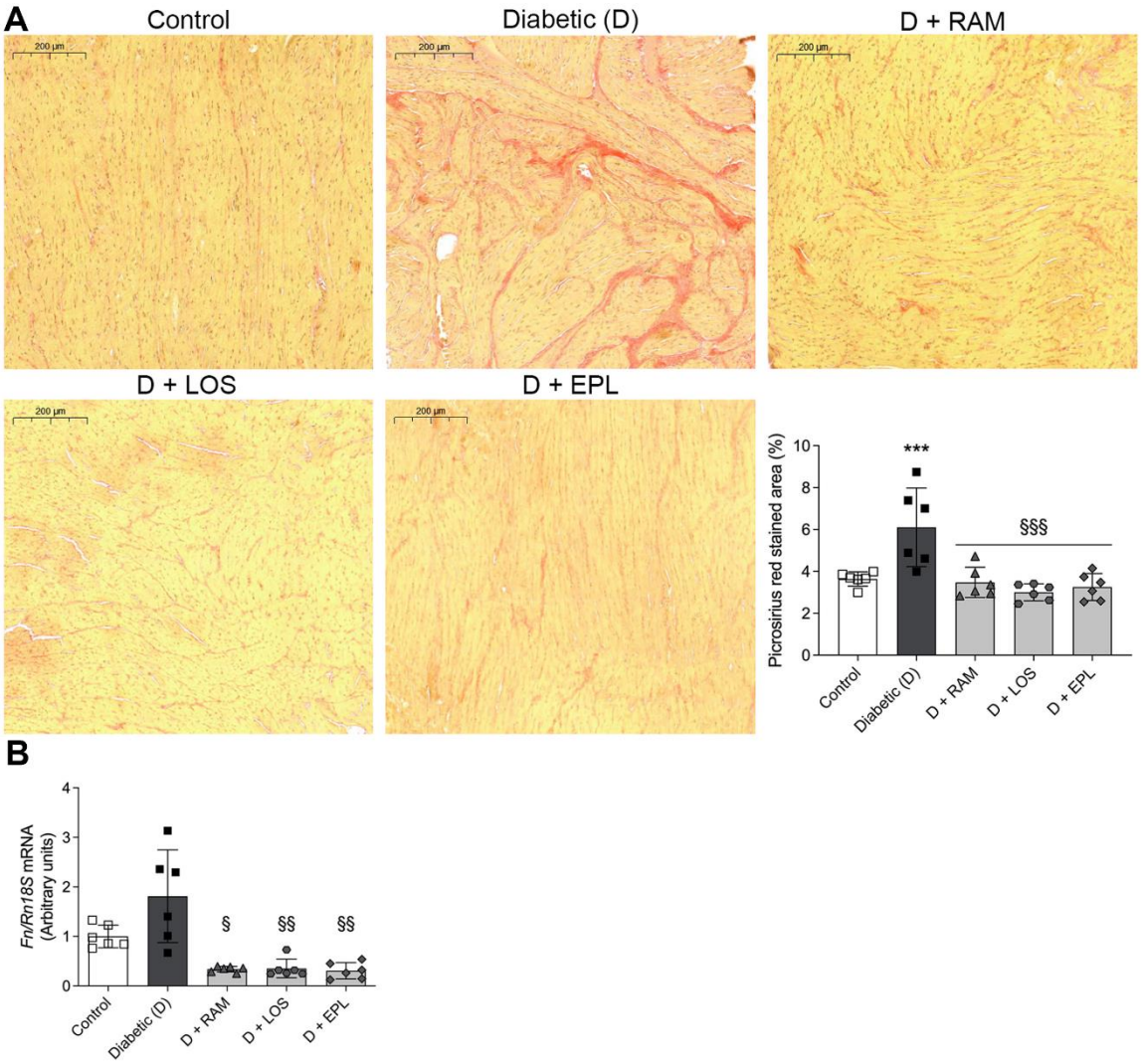

Figure 8 RAASi caused a reduction in the collagen accumulation in the LV of diabetic rats (A) Representative picrosirius red stained heart sections and quantitative evaluation of fibrosis of control, D: diabetic, D + RAM: D + ramipril, D + LOS: D + losartan, and D + EPL: D + eplerenone. The red-stained area was measured based on the red color intensity. The myocardial field of measurement was manually selected with the caution of avoiding the endocardium and perivascular connective tissue. Original

magnification, x400. Scale bar, 50 μm. (B) mRNA expression of fibronectin (*Fn*). mRNA expressions were normalized to Rn18S mRNA expression. Bars indicate means ± SDs. ***p < 0.001 *vs*. control, §p < 0.05 *vs*. diabetic §§p < 0.01 *vs*. diabetic, §§§p < 0.001 *vs*. diabetic (*n* = 6/group).

4.6 DAPA treatment prevented metabolic deterioration in diabetic rats.

After six weeks of T1DM, typical metabolic characteristics of DM manifested, including weight loss and elevated levels of serum glucose, fructosamine, and lipids. Treatment with DAPA significantly improved all metabolic parameters in diabetic rats while exerting no effect on non-diabetic control rats other than inducing glucosuria. 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 (Table 6).

Table 6 Dapagliflozin treatment improved T1DM-induced metabolic changes. C: control, D: diabetic, C + DAPA: dapagliflozin-treated control non-diabetic group, and D + DAPA: dapagliflozin-treated diabetic group. LDL-C: low-density lipoprotein cholesterol, GOT: glutamate-oxaloacetate transaminase, GPT: glutamate-pyruvate transaminase. Values indicate means±SDs. **p < 0.01 *vs.* control, ***p < 0.001 *vs.* control, §§p < 0.01 *vs*. diabetic, §§§p < 0.001 *vs*. diabetic UN: undetectable (*n* = 6 /group).

	Control (C)	C+DAPA	Diabetic (D)	D+DAPA
Body weight (g)	442±35.4	414±34.9	256±29.9***	340±35.0§§
Non-fasting glucose (mmol/L)	6.42±0.58	5.60±0.62	33.3±1.06***	16.2±5.29***§§§
Fructosamine (μmol/L)	143±3.74	143±8.45	277±12.3***	198±34.9§§§
Total cholesterol (mmol/L)	1.96±0.15	1.82±0.21	2.82±0.30***	1.89±0.40§§§
Triglycerides (mmol/L)	1.24±0.51	1.05±0.48	3.12±1.17**	1.00±0.51§§§
GOT (U/L)	127±19.6	195±30.9	382±164***	187±24.1§§
GPT (U/L)	43.0±8.39	49.2±11.3	181±82.1***	80.1±16.1§§
Glucosuria	UN	114±0.60**	346±47.1***	491±94.2***§§

4.7 DAPA hindered cardiac hypertrophy and reduction in heart rate.

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 (Table 7). Notably, DAPA treatment did not affect any parameters within the C+DAPA group, thus ensuring the safety profile of DAPA. Additional molecular investigations were conducted exclusively in control, diabetic, and DAPA-treated diabetic rats.

Table 7 Mean arterial pressure, heart rate, and heart-to-body weight ratio. C: control, D: diabetic, C + DAPA: dapagliflozin-treated control non-diabetic group, and D + DAPA: dapagliflozin-treated diabetic group. Values indicate means±SDs. ***$p < 0.001$ *vs.* control, $^{\S}p < 0.05$ *vs*. diabetic, $^{\S\S}p < 0.01$ *vs.* diabetic ($n = 6$/group).

	Control (C)	C+DAPA	Diabetic (D)	D+DAPA
Mean arterial pressure (mmHg)	88.5±3.66	86.2±3.44	85.9±4.99	77.1±5.69
Heart rate (bpm)	444±12.9	399±24.2	320±25.2***	352±28.3§
Heart-to-body weight ratio (%)	0.29±0.01	0.31±0.05	0.36±0.02***	0.33±0.02§§

4.8 DAPA treatment prevented intima-media thickening.

In *Protocol II*, we also investigated the aortic IMT. Similarly to our previous experiment, control rats showed prominent wavy internal elastic lamina. In diabetic rats, the aorta exhibited a thicker intimal appearance and asymmetrical, 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 (Figure 9).

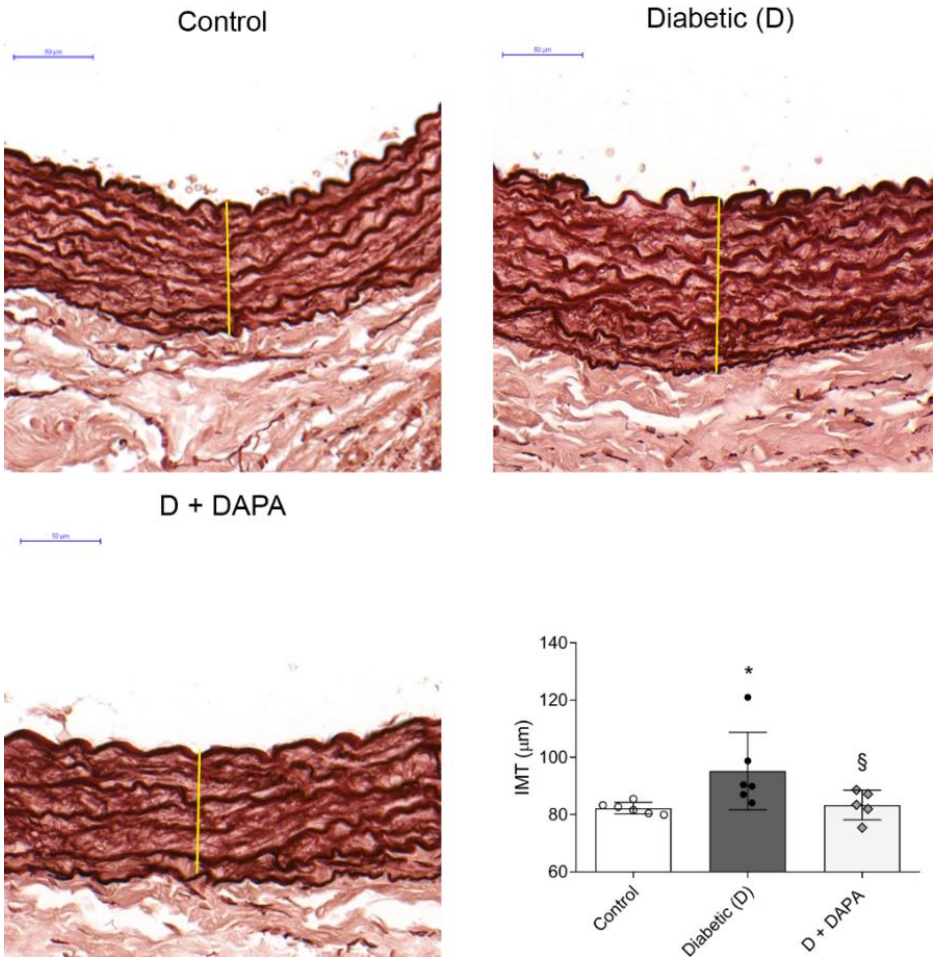

Figure 9 Intima-media thickening is prevented by dapagliflozin. Representative orcein-stained aorta sections and quantitative evaluation of intima-media thickness (IMT) of control, D: diabetic, and D + DAPA: dapagliflozin-treated diabetic group. Original magnification, x200. Scale bar, 50 µm. Elastic fibers are stained brown after orcein staining. They are visualized as either thin fibers or elastic lamella. Bars indicate means±SDs. *$p < 0.05$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic (n = 6/group).

4.9 DAPA mitigated the upregulation of certain biomarkers of myocardial injury.

Natriuretic peptides have a crucial role in the clinical assessment of myocardial injury. Elevated levels of BNP precede the occurrence and duration of DCM and HF (101). Therefore, mRNA expressions of *Nppb* (BNP) were assessed in the LV, revealing significant increases in both markers in diabetic rats. In the DAPA-treated group, the levels of *Nppb* were less elevated compared to the diabetic group (Figure 10A-B).

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 (Figure 10C) (102). In parallel to *Protocol I*, we found that Klotho expression was decreased in the serum of T1DM rats. DAPA treatment halted this reduction (Figure 10D).

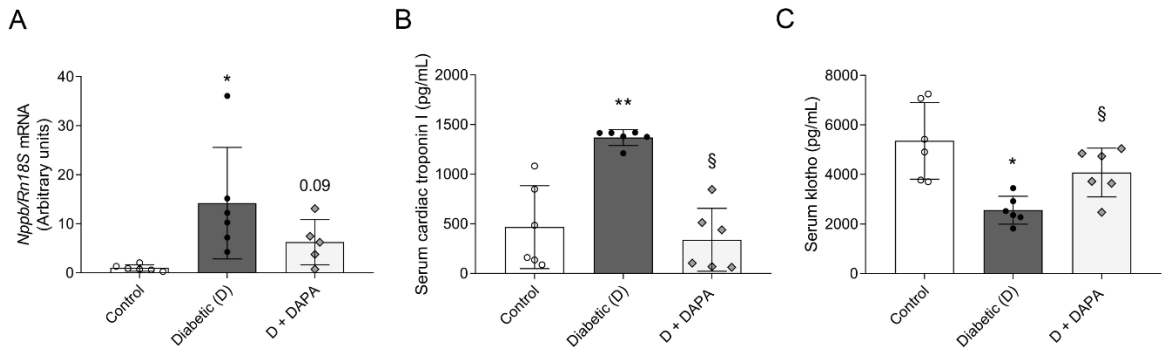

Figure 10 Diabetes-induced myocardial injury biomarker increment is halted by dapagliflozin. mRNA expression of (A) B-type natriuretic peptide (*Nppb*) of control, D: diabetic, and D + DAPA: dapagliflozin-treated diabetic group. mRNA expressions were normalized to *Rn18S* mRNA expression. (B) Serum levels of cardiac troponin I. (C) Serum levels of Klotho. Bars indicate means±SDs. *$p < 0.05$ *vs*. control, **$p < 0.01$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic ($n = 6$/group).

4.10 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 (103). The anti-inflammatory properties of DAPA have been examined by measuring 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 (Figure 11A-D). These findings underscore the anti-inflammatory efficacy of DAPA.

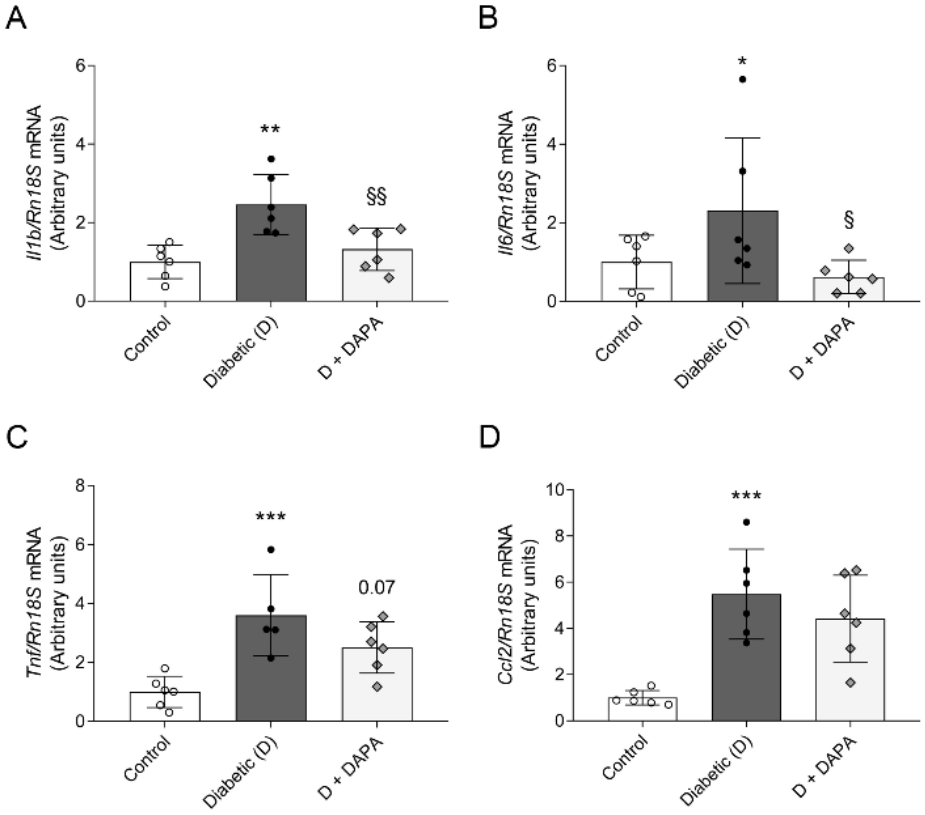

Figure 11 Dapagliflozin mitigated pro-inflammatory response in diabetic rat hearts. mRNA expression of (A) interleukin-1β (*Il1b*), (B) interleukin-6 (*Il6*), (C) tumor necrosis factor (*Tnf*), and (D) monocyte chemoattractant protein 1 (*Ccl2*) of control, D: diabetic, and D + DAPA: dapagliflozin-treated diabetic group. mRNA expressions were normalized to *Rn18S* mRNA expression. Bars indicate means±SDs. *$p < 0.05$ *vs*. control, **$p < 0.01$ *vs*. control, ***$p < 0.001$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic, $^{§§}p < 0.01$ *vs*. diabetic (n = 6/group).

4.11 DAPA mitigated the T1DM-induced cardiac remodeling progression.

4.11.1 Reduction of the pro-fibrotic markers expression in DAPA-treated diabetic rats.

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* (Figure 12A-C).

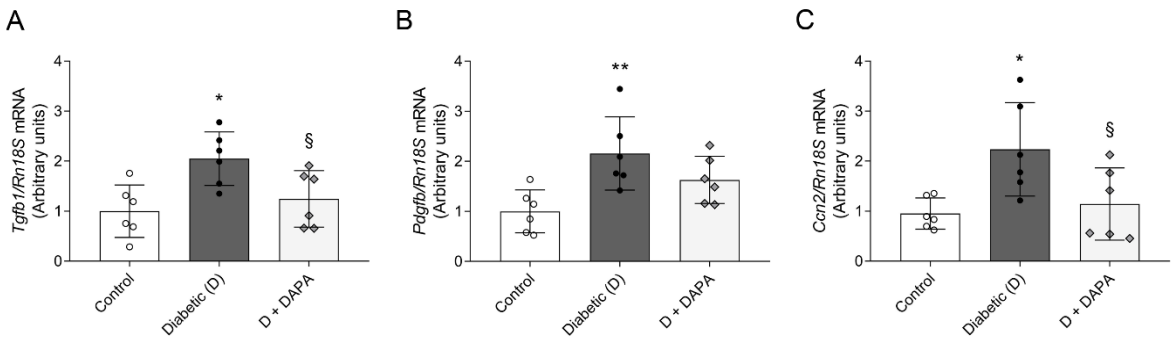

Figure 12 Dapagliflozin attenuated fibrosis in the left ventricle of diabetic rats. mRNA expression of (A) transforming growth factor β (*Tgfb1*), (B) platelet-derived growth factor (*Pdgfb*), and (C) connective tissue growth factor (*Ccn2*) of control, D: diabetic, and D + DAPA: dapagliflozin-treated diabetic group. (D). mRNA expressions were normalized to *Rn18S* mRNA expression. Bars indicate means±SDs. *$p < 0.05$ *vs*. control, **$p < 0.01$ *vs*. control, ***$p < 0.001$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic, $^{§§}p < 0.01$ *vs*. diabetic, $^{§§§}p < 0.001$ *vs*. diabetic (n = 6/group).

4.11.2 Myocardial fibrosis progression is halted in DAPA-treated diabetic rats.

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 (Figure 13A). In parallel, DAPA halted DM-induced higher *Fn* expression, indicating milder myocardial remodeling (Figure 13B).

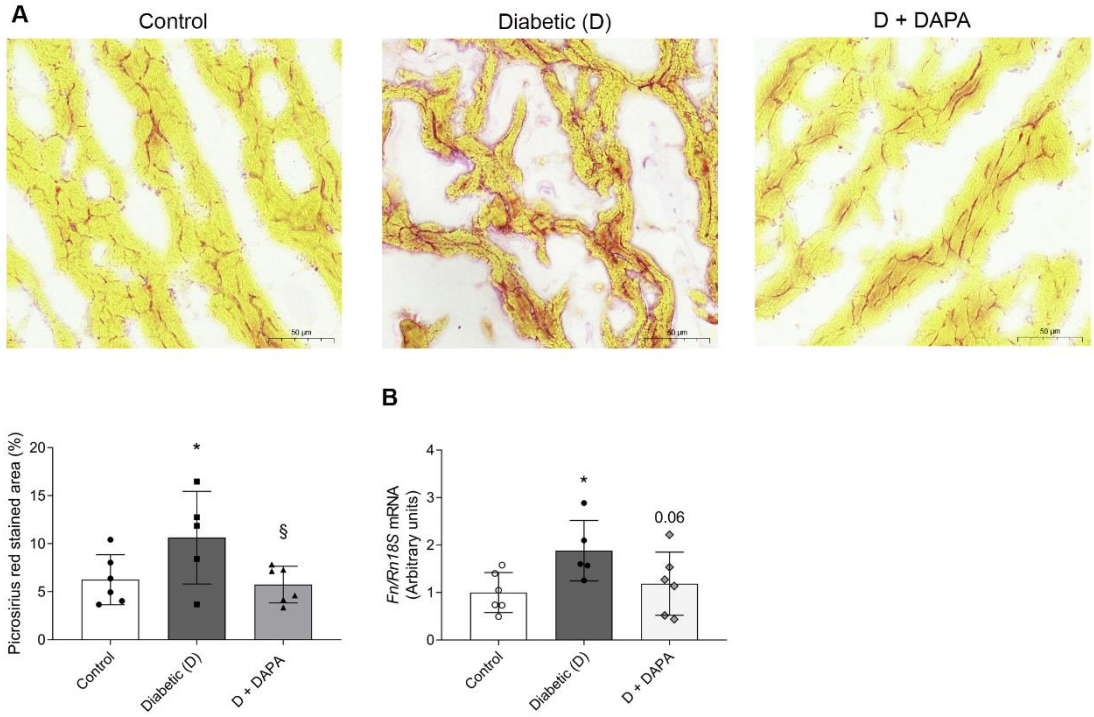

Figure 13 Diabetes-induced cardiac fibrosis is mitigated by DAPA treatment. Representative picrosirius red stained heart sections and quantitative evaluation of fibrosis of control, D: diabetic, and D + DAPA: dapagliflozin-treated diabetic group. The red-stained area was measured based on the red color intensity. The myocardial field of measurement was manually selected with the caution of avoiding the endocardium and perivascular connective tissue. Original magnification, x400. Scale bar, 50 μm. (B) mRNA expression of fibronectin (*Fn*). mRNA expressions were normalized to *Rn18S* mRNA expression. Bars indicate means±SDs. *$p < 0.05$ *vs*. control, $^{§}p < 0.05$ *vs*. diabetic (n = 6/group).

5 DISCUSSION

DM is a major global health problem that affects millions of people and has reached epidemic levels. It causes a wide range of severe health conditions, including blindness, kidney failure, HF, stroke, and lower limb amputation. CVD is two to five times more prevalent in diabetic patients and is the leading cause of death. Individuals in their sixties who have both diseases may face a reduction in life expectancy of up to a decade. This is even more dramatic for younger patients (104).

Both experimental and clinical studies have reported a direct harmful impact of DM on the myocardium. Remodeling in the LV is frequently observed in patients with T1DM and refers to structural changes associated with increased volume, myocyte hypertrophy, myofibroblast proliferation, and interstitial fibrosis. This impact has been observed without clinical CAD, valvular disease, or hypertension leading to the DCM (105). In this thesis, we focused on novel therapeutic strategies that could improve the management of T1DM-induced CVD, particularly the molecular mechanisms behind the protective effect of low-dose RAASi and DAPA.

5.1 The cardioprotective effect of low-dose RAASi treatment.

RAAS activation has been implicated in the pathogenesis of diabetic CV complications, as it promotes myocardial inflammation, fibrosis, and cardiomyocyte loss (106). The AHA and ADA emphasize the importance of maintaining normoglycemia in managing CV risk in DM. For patients with BP above 130/80 mmHg, antihypertensive drugs, like RAASi, should be started to impede and prevent macrovascular and microvascular complications (107).

Concerning the heart and vasculature, apart from a single study demonstrating that low-dose losartan normalized pulse wave velocity in iron-overloaded rats (108), as far as we are aware, these are the first data in T1DM regarding the cardioprotective effect of low-dose RAASi, especially of MR antagonists in monotherapy. Here, we demonstrated that the oral administration of low-dose RAASi alters functional vascular impairment and decreases myocardial injury, including fibrosis. To test whether these cardioprotective

properties are associated with, or limited to, the BP-lowering effects of RAASi, we chose the treatment doses that do not affect BP based on our previous studies (94,109). We confirmed that neither DM nor RAASi affected BP, providing experimental data supporting the non-depressor drug doses used in the current protocol.

T1DM often coexists with other metabolic disorders, such as dyslipidemia, leading to the development of atherosclerosis. Atherosclerosis begins early in life and clinically remains silent for decades, then develops symptoms in adulthood due to the accumulation of fats, cholesterol, calcium, and inflammatory cells in the arterial wall, leading to arterial wall thickening and plaque development (110). This is one of the reasons that T1DM is linked with premature CVD (105). Vascular functional abnormalities are uniform along the whole aorta; however, in DM, the abdominal aorta is where the earliest changes in vascular structure occur. Some reports in STZ-induced T2DM rats on a high-fat diet demonstrated that altered elastin lamellae and increased tissue lipid deposition are mainly confined to the abdominal region. In the same model, others showed segmental differences in functional responses to endothelin and Ang II (111). We have demonstrated that RAASi decreases aortic IMT in diabetic rats, but only eplerenone treatment reached the significance level. In parallel, eplerenone significantly improved cholesterol levels, contributing to reducing the progression of atherosclerotic risk.

Natriuretic peptides and troponins are novel clinical biomarkers used in routine clinical settings for diagnosis, evaluation of cardiomyopathy severity, and prediction of the prognosis of HF. LV mainly expresses BNP under myocardial stress. It exerts crucial systemic (natriuresis/diuresis and vasodilation) and autocrine/paracrine activities in HF. The elevated plasma BNP level precedes the occurrence of DCM as a compensatory protection mechanism (101,112). Troponins regulate the calcium-mediated interaction between actin and myosin. This multiprotein complex comprises various troponin isoforms, including cardiac troponin I, which inhibits actin-myosin interaction. BNP and cardiac troponin I have increased in DM patients and experimental DCM models (113–115). Less is known about the effect of RAASi on alteration in these biomarkers. A study showed that AT1R agonists can prevent DM-induced cardiac troponin I protein kinase C phosphorylation. A recent publication has indicated that captopril and losartan reduce

elevated levels of troponin I and infarct size in STZ-induced diabetic rats following myocardial injury (116). Losartan-treated rats with spontaneous hypertension showed reduced BNP levels (117), while ramipril and eplerenone prevented BNP elevation in studies of acute myocardial infarction injury (118,119). Our study found that BNP and cardiac troponin I increased in T1DM rats, indicating myocardial injury and LV distress. Treatment with RAASi reduced the mRNA expressions of *Nppb* and serum cardiac troponin I, suggesting milder damage.

The PONTIAC I study demonstrated that intensified therapy with angiotensin-converting enzyme inhibitor (ACEi) at the recommended dose in T2DM, who lacked clinical cardiac symptoms but exhibited elevated BNP levels prevented CV outcomes without altering BNP levels (120). The ongoing PONTIAC II study aims to determine whether these advantages are more remarkable in patients with elevated BNP levels than those with normal BNP levels (NCT02817360). The ADOPT study evaluates the efficacy of Ang II type 1 receptor blockers (ARB) or ACEi therapy in preventing CV outcomes in T2DM patients with high CV risk. Furthermore, the MIRAD trial showed that adding high-dose eplerenone to an ARB/ACEi reduced BNP levels, LV mass, and extracellular volume, suggesting an alteration in cardiac remodeling and fibrosis (121).

Recently, there has been increasing interest in Klotho, a protein predominantly synthesized in the kidney, as a potential biomarker for renal disease associated with cardiac complications. Klotho deficiency is a pathogenic CVD factor linked to arterial stiffness, LV hypertrophy, and cardiac remodeling. We observed lower serum Klotho levels in diabetic rats compared to the control group. This correlates with the limited data available from both animal models of T1DM and diabetic patients (122,123). Besides, patients with T1DM have lower Klotho levels than the normal population, which is related to higher IMT (124). In our experiment, only eplerenone reversed the decline of Klotho levels. These findings align with literature where eplerenone blocked Klotho deficiency-related changes (125). The underlying mechanism may involve high aldosterone levels downregulating Klotho transcriptional activity by increasing histone deacetylase expression, and this leads to histone H3K9 deacetylation, a site associated with the Klotho gene promoter. This may explain our results demonstrating the sole

efficacy of eplerenone (126). These findings reinforce the importance of using eplerenone and other MR antagonists as monotherapy. In addition, a recent study in patients at risk for HF with CKD and T2DM showed that adding the selective MR antagonist finerenone to the maximum tolerated dose of RAASi improved CV and renal outcomes regardless of HF history (127).

A modification in the size, shape, or structure of one or more heart chambers is called cardiac remodeling, a key feature of cardiac fibrosis. It is due to the deposition in ECM and the phenotypic change of the cardiomyocytes. Specifically, LV remodeling is characterized by increased volume, myocyte hypertrophy, myofibroblast proliferation, and interstitial fibrosis, which is commonly observed in T1DM patients. Cardiac fibrosis is the final common pathway of the development of HF. Therefore, addressing the reversal or prevention of LV remodeling is crucial in preventing DCM (128). Clinical studies have revealed the presence of myocardial fibrosis in diabetic patients, which may present independently of hypertension or coronary atherosclerosis (129,130). Hyperglycemia-induced Ang II and aldosterone activity contribute to the deposition of ECM proteins, leading to cardiac remodeling and impaired function (131–133). Previous preclinical studies on STZ-induced diabetic models have shown antifibrotic effects of RAASi by decreasing myocardial interstitial fibrosis (134–136). However, these studies used regular or high doses of RAASi (137–139). Here, we found that low-dose RAASi treatment reduced mRNA expressions of profibrotic growth factors, fibronectin, and intramyocardial collagen deposition in the LV of diabetic rats.

Recent findings from the HOMAGE trial, examining the impact of spironolactone on fibrosis and cardiac function in individuals at higher risk of HF, revealed lower levels of serum procollagen type I C-terminal propeptide in patients treated with spironolactone. However, subgroup analyses did not specifically explore whether these effects were observable in the diabetic population (140). These data further strengthen our hypothesis that RAASi might be protective independent of the BP-lowering effect.

In conclusion, our study demonstrates that non-depressor doses of RAASi effectively counteract hyperglycemia-induced CV complications without affecting BP levels.

RAASi used in monotherapy impede cardiac tissue remodeling in STZ-induced CV alterations. Comparative analysis of different RAASi indicates that 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.

5.2 The cardioprotective effect of DAPA treatment.

SGLT2i are oral antidiabetic drugs that regulate glycemic control by inhibiting renal tubular glucose reabsorption. This mechanism leads to glucosuria, resulting in a reduction of blood glucose levels independent of insulin action. They have proven efficacy in treating T2DM and are now recognized as pleiotropic drugs, exhibiting beneficial effects in non-diabetic patients with HFrEF and HFmEF (141). Various studies showed that SGLT2i improved body weight, BP parameters (systolic and diastolic), liver function, and lipid profile, enhancing glucose metabolism (142,143). In line with the literature, our study showed that oral administration of DAPA for six weeks prevented metabolic decline, cardiac hypertrophy, and myocardial injury in STZ-induced T1DM.

The FDA has required proof of CV safety for new glucose-lowering therapies since 2008. Therefore, numerous large clinical studies have evaluated the effects of SGLT2i on T2DM, leading to groundbreaking results in CVD and CKD. EMPA-REG OUTCOME, CANVAS, DECLARE-TIMI 58, and VERTIS-CV studies have shown that SGLT2i reduced the risk of HF in T2DM (59–62). Additionally, these medications enhance CV outcomes and reduce mortality in non-diabetic HF patients undergoing DAPA treatment, as shown in DAPA-HF clinical trials (82). SGLT2i not only reduces BP, decreases body weight, or induces natriuresis but also suggests that their consistent cardioprotective effects extend beyond their antihyperglycemic action. Despite the encouraging results of these trials, the precise molecular mechanisms underlying this cardioprotection remain incompletely understood. Besides, the literary data about the effect of SGLT2i in T1DM is scarce. Therefore, the main goal of our second experiment was to investigate the cardioprotective effect of DAPA in an STZ-induced T1DM rat model.

Premature atherosclerosis is the primary cause of mortality in T1DM, with CV events manifesting over a decade earlier. While atherosclerosis initiates early in life, symptoms

typically arise in adulthood. The accumulation of fats, cholesterol, and calcium in the arterial wall results in thickening and the formation of arterial plaques (110). Hence, averting the onset of atherosclerosis can serve as a pivotal factor in mitigating the risk of CVD in later stages of life. We measured the lipid profile and found that DAPA effectively normalized serum total cholesterol, triglyceride, and LDL-C levels in diabetic rats. These results are consistent with recent reports in murine models of T2DM (144,145). We also measured the aortic IMT of rats. Our findings revealed that diabetic rats exhibited intimal thickening, which was prevented by DAPA treatment. Only two non-randomized and one randomized clinical studies have explored the effects of SGLT2 inhibitors on carotid IMT in patients with T2DM. These studies revealed that empagliflozin reduced IMT, while changes with ipragliflozin (from the FUSION study) and tofogliflozin (from the UTOPIA study) did not achieve statistical significance. (146–148). Nevertheless, it's important to note that these studies have limitations, including small sample sizes, short treatment durations, or the absence of a double-blind, placebo-controlled trial design.

Natriuretic peptides are predominantly produced in the atria and ventricles in response to increased cardiac stretch; thus, they are powerful predictors of ventricular dysfunction and CV outcomes (24). BNP levels are increased in diabetic patients and rat models of prediabetes and T2DM (112,149–151). In our study, mRNA expressions of BNP were significantly increased in the LV of diabetic rats compared with the control group. After DAPA treatment, the mRNA expressions of BNP were decreased, which is consistent with recent findings in the reduction in natriuretic peptide expression in T2DM (152). The DAPA-MODA study demonstrated that DAPA treatment in patients with HF is associated with reduced LV mass and serum level of natriuretic peptides after six months (153). These results suggest that DAPA can moderate cardiac impairment in HF patients.

As previously discussed, circulating cardiac troponin I is critical in detecting myocardial injury cardiac troponin I is elevated in HF patients with DM (113). Canagliflozin treatment effectively delayed the upregulation of high-sensitivity troponin I levels and serum BNP in patients with T2DM for over two years (154). Likewise, we observed an elevation in serum cardiac troponin I level in our experimental model of T1DM, which

was subsequently decreased by DAPA treatment. DAPA treatment delayed the onset of hypertrophy and myocyte damage markers, suggesting that SGLT2 inhibitors may play a protective role in ventricular remodeling induced by T1DM.

Klotho deficiency is an early metabolic consequence of CKD that is strongly associated with a greater risk of CVD events and mortality (100). Recent studies have reported lower levels of Klotho in patients with T2DM and CVD, supporting its potential protective role in cardiorenal diseases (155,156). Moreover, T1DM patients have lower Klotho levels in comparison to non-diabetic patients and are linked to increased IMT (124). The effect of SGLT2i on serum Klotho levels has yet to be comprehensively documented.

Klotho has been reported to inhibit fibrosis induced by TGF-β1 in rat ventricular cardiomyocytes and cardiac fibroblasts *in vitro* (157). A soluble form of Klotho, produced through alternative splicing, has been shown to suppress myofibroblast proliferation and collagen synthesis in cultured mouse cardiac fibroblasts (157). Additionally, Klotho-deficient mice showed cardiac dysfunction, hypertrophy, and fibrosis before reaching 12 weeks of age (158). Our findings demonstrated that DAPA halted the decrease of serum Klotho in T1DM. This suggests that DAPA may slow the progression of cardiac fibrosis by elevating Klotho levels. However, further analysis is needed to elucidate the precise molecular pathway. Interestingly, in a rat model of unilateral ureteric obstruction, empagliflozin increased the expression of renal Klotho (159). Based on this, SGLT2i can improve Klotho levels regardless of DM, indicating a novel mechanism of action for cardiorenal protection.

The link between the pathophysiology of DM and CVD is complex and multifactorial (160). Inflammation is implicated in the pathophysiology of both diseases. According to the “cytokine hypothesis” HF is partly a consequence of activated endogenous cytokine cascades in the heart and circulation (161). Cytokines, such as TNF-α, IL-1β, and IL-6, induce hypertrophy of cardiac cells, impaired contraction, and LV dilation and promote the cardiac interstitial matrix (162). Elevated levels of TNF-α and IL-6 in the serum of DM patients are linked to LV diastolic dysfunction (163). The increased mRNA levels of *Il1b*, *Il6*, *Tnf*, and *Ccl2* in the LV suggest activating inflammatory signaling pathways,

which aligns with results from other T1DM models. (164,165). Our study revealed that treatment with DAPA reduced DM-induced cardiac inflammation. The same anti-inflammatory effect of DAPA was observed when DAPA administration downregulated pro-inflammatory mediators like NF-κB, TNF-α, IL-6, and IL-1β in STZ-induced T1DM in male Sprague Dawley rats (166). This is in line with the anti-inflammatory effect of empagliflozin, and DAPA found in T2DM rodent models (167,168). In addition, SGLT2i reduced inflammation in the hearts of infarcted APOE knockout mice and LPS mouse models, indicating that cardioprotection is not entirely reliant on glucose-lowering effects (92,169,170). Our research and existing literary evidence reinforce the idea that SGLT2i has an anti-inflammatory effect, which may contribute to improving CVD outcomes.

Cardiac fibrosis is the common final pathway HF develops (171). Clinical studies showed the presence of myocardial fibrosis in patients with DM, which may occur independently of hypertension or coronary atherosclerosis (129,130). Hyperglycemia triggers the development of cardiac fibrosis, which causes pathological alterations resulting in the activation of the TGF-β pathway and the accumulation of ECM proteins. This leads to elevated myocardial stiffness and reduced cardiac function (37). Recent studies have demonstrated that SGLT2i reduces cardiac fibrosis in T2DM rodent models and TGF-β-induced activation of human cardiac fibroblasts (172–174). We previously reported that DAPA has antifibrotic effects in T1DM kidneys; therefore, it also seemed plausible that DAPA prevents fibrosis in the heart (175). We found that elevated *Tgfb1* and *Ccn2* expressions were reduced in DAPA-treated rats.

Further, we showed that the accumulation of *Fn*, and collagen was also mitigated after DAPA treatment, supporting the antifibrotic potential of SGLT2i. Consistent with our results, DAPA significantly reduced the elevated *Fn*, Collagen, and *Tgfb* mRNA levels in the ZDF diabetic model (176). Similarly, empagliflozin reduced cardiac interstitial fibrosis and hypertrophy in experimental rats with metabolic syndrome. These effects were associated with the downregulation of cardiac oxidative stress and inflammation (177). These findings indicate that inhibiting SGLT2, regardless of the type of DM, may benefit the cardiac TGF-β pathway, reducing myocardial fibrosis.

In conclusion, this study provides experimental evidence for the cardioprotective effect of DAPA in a T1DM model. Our results indicate that DAPA prevents intimal thickening, cardiac inflammation, and fibrosis. These findings suggest that a complex system underlies the organ protective effects of SGLT2i. Ultimately, various SGLT2i may offer a novel therapeutic opportunity to simultaneously treat and improve outcomes for DM and related disorders.

6 CONCLUSIONS

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.

7 SUMMARY

DM is a major global health problem, impacting millions of individuals worldwide and leading to multiple severe health complications. Among these, CVD is the leading cause of death and disability in DM patients. T1DM patients are younger at the onset of the disease, and they lose more life-years due to CVD. Current therapeutic approaches, which focus primarily on glycemic control, are insufficient to improve patient outcomes significantly. Therefore, it is critically important to explore novel therapeutic strategies and early interventions to improve the management of DM-related CV complications.

RAASi represents the first-line therapy for CV complication prevention in DM patients when hypertension and albuminuria are present. SGLT2i is a new class of antidiabetic drugs. Numerous large randomized clinical trials have evaluated the effects of SGLT2i on T2DM, leading to groundbreaking results in CVD and CKD. These trials have shown that SGLT2 inhibitors decrease MACE. Additionally, these medications improve CV outcomes and reduce mortality in non-diabetic HF patients. Despite these encouraging results, the molecular mechanisms underlying this cardioprotection remain incompletely understood. Moreover, the literary data about the effect of SGLT2i in T1DM is scarce. Therefore, the main goal of our studies was to investigate the cardioprotective and antifibrotic effects of low-dose RAASi and DAPA in an STZ-induced T1DM rat model.

We showed that treatment with low-dose RAASi effectively counteracted T1DM-induced CV complications and cardiac fibrosis without affecting BP levels. Moreover, 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. Furthermore, we showed that DAPA is preventive and protective in experimental T1DM. Specifically, our results highlight that DAPA prevented metabolic decline, intimal thickening, cardiac inflammation, and fibrosis in STZ-induced T1DM rats. Our findings confirm the link between glucose toxicity, inflammation, and fibrosis, forming a detrimental trio that could potentially be addressed by DAPA.

Ultimately, our findings propose that the multifaceted effects of SGLT2i offer a novel therapeutic approach to simultaneously treat and improve outcomes for DM and related disorders.

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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**

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