SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3258.

MOLNÁR ÁGNES

Gyermekkori betegségek klinikuma, élettana és prevenciója

című program

Programvezető: Dr. Szabó Attila, egyetemi tanár, az MTA doktora

Témavezető: Dr. Fekete Andrea, egyetemi tanár, az MTA doktora

THE ROLE OF RENIN-ANGIOTENSIN SYSTEM INHIBITORS IN DIABETES-INDUCED MULTIORGAN DAMAGE

PhD thesis

Ágnes Molnár, MD

Károly Rácz Doctoral School of Clinical Medicine

Semmelweis University Doctoral College

Supervisor: Andrea Fekete, MD, D.Sc.

Official reviewers: Nóra Ledó, MD, Ph.D.
Péter Légrády, MD, habil., Ph.D.

Head of the Complex Examination Committee: Barna Vásárhelyi, MD, D.Sc.

Members of the Complex Examination Committee: András Szabó, MD, D.Sc.
Gergely Kriván, MD, Ph.D.

Budapest

2025

Table of Contents

List of Abbreviations

αSMA	alpha smooth muscle actin
ACE	angiotensin-converting enzyme
ACEi	angiotensin-converting enzyme inhibitor
Ang	angiotensin
ARB	angiotensin receptor 1 blocker
ATR	angiotensin receptor
Bax	B-cell lymphoma 2 associated X protein
Bcl2	B-cell lymphoma 2 protein
BDNF	brain-derived neurotrophic factor
BNP	B-type natriuretic peptide
BSA	bovine serum albumin
C3M	degradation neo-epitope of type III collagen
CKD	chronic kidney disease
CTGF	connective tissue growth factor
CV	cardiovascular
CVD	cardiovascular disease
DCM	diabetic cardiomyopathy
DKD	diabetic kidney disease
DM	diabetes mellitus
ECD	endothelial cell dysfunction
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
FBN-C	C-terminal of fibronectin
FBS	foetal bovine serum
FGF	fibroblast growth factor
GBM	glomerular basal membrane
GDM	gestational diabetes mellitus
GFR	glomerular filtration rate
HbA1c	haemoglobin A1c

HF	heart failure
HG	high glucose
HK-2	human kidney 2 proximal tubular cell line
HLA	human leukocyte antigen
HSP	heat shock protein
ICA	islet cell autoantibody
IFG	impaired fasting glucose
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IL	interleukin
IMT	intima-media thickness
MAP	mean arterial pressure
mBDNF	mature form of brain-derived neurotrophic factor
MMP	matrix metalloproteinase
MODY	maturity onset diabetes of the young
MR	mineralocorticoid receptor
MTT	methyl-thiazolyl-tetrazolium
NFκB	nuclear factor kappa B
NO	nitric oxide
NRK-49F	normal rat kidney 49 fibroblast cell line
OGA	O-linked N-acetylglucosaminidase
O-GlyNAcylation	O-linked N-acetylglucosamine modification
OGT	O-linked N-acetylglucosamine transferase
OGTT	oral glucose tolerance test
p75Ntr	p75 neurotrophin receptor
PCNA	proliferating cell nuclear antigen
pCREB	phospho-cAMP response element-binding protein
PDGF	platelet-derived growth factor
PDGFR-β	platelet-derived growth factor receptor β
pERK	phospho-extracellular signal-regulated kinase
pJNK	phospho-c-Jun N-terminal kinase
proBDNF	precursor brain-derived neurotrophic factor

PRO-C3	formulation neo-epitope of type III collagen
PRO-C4	formulation neo-epitope of type IV collagen
PWV	pulse wave velocity
RAAS	renin-angiotensin-aldosterone system
RAASi	renin-angiotensin-aldosterone system inhibitors
Rn18s	18S ribosomal RNA
SGLT	sodium-glucose cotransporter
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor alpha
TrkB	tyrosine receptor kinase B
TUM	tumstatin
UACR	urine albumine/creatinine ratio
VEGF	vascular endothelial growth factor

1. Introduction

1.1. Diabetes mellitus (DM)

Diabetes mellitus (DM) is a chronic, progressive metabolic disorder which develops by the synergic effect of genetic and environmental factors. In the ageing society of modern times, unhealthy lifestyle, inappropriate diet and decreased physical activity, there is a mean increased exposure to the conventional risk factors like genetic susceptibility, some viral infections, or lower socio-economic status.

DM originates from inadequate insulin secretion of β cells in the pancreas' Langerhans islets or ineffectiveness of the produced insulin (insulin resistance), resulting in hyperinsulinaemia. This relative or absolute insulin depletion leads to the impaired regulation of energy homeostasis. Disturbed carbohydrate metabolism with high blood glucose levels is at the centre of the disease. Still, consequently, it affects salt-water homeostasis, lipid and protein metabolism and acid-base balance as well. (1)

1.1.1. Importance

One of the most significant public health issues in the 21st century is DM, with its plague-like, continuously increasing morbidity and mortality. According to international statistics, 588 million people (every 10th adult) live with DM worldwide, while half of the cases remain undiagnosed. (*Figure 1.*) Considering the increasing incidence and prevalence rates, predictions anticipate 852 million people having DM by 2050. (2)

DM also affects the younger population. The International Diabetes Federation started the Type 1 Diabetes Index program in 2022 to determine accurate estimates of type 1 DM (T1DM). According to their current data, 9.4 million people live with T1DM, of whom 1,66 million are younger than 20. (3)

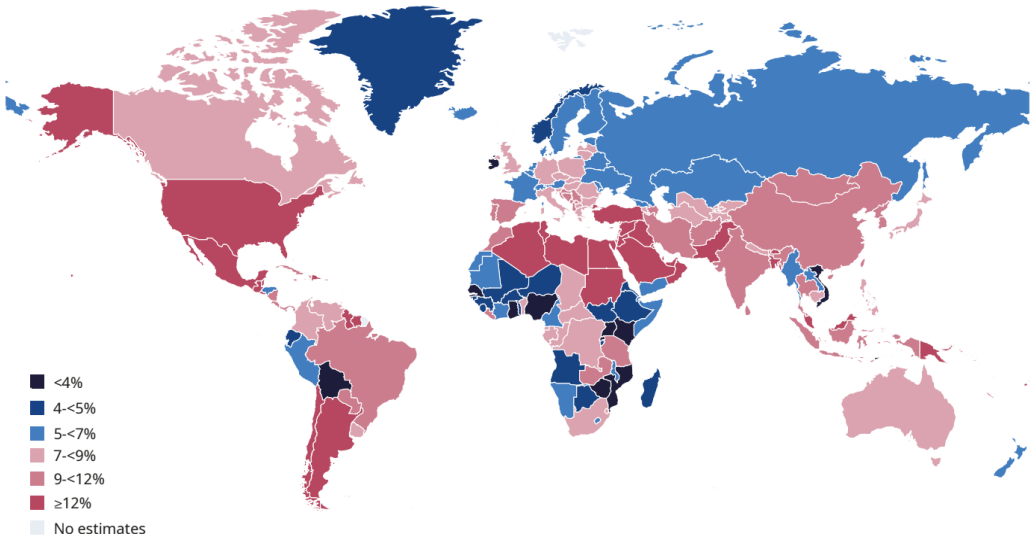


Figure 1. Estimated age-adjusted comparative prevalence of diabetes in adults (International Diabetes Federation Diabetes Atlas 11th edition, 2025) (2)

DM is within the top 10 leading causes of death globally. In 2021, almost 7 million adults were estimated to have died from DM and its complications. (2)

The prevalence of DM has been increasing in Hungary as well. Analysis of the National Health Insurance Fund database revealed that 929 711 patients had type 2 DM (T2DM) in Hungary in 2020, (4) representing 7-9% of the adult population, higher than most European countries. The Hungarian Central Statistical Office stated that between 1999 and 2019, the number of diabetic Hungarians tripled, which means more than 1.1 million adults and 5 thousand children. (5). According to the Type 1 Diabetes Index program's data, 39,130 Hungarian people lived with T1DM in 2024, which is expected to grow to 73,220 by 2040. Hungarian T1DM patients lose 16.3 healthy years on average and have 8.1 years shorter life expectancy. (6)

DM care is a real financial burden, and the treatment, including hospital-related costs, increased from 251 to 699 million Eur in 15 years. (7) In 2019, HUF 63.5 billion was paid out from the Health Insurance Fund due to DM-associated medication and care. (8)

1.1.2. Diagnosis

Diagnosis of DM is based on blood glucose measurement ordered because of typical clinical symptoms, glycosuria, overwhelming family history, or carried out during regular health screenings. The main symptoms appear when the blood sugar level rises because, without insulin, the muscle cells and adipocytes cannot process glucose. A

glucose shortage in tissues caused by hindered uptake enhances the processes of gluconeogenesis in the liver. (9) In T2DM typical hyperglycaemic symptoms may not be present or appear much later.

The main diagnostic criteria of DM is hyperglycaemia; the threshold levels are summarised in *Figure 2*. In short, if random plasma glucose (without considering any food intake) is above 11.1 mmol/L in the presence of classic clinical symptoms, the diagnosis can be made immediately. Haemoglobin A1c (HbA1c) equivalent to 6.5% or higher indicates DM. (10)

If fasting (defined as no caloric intake in the past 8 hours) plasma glucose level is 7 mmol/L or higher, DM can be diagnosed, while with results between 6.1 and 7.0 mmol/L, an oral glucose tolerance test (OGTT) should be carried out. During OGTT, 75g of glucose is consumed orally after fasting for 8 hours, and blood glucose level is measured 120 minutes later. If this value is above 11.1 mmol/L, DM can be diagnosed, while between 11.1 and 7.8 mmol/L, the condition is described as impaired glucose tolerance (IGT). If the OGTT result is normal (below 7.8 mmol/L), impaired fasting glucose (IFG) may be the sign of prediabetes, a transition state between normal glucose regulation and DM. When diagnostic criteria of DM are not met, but glucose regulation is impaired, blood sugar levels are higher than physiologic parameters, IFG and/or IGT are present, and HbA1c levels between 5.7 and 6.4% can also indicate prediabetes. (11)
On the other hand, there are some transient disesases, where blood sugar level can be higher, but the diagnosis of DM can not be verified. Additionally, in T1DM auto-antibody positivity can be detected prior to blood glucose elevation, therefore stage 1 of T1DM can be diagnosed based on antibody screening, as well. (12)

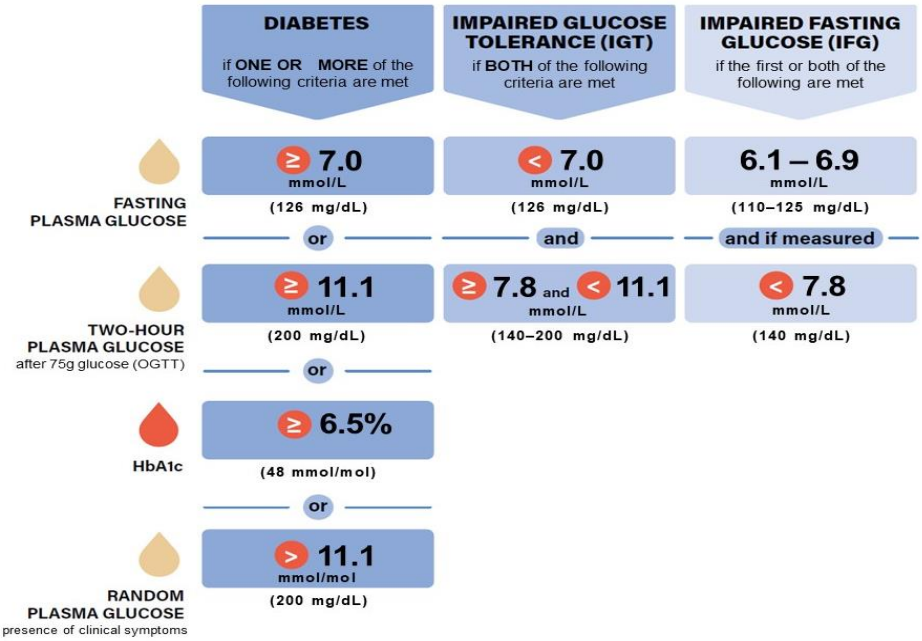


Figure 2. Diagnostic criteria of diabetes (IGT = impaired glucose tolerance; IFG = impaired fasting glucose; OGTT = oral glucose tolerance test; HbA1c = haemoglobin A1c) (Modified figure of International Diabetes Federation Diabetes Atlas, 11th edition, 2025) (2)

1.1.3. Classification

Based on aetiology, DM is classified into four main categories, T1DM, T2DM, gestational DM (GDM), and specific types of diabetes due to other causes. T2DM forms 90% of all DM cases, 20% of them are above 65 years of age, although its prevalence is increasing among children as well. (13) T1DM is one of the most common chronic diseases in childhood, accounting for 5-10% of all DM cases. GDM is diagnosed in 3-5% of pregnancies and foreshadows a higher risk for developing future diabetic conditions. (14)

T1DM usually develops before the age of 35, with classical clinical symptoms, like polyuria (frequent urination caused by osmotic diuresis due to higher urinary glucose content), polydipsia (excessive thirst driving increased fluid intake), sudden unexplained weight loss with constant hunger, as protein and lipid catabolism enhances (causing negative nitrogen balance), fatigue, and blurred vision.

It progresses quickly in children and adolescents and is often accompanied by other autoimmune diseases. In T1DM, autoimmune insulitis causes β-cell damage, eventually resulting in an absolute lack of insulin, even though the exact pathomechanism is still unknown. The autoimmune pathophysiology is confirmed by detecting islet cell-

specific autoantibodies (ICA) before the onset of clinical symptoms. (15) In adult cases of latent autoimmune DM, the slow progression of autoimmune DM can mimic T2DM. Therefore, ICA detection has an essential role in differentiation. The Type 1 Diabetes Index initiative will also be used to determine accurate estimates of T1DM prevalence. (16)

The genetic susceptibility is supported by the fact that 20% of T1DM cases show familial accumulation. In 90% of patients, the human leukocyte antigen (HLA) DR3 and/or DR4 allele is present, and their insulin gene often contains a variable number of tandem repeats polymorphism. (17) As environmental trigger factors, intestinal microbiota, dietary factors (gluten, breastfeeding, cow milk, vitamin D) and viral infections (Coxsackie, mumps, rubella, enteroviruses) are mentioned most often. (18-21)

In T2DM, peripheral insulin resistance is the cause, which results in the relative lack of insulin, leading to hyperglycaemia. After exhaustion of the hyperinsulinaemic phase, the secretory defect results in a low insulin level. Additionally, increased gluconeogenesis in the liver further raises the level of blood glucose. (22) In T2DM, familiar accumulation is even stronger than in T1DM. Single-nucleotide polymorphisms mostly characterise genetic background, but certain environmental factors are crucial for somatic manifestations. Less physical activity, sedentary lifestyle, with or without obesity and smoking, worsen glucose metabolism disturbances. Among further predisposing factors, previous GDM, IGT, IFG, hypertension and dyslipidaemia are the most important. (23)

GDM is diagnosed when carbohydrate metabolism disturbances causing hyperglycaemia are first detected during pregnancy. Higher blood glucose levels during childbearing bring a higher risk for maternal and foetal complications, and GDM in subsequent pregnancies, or developing T2DM. (24)

Besides these most common forms of DM, other rare types also exist. Monogenic DM syndromes (Maturity-onset DM of the young, neonatal DM), secondary (cystic fibrosis, pancreatitis) or steroid-induced DM, type 3c DM associated with Alzheimer's disease, Alström and Wolfram syndrome can be listed as examples. Still, their further detailed explanation is beyond the scope of this dissertation. (25-27)

1.1.4. Multiorgan complications

Pathophysiological changes in DM cause permanent tissue damage to various organs. Due to the increasing incidence and prevalence of DM, its complications are becoming more common as well. DM is the leading cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputations. (28) Macroangiopathic complications involve early atherosclerosis, accelerating the development of cardiovascular diseases (CVD), leading to ischaemic heart disease, peripheral or cerebral artery stenosis, or ischaemic stroke. (29) In microangiopathic complications, thickening of the capillary basal membrane causes retino-, nephro- and neuropathy. (30)

These diseases worsen the severe socio-economic burden in developed countries, including Hungary. (7) Therefore, in everyday diabetic patient care, preventing and treating late-onset complications are the main challenge and tasks.

1.2. Diabetic kidney disease (DKD)

Chronic kidney disease (CKD) occurring in diabetic patients is considered diabetic kidney disease (DKD). DKD is a clinical diagnosis based on albuminuria above 30 mg/ 24 hours, accompanied by decreased glomerular filtration rate (GFR) in patients with diabetes. (31) With time and without treatment, DKD usually progresses and might result in end-stage renal disease (ESRD). ESRD requires renal replacement therapy (dialysis or kidney transplantation), which implies a further significant economic and medical burden on the patient and society. Time after the onset of DM and patient cooperation are essential factors in DKD risk evaluation since worsening glycaemic control enhances disease progression. (32)

1.2.1. Prevalence

Kidney damage develops in about 30% of all diabetic patients. DKD is the leading cause of ESRD in adults, being responsible for 30-40% of the cases, and its prevalence is increasing worldwide. (33) In the case of T1DM, the chance of developing DKD is 10% higher than in T2DM, but renal damage develops later, 10-15 years after the onset of T1DM. In contrast, DKD could be present even at first diagnosis in T2DM patients, due to delays in recognition. The presence of albuminuria means a 95% chance of having DKD in T1DM, but it has less specificity in T2DM, as other causes of chronic

kidney disease can occur within this age group. DKD significantly affects mortality; it is responsible for 55% of the deaths of T1DM patients (34, 35)

1.2.2. Classification and diagnosis

Diagnosis of DKD can be set in diabetic patients, where pathologic albuminuria or decreased renal function are present in 2 out of 3 measurements within 6 months, if other renal disease or other reasons for proteinuria can be excluded. (36)

Classification of DKD is based mainly on the quantity and rate of albuminuria. In the first stage, GFR is increased as hypertrophic glomeruli generate hyperfiltration. Initially, 30-300 mg/day moderately increased albuminuria or a 3-30 mg/mmol urine albumin/creatinine ratio (UACR) refers to early signs of glomerulo- and tubulopathy. The second stage comes with intermittent periods of moderately increased albuminuria and electronmicroscopic histological changes. In the third stage, GFR declines with constant moderately increased albuminuria and increasing blood pressure. Clinically manifested nephropathy occurs with severely increased albuminuria in the fourth stage, where urinary albumin is more than 300 mg/day, and/or UACR greater than 30 mg/mmol. This is accompanied by non-selective proteinuria, significantly decreasing GFR and urinary output, higher serum creatinine levels and hypertension. The fifth stage is ESRD, where the number of working nephrons decreases massively, resulting in uraemia, low or no urine output, oedema, and fatigue. Inappropriate erythropoietin production causes anaemia, cholesterol and triglyceride levels increase; vitamin D production is hindered, enhancing bone loss. (37)

However, newer studies find that decreased GFR values are better predictors of DKD progression than albuminuria, even though both have their limitations in specificity and sensitivity. Therefore, ongoing efforts to develop new biomarkers, especially with non-invasive measurements from urine, will facilitate earlier and more accurate diagnosis of DKD. (38)

1.2.3. Pathogenesis

In the diabetic kidney, endothelial and tubular epithelial cells have pivotal importance as they cannot downregulate their glucose transport even in hyperglycaemic conditions. High intracellular glucose concentration, advanced glycation end products, and reactive oxygen species induce several signal transduction cascades through or without protein

kinase C enzyme activity. (39) Another crucial cellular contributor to glucose toxicity is posttranslational O-linked N-acetylglucosamine modification (O-GlcNAcylation) of proteins through increased hexosamine biosynthesis pathway activity. During the process, a single O-GlcNAc moiety is added to serine/threonine residues of proteins by O-GlcNAc transferase (OGT) and removed by O-GlcNAcase (OGA) enzyme. Therefore, they usually keep the level of glycosylation in a dynamic equilibrium. (40) Hyperglycemia-increased glucose flux activates O-GlcNAcylation, which promotes insulin resistance and disrupts the function and metabolic balance of vascular, neuronal, and renal cells. (41)

These pathways, together with the increased activation of the renin-angiotensin-aldosterone system (RAAS), promote the production of proinflammatory and profibrotic factors such as nuclear factor kappa B (NFκB), transforming growth factor β (TGFβ), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF).

All the above processes lead to the progression of DKD and the development of kidney fibrosis. (42)

One of the first structural changes in the kidney is mesangial matrix expansion due to increased extracellular matrix (ECM) secretion and mesangial cell hypertrophy. In T1DM, mesangial matrix expansion strongly correlates with GFR, albuminuria and hypertonia. Electronmicroscopic changes include glomerular basal membrane (GBM) thickening and podocyte number decrement, resulting in damaged structural integrity of glomerular filtration, leading to proteinuria. In parallel, profibrotic changes like ECM protein accumulation and reorganization of the actin cytoskeleton also affect the tubular structure, leading to tubulointerstitial damage. (43) As DKD progresses, glomerulosclerosis and tubular aggregation of ECM proteins such as collagen I, III, IV and fibronectin lead to fibrotic changes. (44)

1.2.3.1. Fibrosis and its marker molecules

In the pathomechanism of DKD, kidney fibrosis plays a central role. Fibrosis is part of the natural wound healing process, where tissue damage induces an inflammatory response, contributing to normal regeneration or uncontrolled tissue proliferation resulting in progressive fibrosis.

Hyperglycaemia-induced overexpression of profibrotic factors such as TGFβ, CTGF, and PDGF has been implicated in the setting of DKD through the modulation of various signal transduction pathways. Although PDGF and CTGF expression can be activated through TGFβ-dependent and independent pathways (45), they act through PDGF receptor β (PDGFR-β) expressed on fibroblasts. (46-48) These profibrotic factors contribute to the pathogenesis of fibrosis in DKD by enabling fibroblasts to differentiate and migrate, which eventually induces the expression of ECM components, including different collagen types and matrix metalloproteinases (MMPs). (49)

TGFβ1 is the most commonly studied isoform of TGFβ, regulating cell proliferation, differentiation and apoptosis. (50) It is relevant in regular tissue repair and the development of several fibrotic processes. (51) In case of hyperglycaemia or oxidative stress, TGFβ1 is synthesized by all types of renal cells. (52) It promotes ECM deposition by inducing fibroblasts to differentiate and synthesise fibrotic components (53) and directly inhibiting MMPs or activating tissue inhibitors of MMPs. (54)

PDGF protein family consists of homo- and heterodimers. The homodimer PDGF-BB is crucial for producing and maintaining renal ECM, thereby regulating proliferation, migration and mesangial cell survival. (55) Other renal mesenchymal cells, like interstitial fibroblasts or vascular smooth muscle cells, also express PDGF receptors, thus contributing to vascular alterations and interstitial fibrosis. In diabetic kidneys, high glucose induces a persistent increase in PDGFB gene expression, thus stimulating fibroblast proliferation, collagen and ECM production. (56)

CTGF, as a member of the CCN family (cysteine-rich 61, connective tissue growth factor, nephroblastoma overexpressed), is a matricellular protein that promotes angiogenesis, inflammation, tissue repair and fibrosis. (57)

Fibroblasts are the primary effector cells of fibrosis, and their differentiation to myofibroblasts is indicated by alpha-smooth muscle actin (αSMA) production. (58) When activated, they serve as primary collagen-producing cells and contribute to ECM accumulation, where the production of ECM components, including type I and III collagen, and fibronectin increases. (59)

The impaired turnover (formation and degradation) of ECM components leads to the secretion of specific pro-collagen terminal fragments and protein neo-epitopes into the

urine, which have recently been identified as novel, early, non-invasive urinary biomarkers of renal fibrosis. (60) (*Figure 3.*) Two examples of these are neo-epitopes of MMP-9-mediated degradation of type III collagen (C3M) (61) and type IV collagen alpha 3 chain known as tumstatin (TUM). (62)

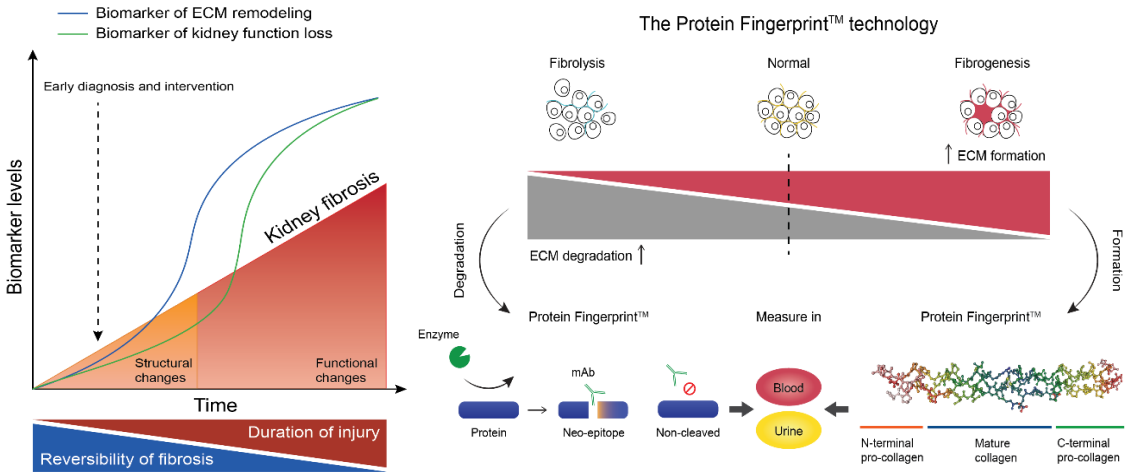


Figure 3. Novel biomarkers of renal fibrosis and concept of their measurement (ECM = extracellular matrix) modified figure of Genovese et al. (60)

1.2.4. Treatment

Current treatment strategies cannot prevent ESRD development; they just slow down the progression of DKD. (63) Basic steps of DKD management include intense blood glucose and blood pressure control, regular screening of albuminuria, avoiding nephrotoxic agents, and dietary restrictions. (64) Guidelines suggest targeting HbA1c level at about 7%, (65) but glycaemic control and restrictions should be considered respecting patients' age, comorbidities and life expectancy.

Currently preferred medications besides antihyperglycaemic agents rely on the beneficial effects of RAAS inhibition, as they reduce blood pressure, microalbuminuria, and the risk of cardiovascular (CV) mortality. Most commonly chosen agents used in monotherapy or combination are angiotensin-converting enzyme (ACE) inhibitors (ACEi) and angiotensin receptor 1 (ATR1) blockers (ARB). (66) However, they seem to be ineffective in stopping the progressive loss of residual renal function and disease progression to ESRD. (67) Monotherapy is often not sufficient to entirely stop RAAS activation. However, the combination of RAAS inhibitors (RAASi) might increase the risk of adverse events such as hyperkalaemia or acute kidney injury. (68) Other newly available treatment options in DKD are sodium-glucose cotransporter 2 (SGLT2)

inhibitors, as well as the non-steroidal mineralocorticoid receptor (MR) antagonist finerenone, and glucagon-like peptide-1 receptor agonist semaglutide.

1.3. DM-associated cardiovascular disease

CV complications in diabetic patients have a particularly high impact on the rapid ascending tendency of morbidity and mortality. Diabetic cardiomyopathy (DCM), which is independent of hypertension or coronary heart disease, is associated with about 30% mortality. (69) Prolonged hyperglycaemia causes accelerated arterial stiffening. (70) Also, non-diabetes specific macroangiopathic complications such as atherosclerosis occur more frequently in T2DM. Still, CV diseases are prevalent in T1DM as well, especially in the presence of albuminuria, due to the microangiopathy of small intramural coronary arteries. (71, 72)

DKD and CVD have a unique bidirectional relationship. DKD is related to cardiac overload, inducing cardiac hypertrophy and fibrosis, which aggravate the worsening of kidney function. On the other hand, CVD, like pre-existing chronic heart failure (HF), also increases the risk of CKD in diabetic patients (cardiorenal syndrome). (73) Their common mechanism involves metabolic disorder, microvascular disease, myocardial fibrosis, cardiac inflammation, and oxidative damage, but the specific pathophysiology is unclear. Definitive treatment options remain suboptimal and often empirical. Thus, novel treatments are needed. (74) (*Figure 4.*)

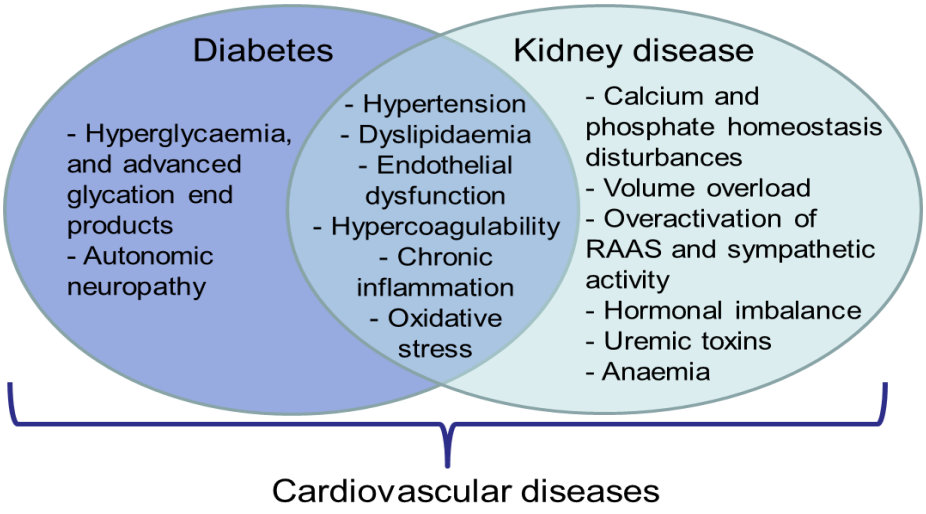


Figure 4. Shared pathogenic features of diabetes and kidney disease leading to a higher risk of cardiovascular complications (RAAS= renin-angiotensin-aldosterone system)

1.3.1. Pathogenesis

Endothelial cell dysfunction (ECD) plays a central role in the pathophysiology of both CV and renal complications of DM. (75) The pathologic metabolic environment with higher glucose levels decreases nitric oxide (NO) production. NO, besides being the most potent vasodilator, is responsible for TGFβ and CTGF downregulation, inhibition of collagen type I and III accumulation, stimulation of fibroblast growth factor 23 (FGF23) production, and activation of MMPs. (76) Other pathophysiologic features of renal ECD, such as increased vascular permeability, result in clinical manifestations like micro- and macroalbuminuria. (77)

In renal and cardiac vascular smooth muscle cells, chronic and uncontrolled hyperglycemia stimulates the production of fibrotic connective tissue elements like collagen or elastin, and their regulators such as CTGF. (78) Cardiac fibroblasts may also transform into myofibroblasts in response to certain pathological stimuli, such as mechanical stretch and neurohormonal disturbances. Their activation, characterised by excessive αSMA production and deposition, leads to myocardial fibrosis and adversely affects cardiac structure and function. (79) Furthermore, the monocyte infiltration in the vascular wall increases the risk of atherosclerosis. (80)

The atherosclerotic process occurs in the first two layers of the artery wall (tunica intima and tunica media), resulting in increased intima-media thickness (IMT). Increased IMT reflects the accumulation of foam cells and the migration and proliferation of smooth muscle cells. (81) Aortic IMT changes progressively with DM duration, indicating its potential as an evolving early biomarker of atherosclerosis. (82)

As another CVD indicator in DM, the stiffness of large elastic arteries is a significant determinant of systolic blood pressure, left ventricular load, oxygen requirements, and pulsatile stress on arterial walls. Stiffening of the aorta increases cardiac workload and reduces coronary flow, leading to left ventricular hypertrophy, coronary ischemia, and HF. (83)

In developing CV complications in DM, hyperglycemia-activated RAAS is essential in controlling arterial pressure, tissue perfusion, and extracellular volume. (84) The increased endothelial angiotensin II (Ang II) and aldosterone activity induce cardiac cell hypertrophy, vascular proliferation and deposition of ECM proteins. This plays a non-

negligible regulatory role in the formation of myocardial hypertrophy, inflammation and interstitial fibrosis, leading to superimposed progression of the diseases. (85)

1.3.2. Diagnosis and prognosis

Early identification of CV dysfunction and efficient disease progression prevention are crucial to improving the expectancy and quality of life of diabetic patients. T1DM and T2DM have a similar impact on CV mortality; however, T1DM patients lose more life-years due to CV complications. (86)

IMT is considered a non-invasive surrogate marker of asymptomatic or subclinical atherosclerosis. Its measurement is recommended for macrovascular risk identification for DKD patients as it allows biologically relevant quantification of disease progression and response to treatment. (87)

Increased aortic stiffness is an independent risk factor for CV morbidity and mortality, and its assessment provides a summative measure of vascular health. (88) Pulse wave velocity (PWV) is suitable for evaluating and following the condition of the vascular system, as the pulse wave travels more slowly in elastic vessels than in sclerotic, rigid arteries. Carotid–femoral PWV is considered the gold standard, non-invasive method for measuring central arterial stiffness; a 1 m/s increment in PWV corresponds to a 15% increased risk of CV events and mortality. (89)

Besides Troponin I and B-type natriuretic peptide (BNP), other serum markers would be needed for the early detection and to monitor the prognosis of CVD. Klotho is a new sensitive and specific cardioprotective marker for CVD and CKD. Klotho proteins are mainly produced in the kidney and form a unique endocrine system that prevents the effects of ageing, oxidative stress, inflammation and fibrosis on the heart and kidney, and reduces the burden of CVD and DKD. (90, 91) Another promising biomarker is FGF23, a key hormone in phosphate homeostasis regulation. Its increased level presents a remarkable correlation with kidney dysfunction and mortality in CKD, and CVD as well. (92)

1.3.3. Treatment

Hypertension management is of crucial importance in reducing CV complications in DKD. Generally, the recommended on-treatment target blood pressure goal is <130/80

mmHg, if it can be safely attained, considering the anticipated benefits and risks for individual patients. Above 65 years of age, the targeted systolic blood pressure is between 130-139 Hgmm. (93)

For T2DM patients with CKD, CVD, or HF, SGLT2 inhibitors are recommended to reduce the risk of adverse CV events. However, for T1DM patients, SGLT2 inhibitors are not recommended because of the possible risk of ketoacidosis. (94)

ACEi and ARBs also reduce the risk of CV events in diabetic patients, even without previous CVD history. (95) MR antagonists also effectively treat hypertension with additional CV benefits, as well as the novel non-steroidal MR antagonist finerenone is recommended in DKD with HF. (96) However, RAASi are recommended for diabetic patients only when macrovascular complications with hypertension, albuminuria, HF, or DKD are present. (97)

1.4. DKD-associated depression

Among diabetic patients, depression worsens the progression of multiorgan complications. (98) Although it occurs often, DKD-associated depression commonly remains unnoticed, and patients do not receive proper treatment. (99) Mood disorders as comorbidities worsen compliance, quality of life and increase mortality. (100) Depressed patients are more likely to perform risky behaviour such as smoking, decreased physical activity or unhealthy eating. (101, 102) In the presence of depression, DM is harder to control, carbohydrate metabolism imbalance worsens, the number of complications increases, and the risk of suicide events grows. (103, 104)

Besides these factors, common molecular pathways link these pathologies. Recent studies suggest the role of immunological processes, oxidative stress, endocrine and neurobiological factors or cerebral structural alterations. (105)

1.4.1. Prevalence

Depression has the highest risk for developing DM among all psychiatric disorders, increasing the likelihood of DM by 40%. (106) Several analyses proved the association in the other direction by showing that the prevalence of depressive symptoms is twice as

high in diabetic patients compared to the non-diabetic population. (107) DM-associated depression is more common and more severe in women than in men. (108)

Depression is the most common psychiatric problem among ESRD patients. The form of renal replacement therapy determines a variance in its pervasiveness; among haemodialysed patients it is around 25%, (109) while in peritoneally dialysed patients it is around 10%. (110) Kidney transplanted patients are in the middle with 20%. (111)

1.4.2. Diagnosis and treatment

Clinical depression is characterized by behavioural, somatic and vegetative symptoms. Major depression is diagnosed, if at least five of the following symptoms is present for at least two weeks: depressed mood, diminished interest, significant change in weight or appetite, slowed down thinking and reduction of physical movement, fatigue, feeling worthless or guilty, diminished ability to concentrate, recurrent thoughts of death. (112) The questionnaires most often used for screening are the Beck depression inventory and Zung self-rating depression scales. (113, 114)

Current common medications are tri- and tetracyclic antidepressants, monoamine-oxidase inhibitors, selective serotonin reuptake inhibitors, and serotonin-norepinephrine reuptake inhibitors. (115) Many patients need multiple medication trials for remission, which is successful in only about 30% of the cases. (116) The therapeutic non-adherence is worsened due to the side effects of the antidepressants (anxiety, insomnia, fatigue, blood pressure changes, sexual dysfunction). (117) Other supportive methods, such as psychotherapy, are essential for complete complex management. (118)

1.4.3. Common pathophysiology of DKD and depression

By investigating DM- and DKD-associated depression, multiple shared bidirectional pathophysiological processes were observed. (119) Common neuroendocrine, oxidative, and inflammatory pathways and neurotrophins, especially brain-derived neurotrophic factor (BDNF), are essential. Since the full description of these factors is beyond the scope of this dissertation, only the most critical information will be summarized.

The over-activation of the hypothalamic-pituitary-adrenal axis can explain the endocrine link between DKD and depression. In DM, negative feedback of adrenal glucocorticoids through MR is damaged, (120) whereas depression is also associated

with higher serum cortisol and corticotropin-releasing hormone levels. (121, 122) The lack of insulin also leads to neurocognitive damage and depression-like symptoms by hindering neuroplasticity in insulin-sensitive regions such as the hippocampus, hypothalamus or amygdala. (123) Conversely, disturbances in amino acid metabolism result in lower tryptophan levels in the central nervous system, holding back serotonin synthesis. (124)

1.4.3.1. Inflammation theory

In the pathophysiological process of both depression and DM, several common inflammatory cytokines and transcription factors were identified. (125) Interleukin 1 (IL-1), IL-6, and tumour necrosis factor alpha (TNFα) levels rise in parallel with pancreas β islet cell death, whereas hyperglycaemia further facilitates the production of these cytokines. (126) The inflammatory mediators protrude into the central nervous system, causing depression-like symptoms. (127) Depression provokes a subclinical systemic inflammatory response, contributing to β-cell damage and insulin resistance. (128) Obesity is common in both diseases, where high levels of adipokines and NFκB augment a multisystemic inflammation. (129)

1.4.3.2. Brain-derived neurotrophic factor (BDNF)

Neuroinflammation and glial activation-induced pathophysiological changes contribute to both DM and neuropsychiatric diseases. (130, 131) Based on the similarities in neurostructural changes in DM and depression, the role of neurotrophins comes into question. (132) This protein family regulates differentiation and survival of neurons, resulting in impaired synaptic plasticity and neuronal survival in inflammation. (133) In depression, the level of BDNF and its receptor, the tyrosine receptor kinase B (TrkB), is lower in the hippocampal and prefrontal regions. (134) BDNF is mainly localised in the central nervous system but has also been identified in endothelial cells of other peripheral organs. (135) Besides being a neurotrophin, it has immuno- and metabotrophic functions. Lower BDNF levels are connected to insulin resistance, worsened glucose metabolism and higher blood sugar. (136)

BDNF is synthesised as a precursor form (proBDNF), which binds to p75 neurotrophin receptor (p75Ntr) and activates apoptosis and long-term synaptic depression through NFκB and phospho-c-Jun N-terminal kinase (pJNK) pathways. (137) However, after a

proteolytic cleavage by furin, plasmin, MMP3 or MMP7, the mature BDNF (mBDNF) form activates TrkB, causing cell survival and long-term synaptic potentiation through phospho-extracellular signal-regulated kinase (pERK) and phospho-cAMP response element-binding protein (pCREB) pathways. (138) Therefore, the two isoforms have biologically distinct physiological features. (139)

1.5. Renin-angiotensin-aldosterone system (RAAS)

Since modulating the RAAS system in different DM-associated pathologies is my dissertation's main topic, its systemic and local pathomechanism will be described in more detail in the following chapter.

The RAAS hormonal cascade is initiated when renin is synthetized from prorenin in the kidney's juxtaglomerular apparatus. (140) As a proteolytic enzyme, it cleaves liver-produced angiotensinogen to Ang I. Further conversion to biologically active, potent vasoconstrictor peptide, Ang II, is made by ACE, mainly on the surface of pulmonary endothelial and renal epithelial cells. ACE also facilitates degradation of the vasodilator peptide bradykinin, which increases systemic vascular tone. (141)

The other homologous form, ACE2, has been identified mainly in the kidney and heart. It does not bind to bradykinin, but hydrolyses Ang I to Ang 1-9 and Ang II to Ang 1-7. These end products have opposing actions to Ang II, by promoting apoptosis and vasodilation. Consequently, as a natural counter-regulator, ACE2 prevents over-activation of Ang II and serves as an endogenous inhibitor of RAAS. (142)

Ang II is a pleiotropic hormone regulating extracellular fluid homeostasis. It has a direct vasoconstrictor effect in precapillary arteries. In the zona glomerulosa of the adrenal cortex, Ang II stimulates aldosterone secretion, which is responsible for renal sodium reabsorption. Ang II also regulates human and rat aortic vascular smooth muscle cells' function and survival. (143, 144)

Ang II can act through two different receptors. ATR1 activation facilitates tubular sodium reuptake, potassium excretion, water intake, vasopressin secretion, increased sympathetic activity, vasoconstriction, and increased blood pressure; it is also involved in inflammation and fibrotic processes. ATR2 generally opposes ATR1-mediated

actions, promotes vasodilation, and has a role in anti-proliferative and anti-inflammatory processes. (145) (*Figure 5*.)

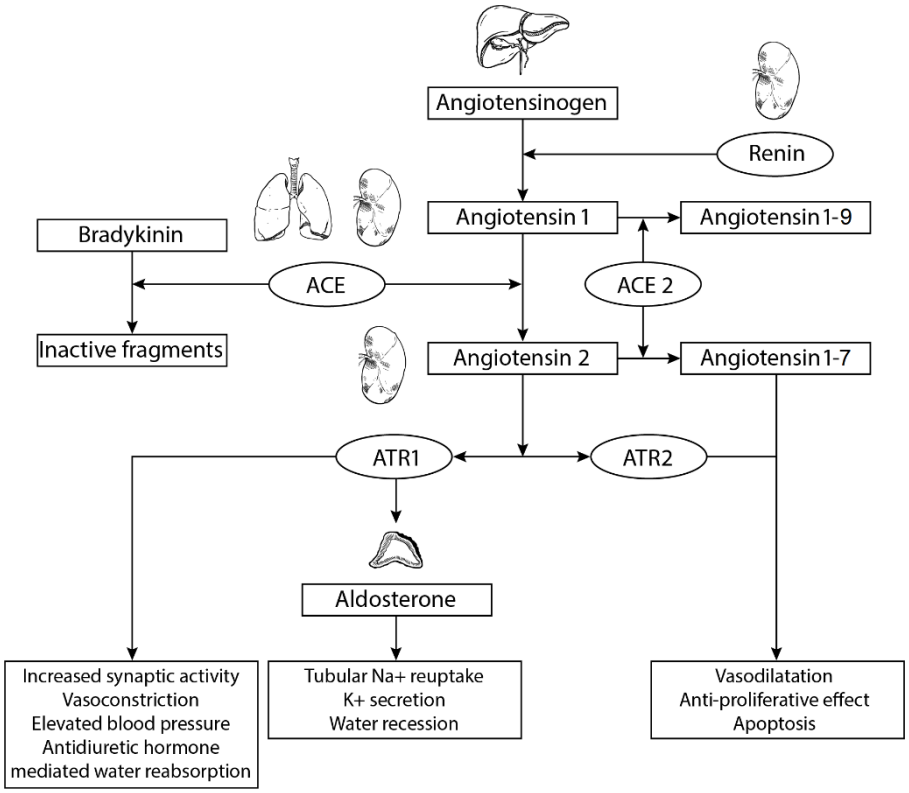


Figure 5. Summary of classical systemic renin-angiotensin-aldosterone system (ACE = angiotensin-converting enzyme, ATR = angiotensin receptor)

1.5.1. Classical and local RAAS

The classical version of RAAS is the systemic, circulating hormonal cascade. It regulates numerous physiological functions such as salt and water homeostasis, endovascular fluid volume, blood pressure, cardiac output, vasoconstriction and vascular wall integrity. Insulin resistance and hyperglycaemia are known to result in RAAS overactivation, and the role of Ang II, ATR1, and aldosterone is especially pivotal. (146) The resulting changes mainly concern the skeletal muscles, liver, heart, vessels, and kidneys. Higher circulating RAAS levels, impaired insulin signalling pathway, and ECD contribute to atherosclerosis and increased blood pressure. (147)

The local (non-classic) RAAS is regulated independently from the circulating systemic hormones. It can be found in the kidney, adrenal cortex, heart, brain, vessels, lungs, and adipose tissue. (148, 149)

1.5.2. Local RAAS in the brain

Two independent local RAAS cascades communicate with each other in the brain. The endogenous system occurs within the blood-brain barrier inside neurons and the synaptic cleft. (*Figure 6.* A) The other cascade mediates the effects of peripheral Ang II through circumventricular organs and cerebrovascular endothelial cells. (150)

Endogenous renin is produced predominantly in hypothalamus and pituitary gland, whereas astrocytes and glial cells synthetize angiotensinogen. (151) The principal effector of local cerebral RAAS is Ang II, generated in the hippocampus, hypothalamus, hypophysis, and amygdala. By extracellular Ang II, neurohormonal functions dominate, whereas neurons can convert angiotensinogen to Ang II and use it as neuro- or co-transmitter. (152) A small amount of aldosterone can pass the blood-brain barrier and bind to MR in the nucleus tractus solitarii and the ventromedial nucleus of the hypothalamus. (153)

Local cerebral RAAS is pivotal in regulating blood pressure, body temperature homeostasis, and locomotor activity. Furthermore, it is crucial in memory formation, behavioural and learning processes. (154, 155) Over-activation of local cerebral RAAS contributes to certain neurological disorders such as Alzheimer’s disease or depression. (156)

1.5.3. Local RAAS in the heart

Classical RAAS plays a role in hemodynamics and tissue remodeling associated with cardiomyocyte- and ECD in the heart, leading to progressive functional impairment. (*Figure 6.* B) With involvement of natriuretic peptides, local RAAS can predispose the onset of HF through different mechanisms involving inflammation, cardiac remodelling, and accumulation of epicardial adipose tissue. (157) Ang II receptors and the intracardiac aldosterone system stimulate cardiac fibroblasts and increase the volume of collagen fraction in the ventricles. (158) Increased level of FGF23 also stimulates local cardiac RAAS, inducing myocardial fibrosis and hypertrophy. (159)

1.5.4. Local RAAS in the kidney

Renal local RAAS is regulated partly independently of the systemic hormones. All components of RAAS are expressed in the kidney, allowing for endogenous Ang II generation. (160) (*Figure 6*. C) Local renal concentration of renin and Ang II is much higher than the systemic levels. (161) Angiotensinogen is also found in renal proximal tubules besides systemic renin production. Ang I can be formed and converted to Ang II on the brush border of proximal tubular cells. (162)

In DKD, hyperglycaemia stimulates local renal RAAS activation. In the early phase, this means selective renin expression increment, then angiotensinogen and Ang II facilitate each other's expression, resulting in a vicious cycle. (163, 164) ATR1 is expressed on the tubular epithelial, vascular endothelial, smooth muscle, and interstitial cell membranes. High Ang II level through this receptor leads to increased vascular resistance, higher blood pressure, decreased renal blood flow and filtration, epithelial cell hypertrophy, mesangial cell contraction and ECM expansion. (165) These, together with Ang II-dependent aldosterone release, eventually induce the production of inflammatory cytokines and growth factors, leading to fibroblast activation, tubulointerstitial inflammation and fibrosis. (166)

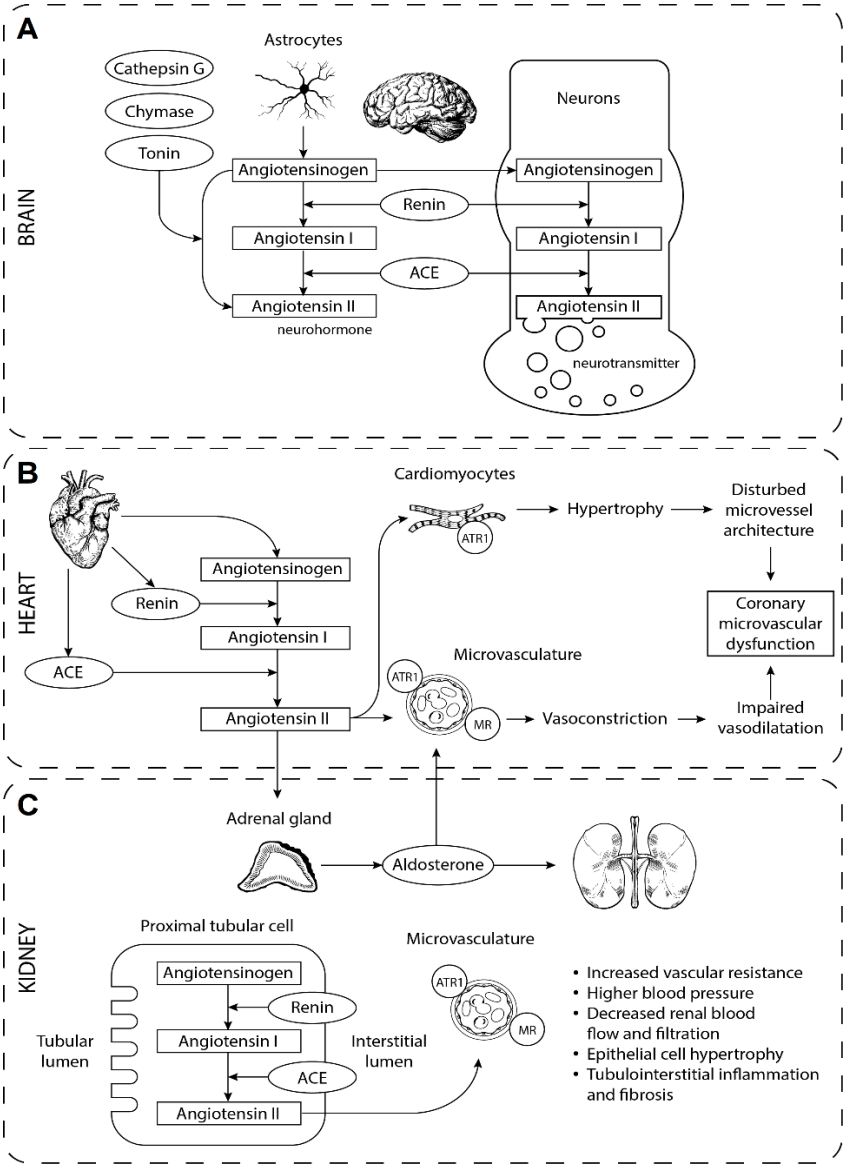


Figure 6. (A) Local brain, (B) local cardiac, and (C) local renal renin-angiotensin-aldosterone system (ACE = angiotensin-converting enzyme, ATR1 = angiotensin receptor 1, MR = mineralocorticoid receptor)

All these literature data and our previous studies suggest that the whole complex of different processes, including glucotoxicity, increased oxidative stress and activated inflammatory pathways, hypoxia and many more, contribute to the development of DM-associated multiorgan damage.

Among these processes, the modulation of the RAAS system as a potential therapeutic target has been investigated in different *in vitro* and *in vivo* models during my PhD studies. We aimed to explore underlying mechanisms of the pleiotropic protective effect of RAASi on the development of diabetic multiorgan complications, including renal, cerebral and cardiac damage.

2. Objectives

Our specific aims are summarized in *Figure 7*. The presented studies assess the changes in specific target organs and explore the protective effect of RAASi treatment against T1DM-induced complications and their underlying mechanisms.

To fulfil these aims, we have set the following specific objectives:

1. To investigate *in vitro* how RAASi influence glucose-induced and profibrotic changes in human proximal tubular and rat kidney fibroblast cells
2. To determine the renoprotective and antifibrotic effect of RAASi in a rat model of T1DM
3. To test the effect of lyophilisation on the reproducibility in fibrotic tissue sample processing
4. To analyse the beneficial effects of RAASi on diabetic vascular impairment, cardiac tissue damage, fibrosis, hypoxic and inflammatory damage in T1DM rat hearts
5. To evaluate the protective potential of RAASi on T1DM-induced neuroinflammation and central BDNF pathway alterations in rat hippocampi

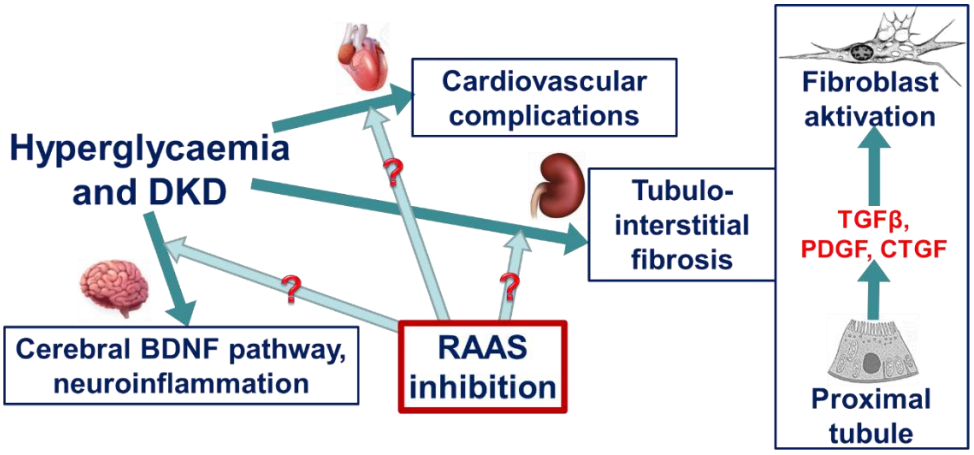


Figure 7. Summarized study objectives: effect of renin-angiotensin-aldosterone system (RAAS) inhibition on multiorgan complications of diabetic kidney disease (DKD) (BDNF = brain-derived neurotrophic factor, TGFβ = transforming growth factor β, PDGF = platelet-derived growth factor, CTGF = connective tissue growth factor)

3. Methods

All chemicals and reagents were purchased from Sigma-Aldrich (Darmstadt, Germany), and all standard plastic laboratory equipment was purchased from Sarstedt (Nümbrecht, Germany) unless stated otherwise.

3.1. *In vitro* experiments

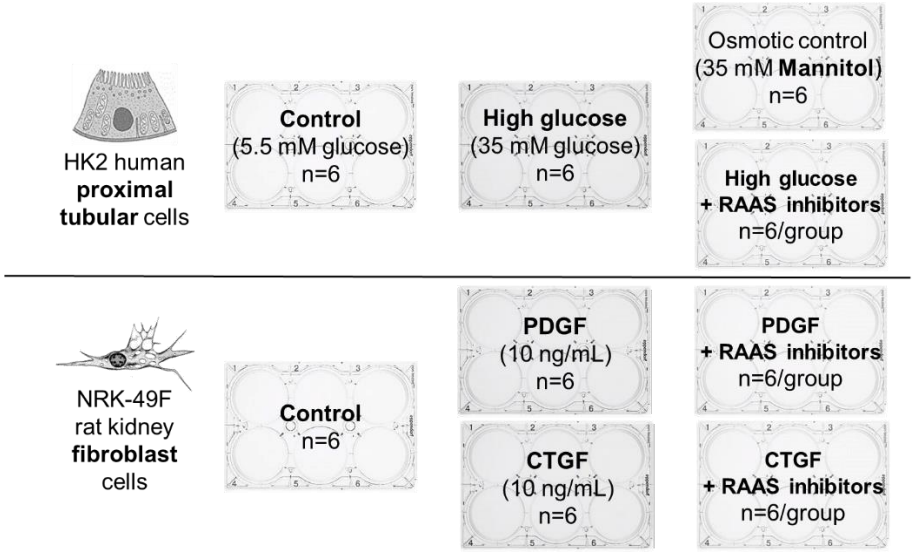
3.1.1. Experimental design and cell culture

Human kidney 2 (HK-2) proximal tubular epithelial cells (LGC Standards, American Type Culture Collection, Manassas, VA, USA) were grown in DMEM containing 5.5 mM D-glucose (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum (FBS) (Gibco, Life Technologies), 1 % L-glutamine, and 1 % antibiotic-antimycotic solution (10,000 IU/mL penicillin, 10 mg/mL streptomycin, 25 mg/mL Amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA)). Cells were incubated at 37 °C in 95% humidified air with 5 % CO_2. Before treatments, cells were detached with 0.25% trypsin-EDTA (Gibco, Life Technologies) and plated in 6-well plates (5×10^5 cells/well), where a growth arrest period was induced for 24 hours in FBS-free medium in all experiments. Three sets of experiments were performed.

HK-2 cells were kept under standard (control; 5.5 mM) or high glucose (HG; 35 mM) conditions for 24 or 48 hours. To differentiate between the direct glucotoxic and the osmotic effect of HG, HK-2 cells were cultured in isosmotic control mannitol (5.5 mM glucose + 29.5 mM mannitol) containing media as well. HG groups were treated with either 10 μM ramipril (HG + Ramipril), 10 μM losartan (HG + Losartan), or 10 μM eplerenone (HG + Eplerenone). Drug doses were adopted from the literature. (167, 168)

Normal rat kidney fibroblast (NRK-49F) cells were grown in DMEM containing 25 mM D-glucose supplemented with the same additional ingredients as HK-2 cells, and identical incubation and growth arrest protocols were applied. NRK-49F cells were treated with PDGF (10 ng/mL) or CTGF (10 ng/mL) and with various RAASi as described above. (*Figure 8.*)

Control cells were treated with vehicle (dimethyl sulfoxide) alone (n = 6 wells/group). Cells were subsequently incubated at 5 % CO_2 and 37 °C, detached with 0.25 % trypsin-EDTA and RNA and protein were isolated with the same buffers as tissue homogenates.



RAAS-inhibitors	*In vitro* dose
Ramipril	10 µM
Losartan	10 µM
Eplerenone	10 µM

Figure 8. Study design and experimental protocol for *in vitro* experiments (PDGF = platelet-derived growth factor, CTGF = connective tissue growth factor, RAAS = renin-angiotensin-aldosterone system)

3.1.2. Cell viability and proliferation assays

Before the experiments, the non-toxic properties of RAASi doses were confirmed by cell viability assays. Cell viability was determined using a methyl-thiazolyl-tetrazolium (MTT) assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Cells were detached with 0.25 % trypsin-EDTA by trypan blue exclusion and resuspended in medium diluted 1:1 with trypan blue solution. Live cells from triplicate wells were counted in a Bürker chamber.

In pilot studies, the most effective proliferative doses of PDGF and CTGF on NRK-49F cells were determined for different concentration series by proliferative assays.

3.1.3. Immunocytochemistry

For immunocytochemical examination, NRK-49F cells were cultured in tissue culture chambers. After repeated washes with phosphate-buffered saline, cells were fixed in 4% buffered formalin, rewashed, and permeabilised with Triton X-100. After blocking with 5 % bovine serum albumin (BSA), samples were incubated for 2 hours with specific anti-PDGFR-β (#sc-432, Santa Cruz Biotechnology, Dallas, TX, USA) primary antibody diluted 1:100. After repeated washes, slides were incubated with specific goat anti-rabbit Alexa fluor 568 (#A-11036, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody diluted 1:100. F-actin was immunostained by incubation with phalloidin-TRITC diluted 1:300 for 1 hour at room temperature. Samples were washed

and nuclei were counterstained with Hoechst 33342 (#4082S, Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000. Appropriate controls were prepared omitting the primary antibody to assure specificity and to avoid autofluorescence. After drying, sections were fixed with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Coverslipped slides were analysed with a Zeiss LSM 510 Meta Image Examiner confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) with 63× or 100× magnification objectives or, for phalloidin-stained slides, with an Olympus IX81 fluorescence microscope (Olympus Europa, Hamburg, Germany) at 100× magnification.

3.2. *In vivo* experiments

All animal procedures and handling were approved by the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at Semmelweis University, Budapest, Hungary (PEI/001/380-4/2013). All experiments were conducted following international ethical principles and regulations. Power analysis before the *in vivo* experiments determined the minimal required animal numbers.

3.2.1. Experimental design and animal model

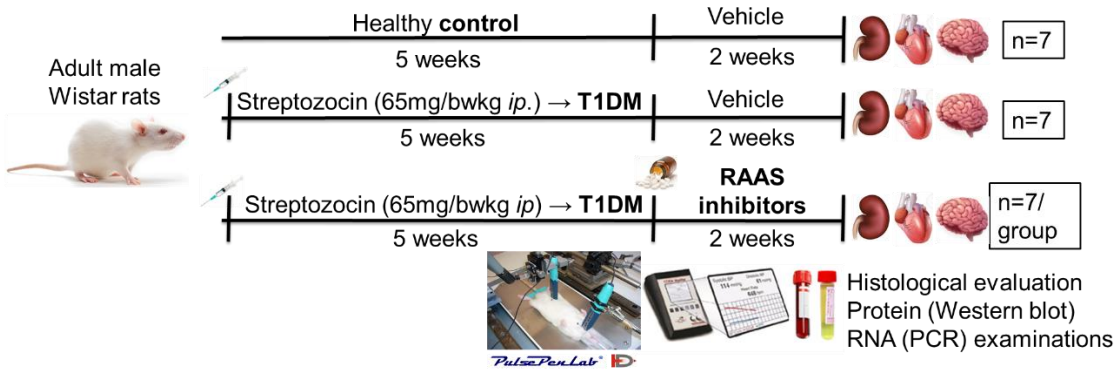
Eight-week-old, male Wistar rats (*Rattus norvegicus*) weighing 200 ± 10 g were purchased from "Toxi-Coop" Toxicological Research Centre (Dunakeszi, Hungary) and housed in groups of three in plastic cages under a 12-hour dark/light cycle at constant temperature (24 ± 2 °C) and humidity (55 %) with *ad libitum* access to standard rodent chow and drinking water.

T1DM was induced chemically, with a single intraperitoneal injection of 65 mg/bwkg streptozotocin (STZ), dissolved in 0.1 M citrate buffer (pH 4.5). After overnight fasting, blood glucose level was measured three times from the tail vein with a Dcont IDEAL device (77 Elektronika, Budapest, Hungary). Rats were considered diabetic and enrolled in the study if their peripheral blood glucose value was above 15 mmol/L 72 hours after the STZ injection and remained elevated.

Five weeks after the onset of DM, rats were randomized into four groups considering equal average blood glucose values (n = 7 animals/group) and were treated daily for 2 weeks *per os* by oral gavage either with: (i) isotonic saline as vehicle (D); or (ii) ACEi

ramipril (D + Ramipril, 10 µg/bwkg/day); or (iii) ARB losartan (D + Losartan, 20 mg/bwkg/day); or (iv) MR antagonist eplerenone (D + Eplerenone, 50 mg/bwkg/day). (*Figure 9.*) The RAASi drug doses were adopted from our previous experiments in line with the literature, where effective blockade of RAAS was reached without changes in systemic blood pressure. (169-172)

Non-diabetic, age- and body weight-matched healthy animals (Control; n = 7 animals/group) received an equivalent volume of citrate buffer without STZ once, and the same amount of saline by oral gavage daily, at the same time as the diabetic animals throughout the 2-week treatment period.



RAAS-inhibitor	Ramipril	Losartan	Eplerenone
***In vivo* dose**	10 µg/bwkg/day	20 mg/bwkg/day	50 mg/bwkg/day

Figure 9. Study design and experimental protocol for the *in vivo* experiments (T1DM = type 1 diabetes mellitus, RAAS = renin-angiotensin-aldosterone system)

Diabetic rats did not receive insulin treatment. Therefore, their physical state gradually decreased in parallel with the metabolic changes. The general well-being of the animals was monitored daily, and those whose pain signals elevated significantly were anaesthetised. At the end of the experimental protocol, rats were placed into metabolic cages to collect urine for 24 hours. Based on protein excretion rates, in this experimental model, animals show signs of chronic renal damage after 5 weeks of untreated DM.

Lastly, at 7 weeks of the experimental period, rats were anaesthetized by a mixture of 60 mg/bwkg ketamine (Richter Gedeon, Budapest, Hungary) and 5 mg/bwkg xylazine (Medicus Partner, Biatorbagy, Hungary) administered intraperitoneally and sacrificed by terminal blood drawn from the abdominal aorta. Blood, urine, kidney, heart, aorta,

and hippocampal samples were collected, immediately snap-frozen or placed in formalin and stored for further investigations.

3.2.2. Measurement of arterial blood pressure and pulse wave velocity

After two weeks of treatment, PWV and blood pressure measurements were taken under isoflurane anaesthesia (4% inducing concentration then 1.5% for maintenance) on a 37 °C heating pad. A CODA tail cuff standard monitor system (EMKA Technologies, Paris, France), which uses clinically validated proprietary volume pressure recording technology (173), was used to non-invasively measure systolic and diastolic blood pressure in the tail vein. Mean arterial pressure (MAP) was calculated from these values. Non-invasive PWV registration was carried out using PulsePenLab (DiaTecne, Milan, Italy) applanation tonometry device while recording an electrocardiogram. Pulse waves were detected simultaneously over the carotid and femoral arteries on the same side. The time difference between the two waves was divided by 80 % of the direct carotid-femoral surface distance, measured with a high-precision digital calliper. (174)

3.2.3. Metabolic and renal function parameters

Blood and urine samples were centrifuged for 6 minutes at 3600 rpm. Serum metabolic (glucose, fructosamine, total cholesterol) and renal (blood urea nitrogen, serum creatinine, and creatinine from urine collected for 24 hours in metabolic cage) parameters were determined photometrically with commercially available kits on a Hitachi 912 chemistry analyser (Roche, Basel, Switzerland). Creatinine clearance was calculated using the formula: (urine creatinine x urine volume/serum creatinine) / urine collection time minutes/bodyweight.

3.2.4. Enzyme-linked immunosorbent assay (ELISA)

Collected rat blood samples were centrifuged at 3600 rpm for 6 minutes and separated serum samples were diluted to 1:10. Serum Cardiac Troponin I and Klotho levels were measured by using commercially available rat-specific sandwich ELISA kits (Abcam, Cambridge, UK and ABclonal, Woburn, MA, USA, respectively), following the manufacturer's protocols. Concentration was calculated based on absorbance measurement at 450 nm with wavelength correction at 650 nm using SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany).

3.2.5. Histology

Kidney, left ventricular muscle, and aorta sections were dissected, fixed in 4 % buffered formalin, dehydrated in a graded series of alcohols, and embedded in paraffin. 5 µm-thick sections were cut and deparaffinized in xylene, rehydrated and mounted on coated glass microscope slides. On specific stained sections, images were taken with an AxioImager A1 light microscope (Zeiss, Jena, Germany) and histological examination was performed under ×200 magnification using Panoramic Viewer software version 1.15.2. (3DHISTECH, Budapest, Hungary). Analysis was performed double-blinded with computer-assisted morphometry using ImageJ software version 1.5 (National Institutes of Health, Bethesda, MD, USA).

Picrosirius red staining was performed on the kidney cortex and left ventricular muscle to evaluate fibrosis. Ten areas from each kidney cortex and heart left ventricular muscle cross-section were randomly selected, and the specific stained fibrotic area was measured. The number of pixels containing stained fibrotic tissue was divided by the total number of pixels in the area to obtain the percentage of fibrosis and collagen deposition.

For IMT measurement, aorta sections were immersed in 1 % orcein at 60 ºC 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 washed with distilled water. Orcein-stained elastic fibres made IMT measurement possible on cross sections of each aorta, and the mean value of ten measurements was calculated.

Masson's trichrome staining was performed to evaluate the fibrosis of the media layer. The specifically stained interstitial areas were measured within the intima-media layer of each aorta cross-section. The number of pixels containing blue-stained fibrotic tissue was divided by the number of other pixels in the area to obtain the ratio of medial fibrosis.

3.2.6. Lyophilisation

Collected kidney and heart tissue samples were stored at -80 °C in 2 ml Eppendorf tubes, and optimally arranged to approach the most significant surface exposure for lyophilisation. Freeze-drying was performed with the ScanVac CoolSafe Touch Superior device (LaboGene A/S, Allerod, Denmark). Tubes remained open through the

whole process. After one hour pre-freezing at -40 °C, pressure was lowered to 0.07 hPa, then primary drying consisted of six 2-hour steps at 0.22 hPa with gradually increasing temperature up to 30 °C; secondary drying was carried out on 0.1 hPa at 40 °C for 3 hours. Dried tissue products were manually smashed with 20-Gauge needles and pulverized with 5 mm stainless steel balls using TissueLyser LT (Qiagen GmbH, Hilden, Germany). Powdered dry tissue samples were stored at 4 °C until further processing and measurements.

3.3. Measurement of extracellular matrix formation and degradation biomarkers

C3M, PRO-C3 (measuring type III collagen formation) and TUM biomarkers were measured in rat urine samples. FBN-C (measuring C-terminal of fibronectin turnover) and PRO-C4 (measuring type IV collagen formation) were measured in HK-2 and NRK-49F cells' supernatant samples. For both, competitive ELISA kits were developed by Nordic Bioscience (Herlev, Denmark). Urinary biomarker levels were divided by urinary creatinine levels measured with the QuantiChrom™ Creatinine kit (BioAssay Systems, Hayward, CA, USA) to normalize for urine output. The assays were accomplished at Nordic Bioscience Laboratories following previously described protocols. (61, 62, 175-177) Briefly, streptavidin-precoated 96-well ELISA plates (Roche, Basel, Switzerland) were incubated with 100 µL biotinylated peptide for 30 minutes. Plates were washed five times with washing buffer, and then incubated with 20 µL standard peptide or sample together with HRP-conjugated monoclonal antibody for 20 hours at 4 °C (C3M, PRO-C3, FBN-C) or 1 hour at 20 °C (TUM, PRO-C4). Repeated washing steps were followed by incubation with 100 µL 3,3′,5,5-tetramethylbenzidine (Kem-En-Tec, Taastrup, Denmark) for 15 minutes in the dark. The reaction was stopped by adding 1% sulfuric acid solution, and density values were measured with an ELISA reader at 450 nm, with 650 nm as reference (VersaMax, Molecular Devices, CA, USA).

3.4. Quantitative real-time reverse transcript polymerase chain reaction

Total RNA was extracted from HK-2 and NRK-49F cells, rat kidney, heart, and hippocampal samples using the Total RNA isolation Mini Kit (Geneaid Biotech, New Taipei City, Taiwan). The quality and quantity of isolated RNA were measured with a NanoDrop ND-1000 spectrophotometer (Baylor College of Medicine, Houston, TX, USA). 250 ng of RNA was reverse-transcribed using Maxima First Strand cDNA

Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA). Specific mRNA expressions of interest were determined in duplicates from 1 µL cDNA samples obtained by qPCR using SYBR Green I Master enzyme mix (Roche Diagnostics, Mannheim, Germany) and 10 pmol/µL of each specific primer (Invitrogen, Budapest, Hungary), following sequences designed based on nucleotide sequences from National Center for Biotechnology Information's nucleotide database (For primer sequences and details see *Table 1*). Results were analysed by LightCycler 480 software version 1.5.0 (Roche Diagnostics, Mannheim, Germany). The mRNA expressions of interest were normalised to the mRNA expression of the 18S ribosomal RNA housekeeping gene from the same samples as a reference transcript.

Table 1. Sequences of primer pairs for RT-qPCR

Gene name	**Regular name**	**NCBI ID**	**Primer pairs**		**Product length**
TGFB1	human TGFβ1	NM_ 000660.7	Forward:	5′-GCG TGC GGC AGC TGT ACA TTG ACT-3′	174 bp
			Reverse:	5′-CGA AGG CGC CCG GGT TAT GC-3′	
PDGFB	human PDGFB	NM_ 002608.4	Forward:	5′-AGA TGG GGC CGA GTT GGA CCT GAA-3′	163 bp
			Reverse:	5′-GCG CCG GGA GAT CTC GAA CAC CT-3′	
CCN2	human CTGF	NM_ 001901.3	Forward:	5′-GTC CAC CCG GGT TAC CAA TGA CAA-3′	228 bp
			Reverse:	5′-CAG GAT CGG CCG TCG GTA CAT ACT-3′	
RN18S	human 18S ribosomal RNA	NM_ 003286.4	Forward:	5′-GGC GGC GAC GAC CCA TTC-3′	136 bp
			Reverse:	5'-TGG ATG TGG TAG CCG TTT CTC AGG-3'	
Pcna	rat PCNA	NM_022381.3	Forward:	5'-AGG GCT GAA GAT AAT GCT GAT ACC-3'	238 bp
			Reverse:	5'-AAA ACT TCA CCC CGT CCT TTG-3'	
Mki67	rat Ki67	NM_001271366.1	Forward:	5'-GCC CAT TAG CAG TTG GCA AAA-3'	72 bp
			Reverse:	5'-GGG TTC TAA CTG GTC TTC CTG G-3'	
Acta2	rat αSMA	NM_031004.2	Forward:	5'-GAG CGT GGC TAT TCC TTC GTG-3'	106 bp
			Reverse:	5'-CAG TGG CCA TCT CAT TTT CAA AGT-3'	

Nppb	rat BNP	NM_031545.1	Forward:	5′-CAG CTC TCA AAG GAC CAA GG-3′	192 bp
			Reverse:	5′-CTA AAA CAA CCT CAG CCC GT-3′	
Pdgfb	rat PDGF	NM_031524.1	Forward:	5'-TCG ATC GCA CCA ATG CCA ACT TCC-3'	236 bp
			Reverse:	5'-CAC GGG CCG AGG GGT CAC TAC TGT-3'	
Ccn2	rat CTGF	NM_022266.2	Forward:	5'-TCC ACC CGG GTT ACC AAT GAC AAT AC-3'	195 bp
			Reverse:	5'-TTC CTT ATT GGG GTC AGC AC-3'	
Il6	rat IL-6	NM_012589.2	Forward:	5'-GCC ACT GCC TTC CCT ACT TC-3'	153 bp
			Reverse:	5'-GCC ATT GCA CAA CTC TTT TCT C-3'	
Tnf	rat TNF	NM_012675.3	Forward:	5'-GGG GCC ACC ACG CTC TTC TGT-3'	180 bp
			Reverse:	5'-CTC CGC TTG GTG GTT TGC TAC GAC-3'	
Hspa1b	rat HSP72	NM_031971.2	Forward:	5'-GGC TGA GAA AGA GGA GTT CG-3'	216 bp
			Reverse:	5'-CCA CCC ATC TGT CTC CTA GA-3'	
Il1a	rat IL-1α	NM_017019.1	Forward:	5'-TCT GCC ATT GAC CAT CTG TCT CTG-3'	152 bp
			Reverse:	5'-ACC ACC CGG CTC TCC TTG AA-3'	
Rn18s	rat 18S ribosomal RNA	NM_046237.1	Forward:	5'-GCG GTC GGC GTC CCC CAA CTT CTT-3'	105 bp
			Reverse:	5'-GCG CGT GCA GCC CCG GAC ATC TA-3'	

3.5. Western blot analysis

For Western blot analysis, all reagents and equipment were obtained from Bio-Rad Laboratories (Hercules, CA, USA) unless stated otherwise. Total protein was extracted from homogenised HK-2 and NRK-49F cells, rat kidney, heart, and hippocampi samples at 4°C in lysis buffer (1 M Tris, 0.5 M EGTA, 1 % Triton X-100, 0.25 M NaF, 0.5 M phenylmethylsulfonyl fluoride, 0.5 M sodium orthovanadate, 5 mg/mL leupeptin, and 1.7 mg/mL aprotinin, pH 7.4) using TissueLyser LT (Qiagen, Hilden, Germany) homogenisator. For the measurement of O-GlcNAc, 40 μM O-(2-acetamido-2-deoxy-d-glucopyranosylidene)-amino-N-phenyl-carbamate (Sigma-Aldrich) was added to lysates

to inhibit OGA activity. Lysates were centrifuged at 13000 rpm, 4 °C for 10 minutes. Protein concentration of supernatant samples was measured with a detergent-compatible Bradford dye-binding method protein assay kit in 96-well microplates at 650 nm using SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany). Protein samples were mixed 1:3 with Laemmli buffer (12.5 mM Tris-HCl, 4 % sodium dodecyl sulphate, 4 % mercaptoethanol, 15 % glycerol, 0.01 % bromophenol blue) and heated at 95 °C for 5 minutes for denaturation.

Solubilised sample lysates of the appropriate amount of protein/lane (25 µg for tissues and 18 µg for cells) were loaded onto 4-20 % gradient Mini-PROTEAN TGX polyacrylamide precast gels, electrophoretically separated, and transferred to nitrocellulose membranes with high-efficiency semi-dry Trans-Blot Turbo Transfer System. Ponceau S staining was performed for protein transfer verification and total loaded protein evaluation. Membranes were blocked in 5 % w/v non-fat dried milk or BSA in Tris-buffered saline for 1 hour at room temperature and immunoblotted with specific primary antibodies at 4 °C overnight. After repeated washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. (Details of antibodies are listed in *Table 2*.) Chemiluminescence was detected using Luminata Forte (Millipore, Billerica, MA, USA) substrate and immunoreactive bands of interest were quantified densitometrically on Versadoc, Quantity One Analysis software (Bio-Rad, Budapest, Hungary). After subtraction of background, the integrated optical density was factored for Ponceau S staining to correct any variations in total protein loading. Blots were also normalised to an internal control to compare bands on separate membranes.

Table 2. Properties of antibodies used for Western Blot

Primary target protein	**Manufacturer**	**Catalogue number**	**Primary dilution**	**Secondary antibody**	**Secondary dilution**
O-GlcNAc	Sigma-Aldrich, St. Louis, MO, USA	07764	1:2000	goat anti-rabbit	1:2000
OGT	Sigma-Aldrich, St. Louis, MO, USA	O6264	1:1000	goat anti-rabbit	1:2000
OGA	Proteintech Europe, Manchester, UK	14711-1-AP	1:1000	goat anti-rabbit	1:3000
αSMA	Sigma-Aldrich, St. Louis, MO, USA	A2547	1:500	goat anti-mouse	1:6000

FGF23	Abcam, Cambridge, MA, USA	ab56326	1:1000	donkey anti-goat	1:4000
NFκB	Cell Signaling, Danvers, MA, USA	C22B4	1:1000	goat anti-rabbit	1:2000
VEGF	Abcam, Cambridge, MA, USA	ab46154	1:1000	goat anti-rabbit	1:3000
BDNF	Abcam, Cambridge, MA, USA	108319	1:1000	goat anti-rabbit	1:3000
Furin	Abcam, Cambridge, MA, USA	183495	1:2000	goat anti-rabbit	1:3000
MMP3	Abcam, Cambridge, MA, USA	52915	1:1000	goat anti-rabbit	1:3000

3.6. Statistical analysis

Analyses were performed using GraphPad Prism software version 7.0 (GraphPad Software, San Diego, CA, USA). The minimum required number of items per group was determined with power analysis before the experiments to reach an appropriate statistical strength. A Kolmogorov-Smirnov normality test was performed to test if the values were from a Gaussian distribution.

A linear regression fit was applied to the dataset pairs to test correlation.

For comparison of two groups, data were analysed by two-tailed paired t-tests for all parametric comparisons, or in the case of nonparametric data, they were analysed using the Wilcoxon test on ranks. To test homogenisation effectiveness, variances within each group were compared using Levene's test, which was conducted in Microsoft Office Excel (Microsoft, Redmond, WA, USA). Single-factor ANOVA was applied to the absolute differences of values from the mean. Data are plotted in a scatter plot with mean and standard deviation.

For comparing more groups, data were analysed by one-way ANOVA, followed by Bonferroni's multiple-comparison post hoc test for all parametric comparisons, or in the case of nonparametric data, Kruskal-Wallis ANOVA on ranks, followed by Dunn correction. Significance was set a priori at $P < 0.05$, corrected for multiple comparisons. Data are presented using a box and whisker plot with means and 95 % confidence intervals.

4. Results

4.1. *In vitro* experiments

Before investigating the multiorgan damage associated with DKD, we wanted to explore further details of the underlying molecular mechanisms causing renal damage in DM. Therefore, we assessed the direct effect of glucose and profibrotic growth factors and the protective role of RAASi on renal proximal tubular cells and fibroblasts.

To ensure that the chosen RAASi concentrations are not toxic and do not affect cell viability in our experimental setup, MTT assays were performed initially. No RAASi treatments in the selected concentration of 10 μM affected cell viability in HK-2 or NRK-49F cells. (*Figure 10.* A and B, respectively)

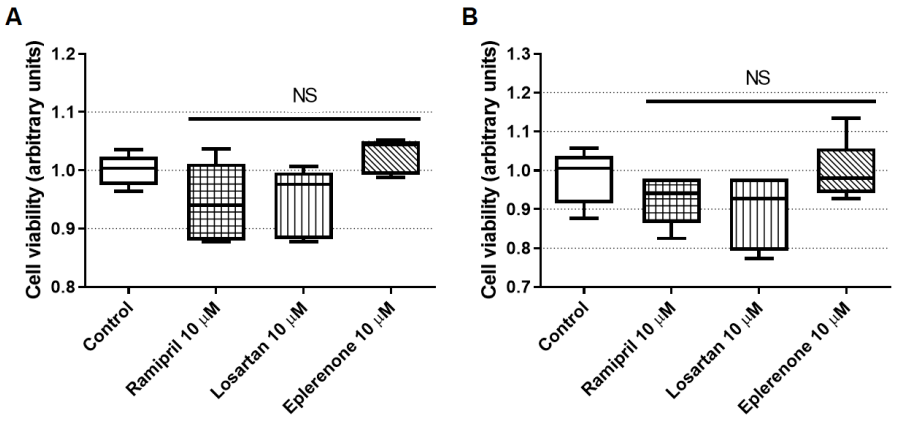


Figure 10. Cell viability assay of various renin-angiotensin-aldosterone inhibitor treatments on (A) human proximal tubular cells cultured in 35 mM glucose and (B) normal rat kidney fibroblast cells kept on standard 25 mM glucose.
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; ^{NS} not significant *vs.* Control.

To assess how growth factor treatment affects NRK-49F cells, first, we verified that our PDGF and CTGF treatment doses induce cell proliferation. Therefore, cell proliferation assays were performed with various doses of PDGF and CTGF (from 0.078 ng/mL to 10 ng/mL), which were selected based on the literature (178-181). 10 ng/mL PDGF and CTGF doses sufficiently caused cell proliferation of NRK-49F and were not toxic either (*Figure 11.)*

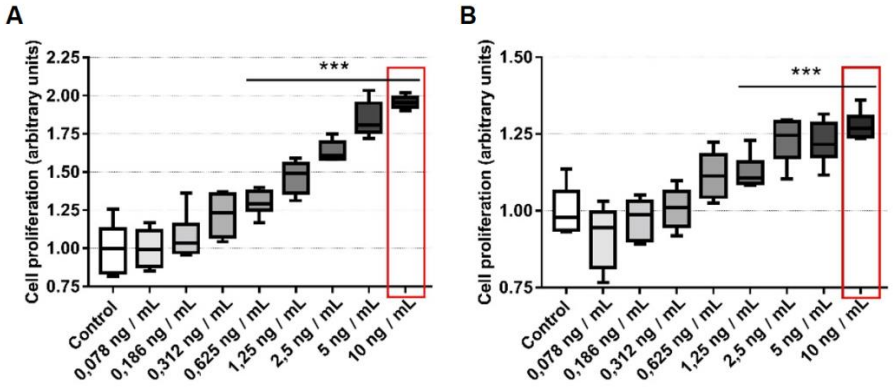


Figure 11. Cell proliferation assay in normal rat kidney fibroblast cells. (A) Dose-response curve of platelet-derived growth factor treatment. (B) Dose-response curve of connective tissue growth factor treatment.
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; ***p < 0.001 *vs.* Control

4.1.1. RAAS inhibitors (RAASi) prevent glucose-induced changes in tubular cells

To distinguish the *per se* effect of hyperglycaemia and hyperosmolarity on proximal tubules, HK-2 cells were cultured in standard, HG or mannitol-containing isosmotic media conditions. Upon hyperglycaemia, profibrotic changes were observed, as mRNA expressions of TGFβ1, PDGFB and CTGF were increased in HG-treated HK-2 cells. While modelling hyperosmolarity without hyperglycaemia, mannitol treatment did not change TGFβ1 and PDGFB, but enhanced the CTGF mRNA expression. (*Figure 12.)*

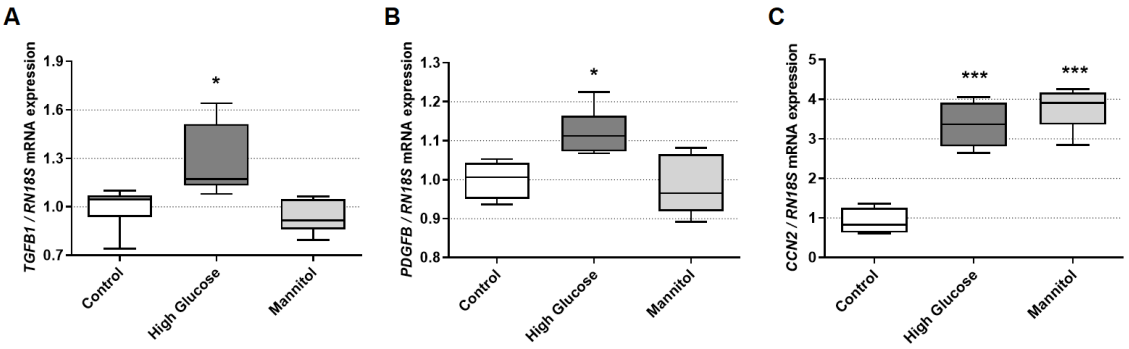


Figure 12. Expression of (A) transforming growth factor β1 (TGFB1), (B) platelet-derived growth factor B (PDGFB), and (C) connective tissue growth factor (CCN2) in human kidney 2 proximal tubular epithelial cells on standard, high glucose, or osmotic control mannitol treatment (182)
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni′s multiple-comparison post hoc test; *p < 0.05, ***p < 0.001 *vs.* Control

Regarding the fact that growth factors produced by proximal tubular cells under hyperglycaemic conditions contribute to fibrosis, we wanted to explore the possible protective effect of RAASi on profibrotic changes of HK-2 cells. In HG-treated cells, RAASi significantly reduced both PDGFB and CTGF expression to the level of controls, but no effect was observed on TGFβ1. (*Figure 13*.)

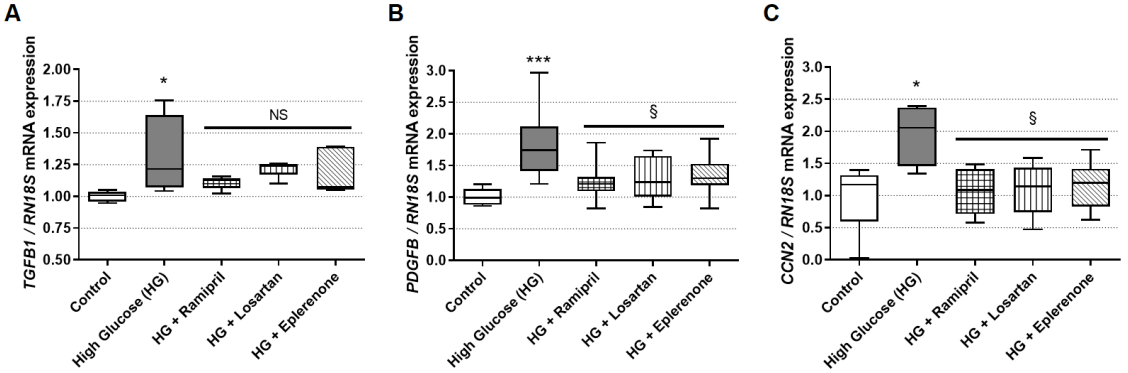


Figure 13. Expression of (A) transforming growth factor β1 (TGFB1), (B) platelet-derived growth factor B (PDGFB), and (C) connective tissue growth factor (CCN2) in human kidney 2 proximal tubular epithelial cells on standard, high glucose (HG), and renin-angiotensin-aldosterone system inhibitor treatment (182)
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni′s multiple-comparison post hoc test; *$p < 0.05$, ***$p < 0.001$ *vs*. Control; $^{§}p < 0.05$, ^{NS} not significant *vs*. HG

As a novel, non-invasive biomarker, we measured secreted collagen formation and degradation by-products from supernatants. Hyperglycaemia reduced the level of FBN-C (a biomarker of decreased fibronectin turnover), which was restored to the level of controls by RAASi treatments. The amount of internal epitope of the 7S domain of type IV collagen (PRO-C4) secreted into the cells' supernatant was the same in all groups, suggesting no changes in type IV collagen formation in HK-2 cells. (*Figure 14*.)

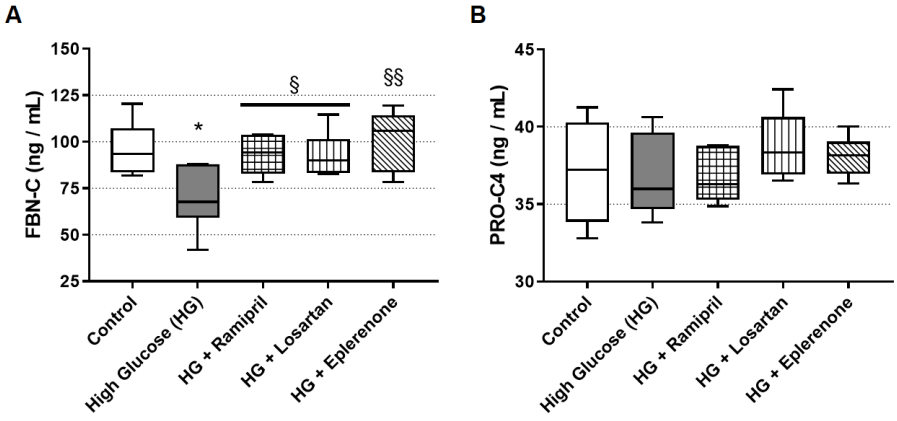


Figure 14. (A) C-terminal of fibronectin (FBN-C) turnover marker secretion by human kidney 2 (HK-2) proximal tubular epithelial cells on high glucose (HG) and renin-angiotensin-aldosterone inhibitor (RAASi) treatment. (B) Type IV collagen formation biomarker (PRO-C4) secretion by HK-2 cells on HG and RAASi treatment (182)
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni′s multiple-comparison post hoc test; *p < 0.05 *vs*. Control; §p < 0.05, §§p < 0.01 *vs*. HG

Time-dependent effects of hyperglycaemia-induced protein O-GlcNAcylation and expression of OGT and OGA enzymes were also investigated in HK-2 cells. Protein O-GlcNAcylation level was elevated after 24 and 48 hours of HG treatment, compared to control cells cultured in standard glucose. OGT was increased after 24 hours of HG treatment, but compared to the 24-hour value, it decreased after 48 hours. OGA changed oppositely, decreased below the level of control after 24 hours, but increased after 48 hours. (*Figure 15.*)

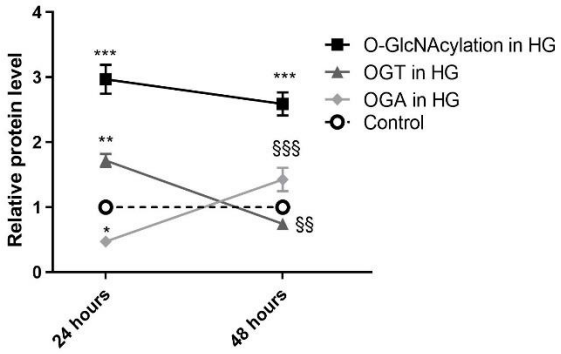


Figure 15. Relative protein O-linked N-acetylglucosamine modification (O-GlcNAcylation) and O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) protein levels in control, or high glucose (HG) treated human kidney 2 proximal tubular cells at 24 and 48 hours.
Values are presented as means ± SEM; n = 6 wells/group; two-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001 *vs*. Control; §§p < 0.01, §§§p < 0.001 *vs*. HG 24 hours

RAASi did not influence hyperglycaemia-induced protein O-GlcNAcylation or OGT enzyme level at 24 hours of HG treatment in HK-2 cells, whereas OGA was increased by RAASi treatments. (*Figure 16.*)

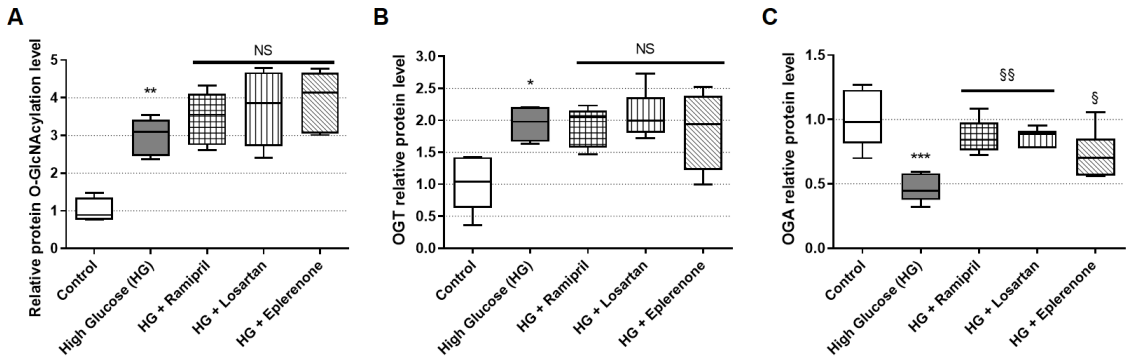


Figure 16. (A) Relative protein O-linked N-acetylglucosamine modification (O-GlcNAcylation) and (B) O-GlcNAc transferase (OGT) and (C) O-GlcNAcase (OGA) protein levels in control, high glucose (HG), and renin-angiotensin-aldosterone system inhibitor-treated human kidney 2 proximal tubular cells.
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni′s multiple-comparison post hoc test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. Control; $^{§}p < 0.05$, $^{§§}p < 0.01$, ^{NS} not significant *vs*. HG

4.1.2. RAASi ameliorate profibrotic changes in renal fibroblasts

Since profibrotic factors, produced by proximal tubular epithelial cells, can act directly on renal fibroblasts, the effect of growth factor treatment on NRK-49F cells was also evaluated. Since we could not detect significant changes in TGFB1 expression in HK-2 cells, we focused our further analyses on PDGFB and CTGF treatment.

Initially, we proved by fluorescent immunocytochemistry that NRK-49F cells express PDGFR-β, which is essential for the action of PDGF and CTGF (*Figure 17*).

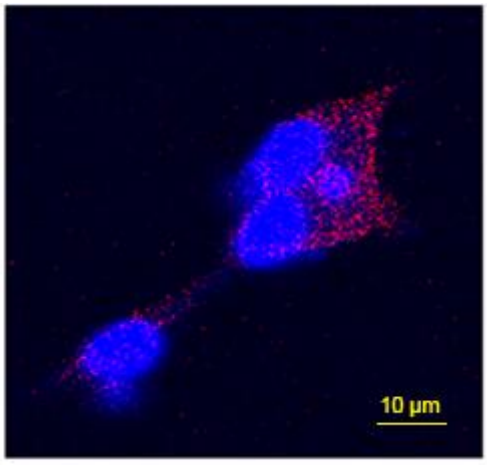


Figure 17. Representative picture of platelet-derived growth factor receptor β (PDGFR-β) stained normal rat kidney fibroblast cell. 1000x magnification; red – PDGFR-β, blue – nucleus; scale bar = 10 µm (182)

MTT assay was used to assess the effects of various RAASi on fibroblast proliferation. PDGF and CTGF treatment induced cell proliferation, but no RAASi changed it. (*Figure 18.* A, D)

Proliferating cell nuclear antigen (PCNA) and Ki67, which are more specific markers, were measured additionally. These factors are the most established components of cell proliferation. PCNA has a role in DNA synthesis and repair and is one of the regulators of the cell cycle. Ki67 can only be detected in nuclei of proliferating cells. (183) In our study, there was an increasing tendency in proliferation marker expression after both growth factor treatments. However, the elevation reached the significance level only in PDGF-treated NRK-49F cells. RAASi decreased PCNA expression, but not Ki67. (*Figure 18.* B-C, E-F)

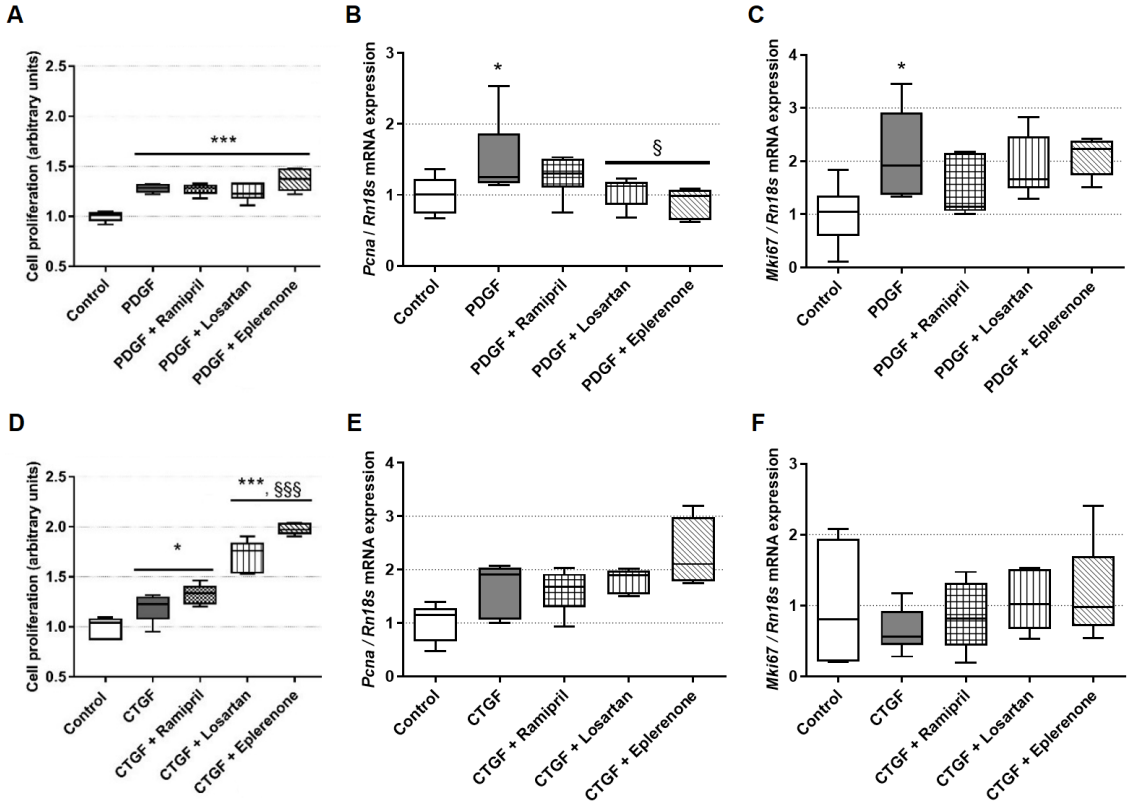


Figure 18. (A) Cell proliferation assay in normal rat kidney fibroblast (NRK-49F) cells on platelet-derived growth factor (PDGF) and renin-angiotensin-aldosterone system inhibitor (RAASi) treatment. (B) Proliferating cell nuclear antigen (Pcna) and (C) Ki67 (Mki67) proliferation marker mRNA expression in NRK-49F cells treated with PDGF and RAASi (D) Cell proliferation assay in NRK-49F cells on connective tissue growth factor (CTGF) and RAASi treatment. (E) PCNA and (F) Ki67 (Mki67) proliferation marker mRNA expression in NRK-49F cells treated with CTGF and RAASi

Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p <0.05, ***p < 0.001 *vs.* Control; $^{§}p < 0.05$ *vs.* PDGF; $^{§§§}p < 0.01$ *vs.* CTGF

Next, we investigated morphological changes/cytoskeleton rearrangements of NRK-49F cells caused by PDGF or CTGF treatment by phalloidin staining. This highly selective bicyclic peptide is used for staining actin filaments (F-actin).

Control cells were slightly elongated in shape and contained a diffuse network of thin actin filaments. CTGF and PDGF changed fibroblast cytoskeleton morphology, specifically the reorganisation of stress fibres along a longitudinal axis, parallel to forming F-actin bundles. However, elongation of cells and F-actin bundles were more pronounced after PDGF treatment. In addition, PDGF and CTGF caused actin-clump formation (marked with white-arrows), especially at the edges of fibroblasts. (*Figure 19.*)

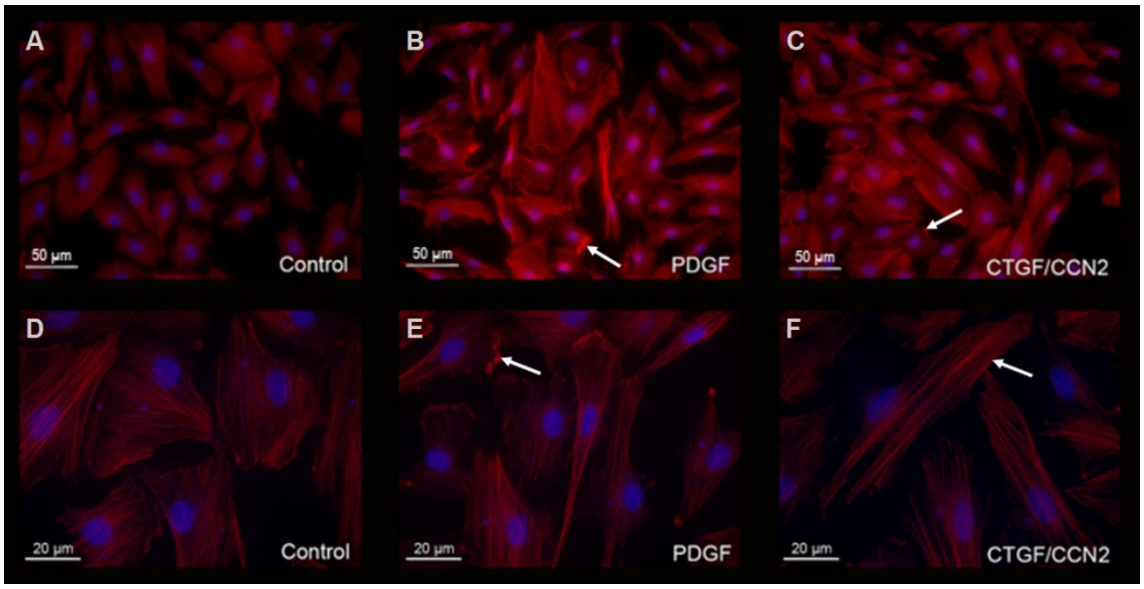


Figure 19. Representative pictures of phalloidin-TRITC immunostained (A, D) normal rat kidney fibroblast cells (Control), treated with (B, E) platelet-derived growth factor (PDGF) or (C, F) connective tissue growth factor (CTGF). Red – F-actin; blue – nucleus. Arrows mark actin clumps. 400x and 1000x magnification. Scale bar = 50 and 20 µm, respectively (182)

Lastly, we measured αSMA content and PRO-C4 secretion of NRK-49F cells. PDGF and CTGF treatments increased αSMA protein levels. All RAASi hindered these changes by diminishing αSMA production to the level of controls. (*Figure 20.* A, B) Type IV collagen formation did not change due to growth factor treatment or RAASi. (*Figure 20.* C, D)

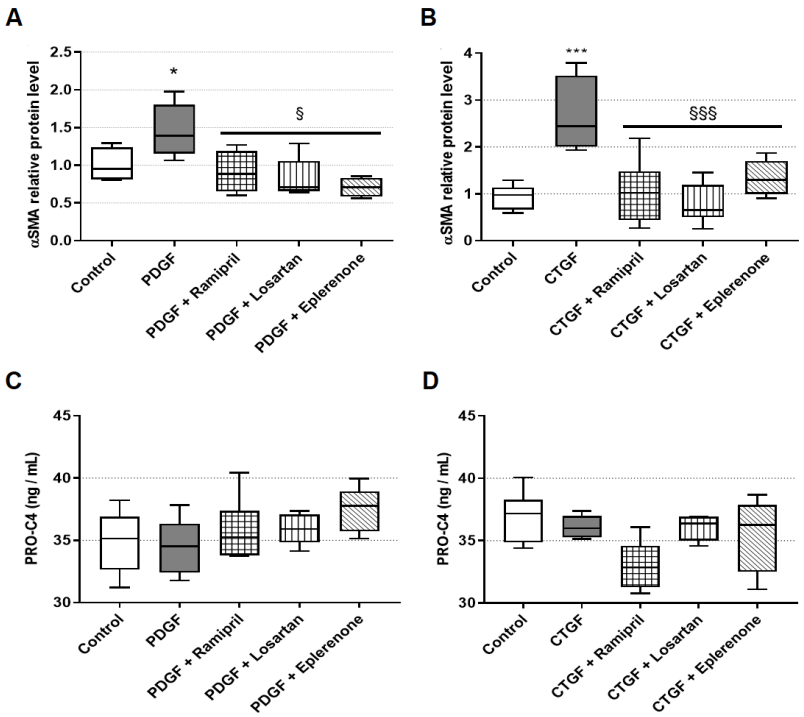


Figure 20. (A-B) Relative alpha-smooth muscle actin (αSMA) protein levels in normal rat kidney fibroblast (NRK-49F) cells treated with (A) platelet-derived growth factor (PDGF) or (B) connective tissue growth factor (CTGF) and renin-angiotensin-aldosterone system inhibitors (RAASi)
(C-D) Type IV collagen formation biomarker (PRO-C4) secretion of NRK-49F cells on (C) PDGF or (D) CTGF and RAASi treatment (182)
Values are presented as means ± 95% confidence intervals; n = 6 wells/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p <0.05, ***p < 0.001 *vs.* Control; §p < 0.05 *vs*. PDGF; §§§p < 0.01 *vs*. CTGF

4.2. *In vivo* experiments

Investigation of the *per se* effect of monotherapeutic RAASi on multiorgan complications of DM was conducted with non-depressor drug doses adopted from previous studies in line with the literature. (169-172)

Seven weeks after the induction of T1DM, diabetic rats had impaired weight gain, elevated serum glucose, fructosamine and total cholesterol levels, demonstrating the development of DM. MAP remained unaltered in all groups, confirming that the examined effects of RAASi are independent of their antihypertensive properties. RAASi did not affect body weight or metabolic parameters, either. (*Table 3.)*

Table 3. Physiological and metabolic parameters of control, diabetic (D), and renin-angiotensin-aldosterone system inhibitor-treated rats. Values are presented as means ± standard deviations; n = 7 rats/groups; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p<0.05, **p<0.01 *vs.* Control

Parameters	Control	Diabetes (D)	D + Ramipril	D + Losartan	D + Eplerenone
Mean arterial pressure (Hgmm)	74.7 ± 7.51	73.9 ± 8.78	72.3 ± 5.46	73.8 ± 14.0	77.3 ± 13.2
Body weight (g)	418 ± 23.4	276 ± 28.9*	265 ± 12.8*	258 ± 24.8*	288 ± 20.9*
Serum glucose (mmol/L)	12.7 ± 1.15	47.3 ± 7.80*	44.1 ± 3.67*	43.8 ± 4.79*	34.8 ± 3.57
Fructosamine (µmol/L)	153 ± 8.28	243 ± 16.3**	246 ± 11.5**	256 ± 17.6**	239 ± 16.5**
Total cholesterol (mmol/L)	1.90 ± 0.05	2.28 ± 0.06*	2.21 ± 0.10	2.06 ± 0.08	1.88 ± 0.14

4.2.1. RAASi improve renal function and diminish kidney fibrosis

DM-induced renal impairment is presented in higher blood urea nitrogen levels and lower creatinine clearance. These functional parameters of DKD were ameliorated by RAASi treatment. (*Figure 21.* A, B) Deteriorated renal function (lower creatinine clearance) was associated with higher blood sugar levels. (*Figure 21.* C)

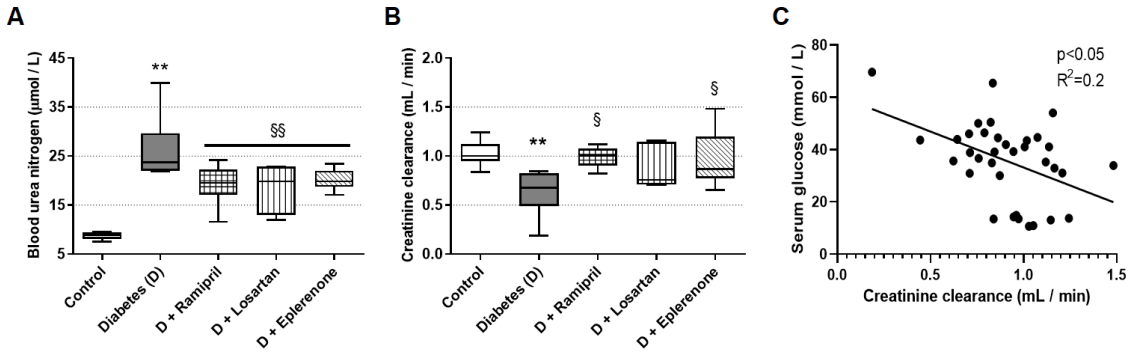


Figure 21. Renal functional status estimated with (A) blood urea nitrogen level and (B) creatinine clearance values measured in control, diabetic (D), and renin-angiotensin-aldosterone system inhibitor-treated rats. Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; **p <0.01 *vs.* Control; §p < 0.05, §§p < 0.01 *vs.* Diabetes (182)
(C) Correlation of creatinine clearance with serum glucose levels in all rats. (Linear regression fit; $p < 0.05$; $R^2 = 0.2$)

Besides functional decline, massive collagen accumulation was observed in untreated diabetic rats, as a key histological feature of renal fibrosis. All of the RAASi minimised fibrosis as reflected by fewer Sirius Red-positive areas. (*Figure 22.* A)

As novel, non-invasive biomarkers of kidney fibrosis, urinary markers of ECM remodelling showed increased collagen IV turnover (TUM) and elevated rates of collagen III formation (PRO-C3) and degradation (C3M) in diabetic animals. Eplerenone treatment decreased the urinary level of C3M. (*Figure 22.* B-D)

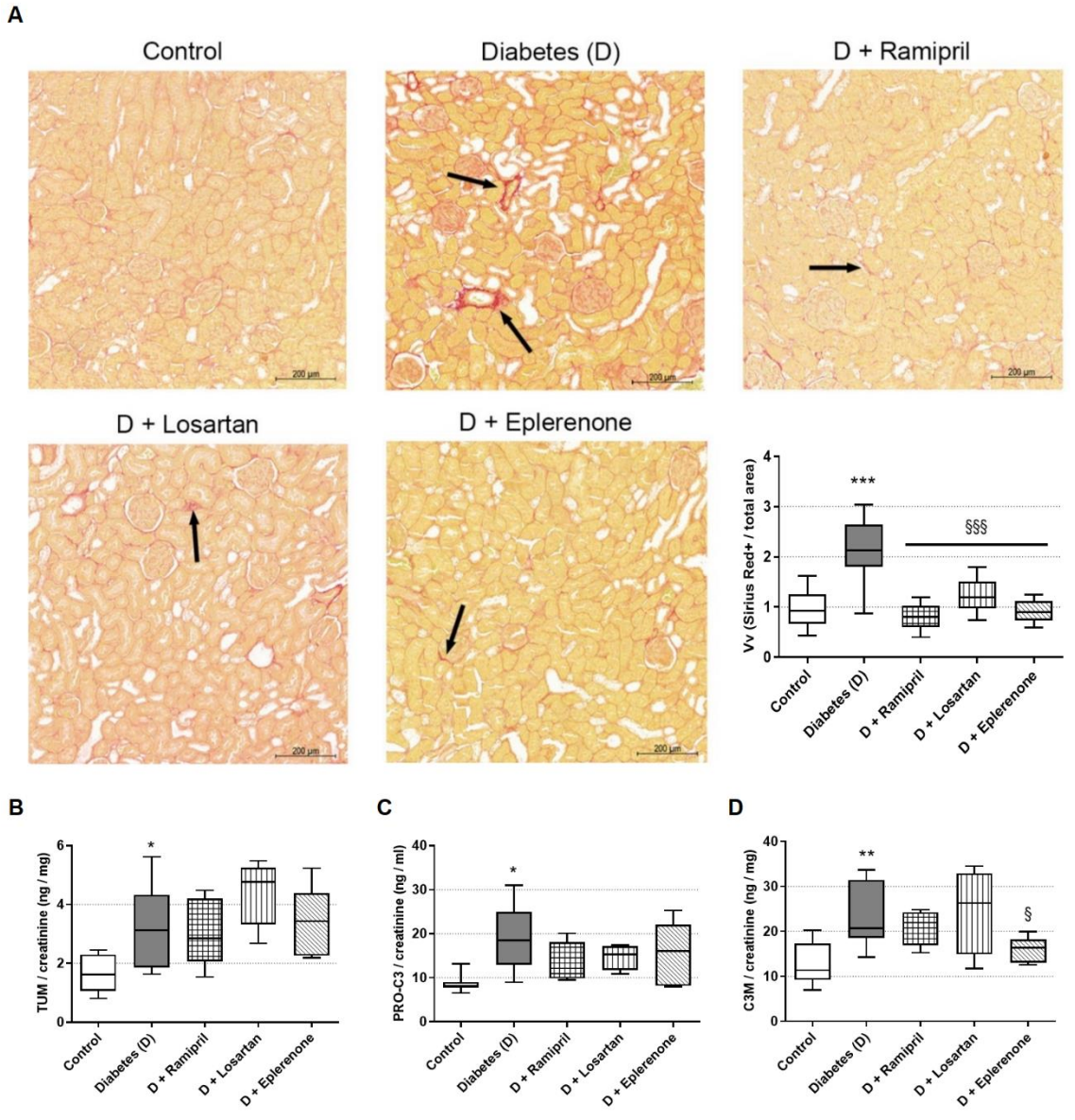


Figure 22. Renal fibrosis evaluation in control, diabetic (D), and renin-angiotensin-aldosterone system inhibitor-treated rats (182)
(A) Representative pictures and quantitative evaluation of Sirius Red-stained fibrotic tissue accumulation in the kidneys (20x magnification, scale bar = 200µm)

(B-D) Urinary biomarkers of (B) collagen IV turnover (TUM), (C) collagen III formation (PRO-C3), and (D) collagen III degradation (C3M)
Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p<0.05, **p <0.01, ***p<0.001 *vs*. Control; §p < 0.05, §§§p < 0.001 *vs*. Diabetes

4.2.2. Lyophilisation improves reproducibility in fibrotic tissues

During our experiments, we often found that fibrotic tissue accumulation was inhomogeneous in the samples, and significant differences were seen between some areas of the kidney (*Figure 23.* A) and the heart. Depending on the tissue region used for protein extraction, this caused higher scatter and more outlier data points within groups and between different isolations (*Figure 23.* B).

Therefore, we introduced lyophilisation to our tissue preparation protocol as an additional method. To test whether the problem of focality can be resolved, protein and RNA were extracted 4 times from lyophilized, pulverized homogenates and from four parts of the same frozen samples. (*Figure 23.* C) Both mRNA and protein levels of αSMA showed lower variances in the lyophilized, homogenized samples than within the conventionally processed group from frozen samples (*Figure 23.* D-E).

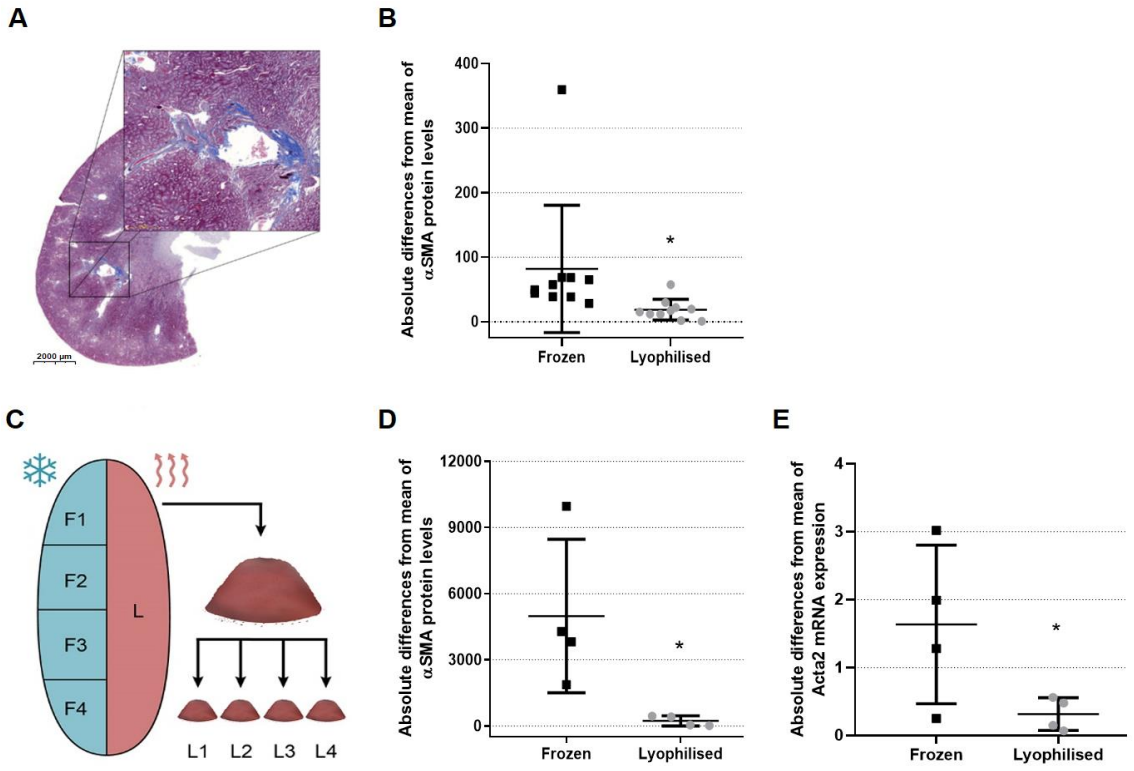


Figure 23. Comparison of scatter in conventionally processed frozen and lyophilised, pulverized samples from fibrotic tissue (184)

(A) Representative Masson's trichrome-stained section, where the enlarged area shows examples of focal fibrosis in the diabetic rat kidney. (10-200x magnification, scale bar = 2000μm, 50μm)
(B) Absolute differences of alpha smooth muscle actin (αSMA) protein values to mean in diabetic rat kidneys and hearts processed conventionally (Frozen) or homogenized after lyophilisation (Values are presented as scatter plot with mean and standard deviation; n=10/group; variances compared by Levene's test, single factor ANOVA applied to absolute differences of values to mean; *p<0.05 *vs*. Frozen)
(C) Schematic presentation of sample processing for homogeneity investigations. Half of each kidney was lyophilised (L), pulverised, and protein and RNA were isolated from 4 portions of homogenous powder (L1-L4). The other half underwent conventional frozen sample processing: 4 pieces were cut for the isolations (F1-F4).
(D-E) Absolute differences of (D) αSMA protein and (E) alpha smooth muscle actin mRNA (*Acta2*) values to mean in frozen parts *vs.* lyophilised homogenous powder of the same diabetic rat kidney samples. (Values are presented as scatter plot with mean and standard deviation; n=4 frozen parts and four powder portions of the same organs; variances compared by Levene's test, single factor ANOVA applied to absolute differences of values to mean; *p<0.05 *vs*. Frozen)

4.2.3. RAASi ameliorate diabetic vascular impairment

To evaluate changes in arterial stiffness, as the earliest detectable manifestations of pathological vascular remodelling, PWV was measured in diabetic rats. PWV values correlated with the animals' blood pressure and body weight. (*Figure 24.* A-B) Since there was no significant difference in MAP values between the groups, PWV was corrected for body weight. Weight-corrected PWV was elevated in DM, reflecting the increased arterial stiffness. (*Figure 24.* C)

Aortic histological changes were evaluated with Orcein and Masson's trichrome staining. IMT was measured on Orcein-stained aortic sections, where diabetic rats exhibited intimal thickening, irregularities, and diffused elastic membranes. (*Figure 24.* D) Ramipril and eplerenone returned the increased thickness in diabetic animals to the control value. (*Figure 24.* E) Aortic media layer thickness correlated with serum total cholesterol. (*Figure 24.* F). Masson's trichrome staining showed disorganized elastic lamina arrangement (*Figure 24.* G) and increased ratio of blue-stained medial fibrotic ECM components in diabetic aortas which was decreased by eplerenone. (*Figure 24.* H)

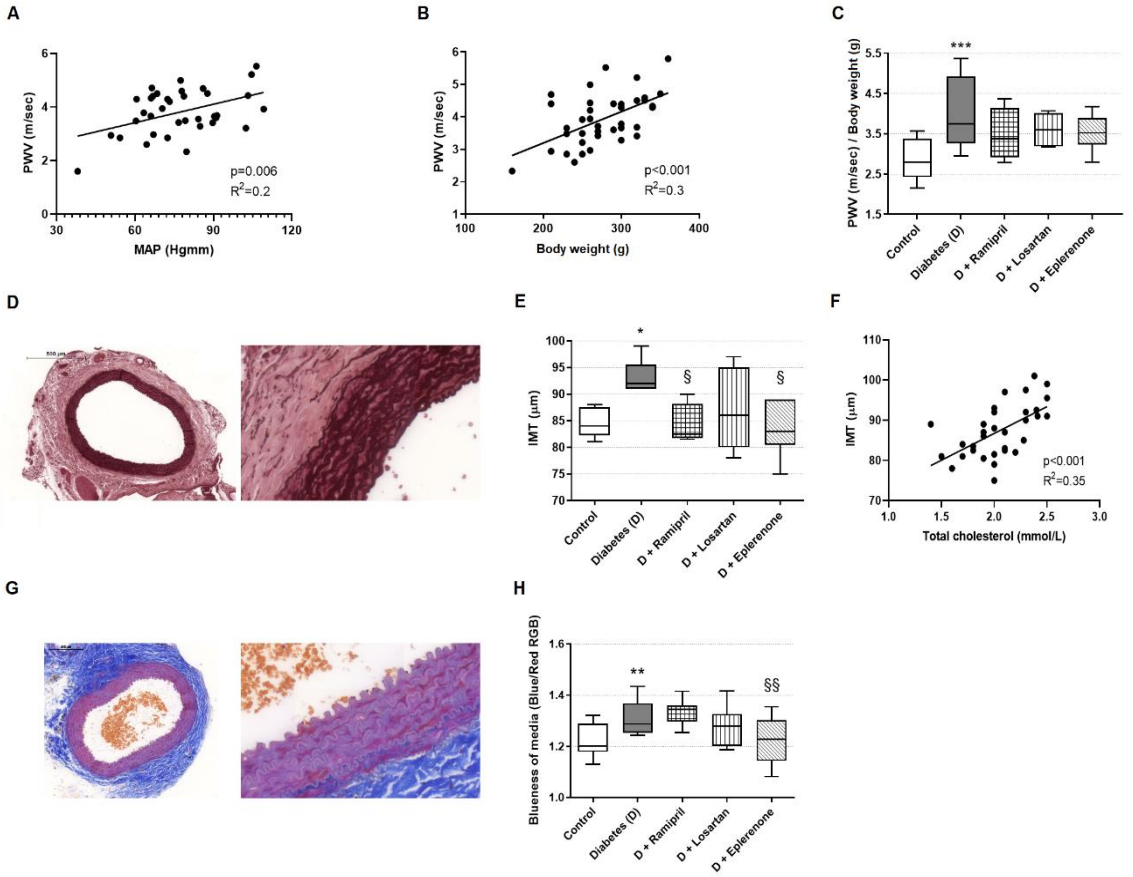


Figure 24. Vascular functional and structural parameters and their correlations with physiological and metabolic values in control, diabetic, and renin-angiotensin-aldosterone system inhibitor-treated rats (185)

(A-B) Correlation of pulse wave velocity (PWV) with (A) mean arterial pressure (MAP) and (B) body weight (Linear regression fit; $p<0.01$; $R^2 = 0.2$ and 0.3, respectively)

(C) Statistical evaluation of body weight-corrected PWV values (Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; ***$p<0.001$ *vs*. Control)

(D) Representative pictures and (E) quantitative evaluation of Orcein-stained histological aortic sections for visualization of elastin fibres and measuring intima-media thickness (IMT) (40x magnification; scale bar = 500µm) (Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *$p<0.05$ *vs*. Control; $^{§}p<0.05$ *vs*. Diabetes)

(F) Correlation of IMT with serum total cholesterol values (Linear regression fit; $p<0.001$; $R^2 = 0.35$)

(G) Representative pictures of Masson's trichrome stained aortic histological sections for connective tissue visualization and (H) quantitative evaluation of medial fibrosis (40x magnification; scale bar = 500µm) (Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; **$p<0.01$ *vs*. Control; $^{§§}p<0.01$ *vs*. Diabetes)

4.2.4. RAASi minimize cardiac tissue damage and fibrosis

Serum troponin I level, a routine clinical marker of cardiac tissue damage, was elevated in diabetic rats, indicating myocardial injury. mRNA expression of BNP (specific to chronic left ventricular expansion) was also increased in diabetic hearts. All RAASi decreased troponin I and BNP to normal control values. (*Figure 25.* A-B)

Klotho, a newly recognized protective marker regulating fibrosis, oxidative stress and inflammation, showed decreased serum levels in diabetic rats, which was compensated by eplerenone. (*Figure 25.* C) On the same pathway, the counter-regulatory FGF23 protein was elevated in the cardiac muscles of diabetic animals, and all RAASi minimized this response. (*Figure 25.* D)

Left ventricular fibrosis was assessed on Sirius Red stained sections. Fibrotic connective tissue was increased in diabetic hearts, while all RAASi decreased it to control levels. (*Figure 25.* E) Another specific marker of cardiac fibrosis, αSMA protein, was elevated in diabetic rat hearts, while ramipril and eplerenone decreased it. (*Figure 25.* F) Since DCM-associated growth factors induce fibrotic differentiation, we also measured the expression of certain profibrotic factors. Increased heart CTGF and PDGF mRNA expressions were observed in DM, which were reduced by all RAASi treatments. (*Figure 25.* G-H)

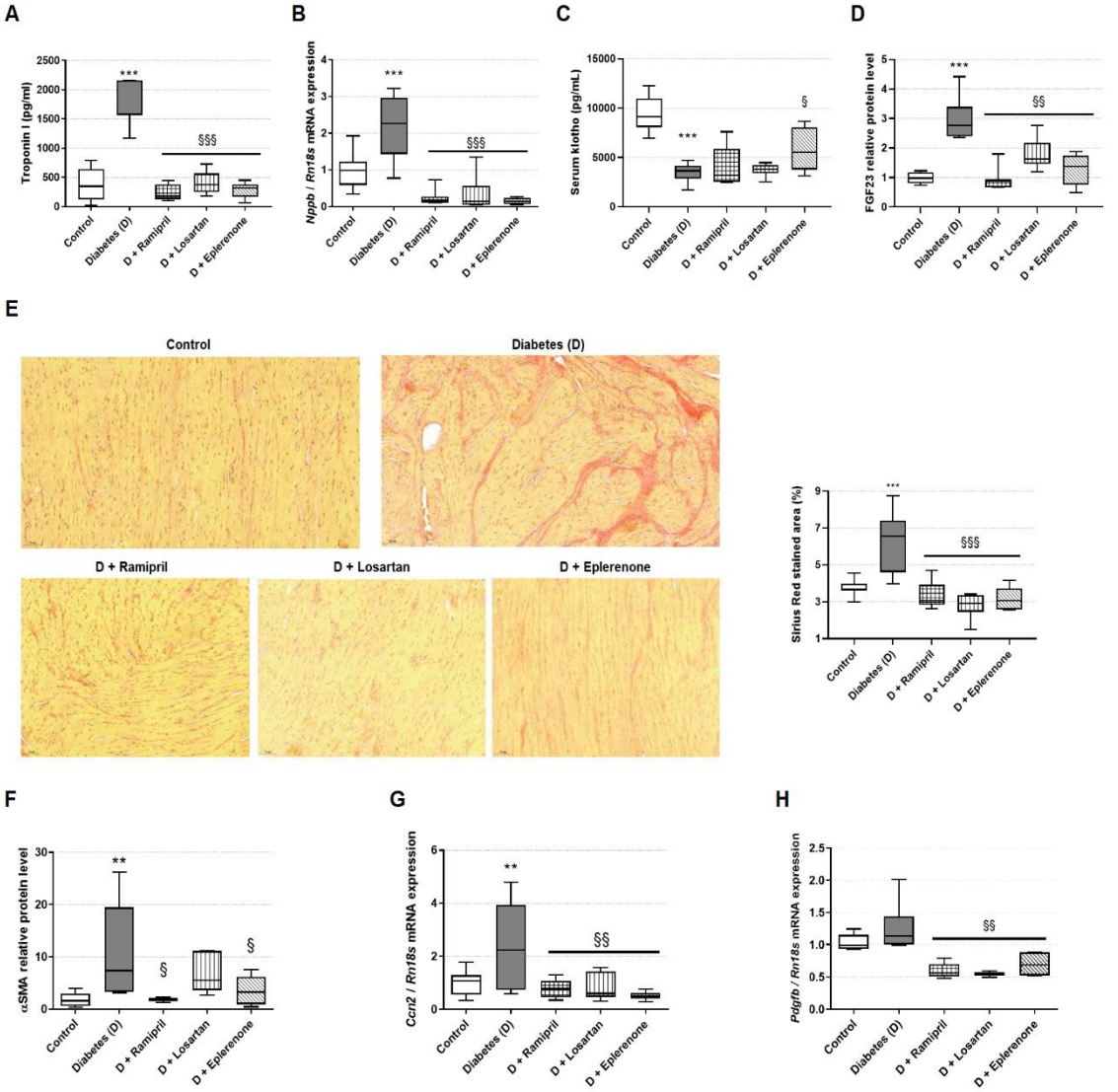


Figure 25. Makers of cardiac tissue damage and fibrosis in control, diabetic, and renin-angiotensin-aldosterone system inhibitor-treated rats (185)

(A-D) Markers of cardiac damage: (A) serum troponin I protein levels, (B) B-type natriuretic peptide (*Nppb*) mRNA expression in left ventricular heart muscles, (C) serum klotho protein levels, (D) fibroblast growth factor 23 (FGF23) protein level in cardiac left ventricular muscles (Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; ***p<0.001 *vs*. Control; §p<0.05, §§p<0.01, §§§p<0.001 *vs*. Diabetes)

(E) Representative pictures and quantitative evaluation of Sirius Red stained left ventricular muscle sections for fibrotic tissue accumulation measurement (200x magnification; scale bar = 50µm; values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; ***p<0.001 *vs*. Control; §§§p<0.001 *vs*. Diabetes)

(F-H) Fibrotic markers measured in left ventricular heart muscles: (F) alpha smooth muscle actin (αSMA) protein level, (G) connective tissue growth factor (*Ccn2*) and (H) platelet-derived growth factor (*Pdgfb*) mRNA expression (Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; **p<0.01 *vs*. Control; §p<0.05, §§p<0.01 *vs*. Diabetes)

4.2.5. RAASi decrease cardiac hypoxic and inflammatory injury

While exploring the inflammatory aspects of cardiac tissue damage, we detected higher mRNA expression of IL-6 and NFκB protein levels in diabetic heart muscles that were decreased by losartan treatment. (*Figure 26.* A-B) Conversely, TNFα expression was lower in DM, while ramipril and losartan treatment compensated this change. (*Figure 26.* C) In line with TNFα changes, the mRNA expression of its suppressor heat shock protein 72 (HSP72) was doubled in DM and decreased by all RAASi. (*Figure 26.* E)

In the hypoxic pathway, the vascular endothelial growth factor (VEGF) protein level was elevated in DM and decreased by RAASi. (*Figure 26.* D)

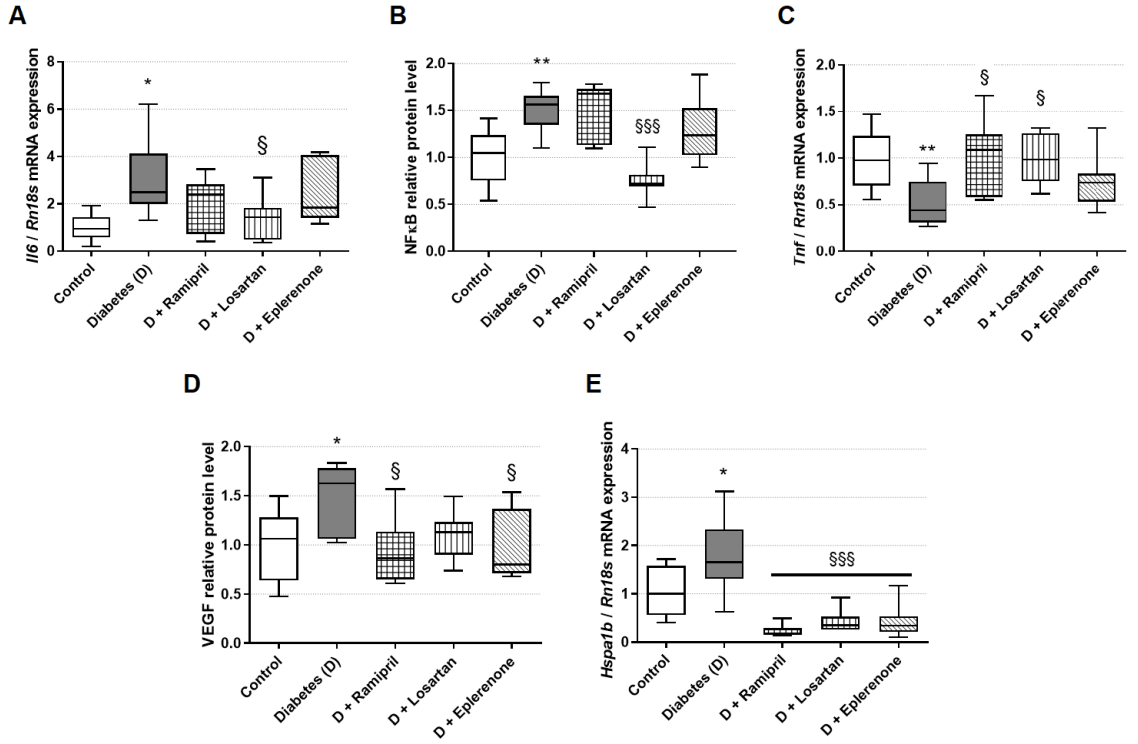


Figure 26. Inflammatory and hypoxic markers in hearts of control, diabetic, and renin-angiotensin-aldosterone system inhibitor-treated rats

(A-C) Inflammatory cytokines and transcription factors: (A) interleukin-6 (*Il6*) mRNA expression, (B) nuclear factor kappa B (NFκB) protein level, (C) tumor necrosis factor (Tnf) mRNA expression

(D-E) Hypoxia activated pathway: (D) vascular endothelial growth factor (VEGF) protein level, (E) heat shock protein 72 (*Hspa1b*) mRNA expression

(Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p<0.05, **p<0.01 *vs*. Control; §p<0.05, §§§p<0.001 *vs*. Diabetes)

4.2.6. RAASi mitigate DM-induced inflammation in hippocampi

Inflammatory processes are strongly associated with depression and DM. Therefore, we measured proinflammatory cytokine expression in the hippocampi of diabetic and

RAASi-treated rats. Expression of IL-1α, IL-6 and TNFα was increased in the hippocampi of diabetic rats, and was diminished by RAASi (*Figure 27.* A-C). The protein level of the main transcription factor NFκB, associated with IL-1α and TNF signaling pathways, also increased. All RAASi decreased that back to control level (*Figure 27.* D).

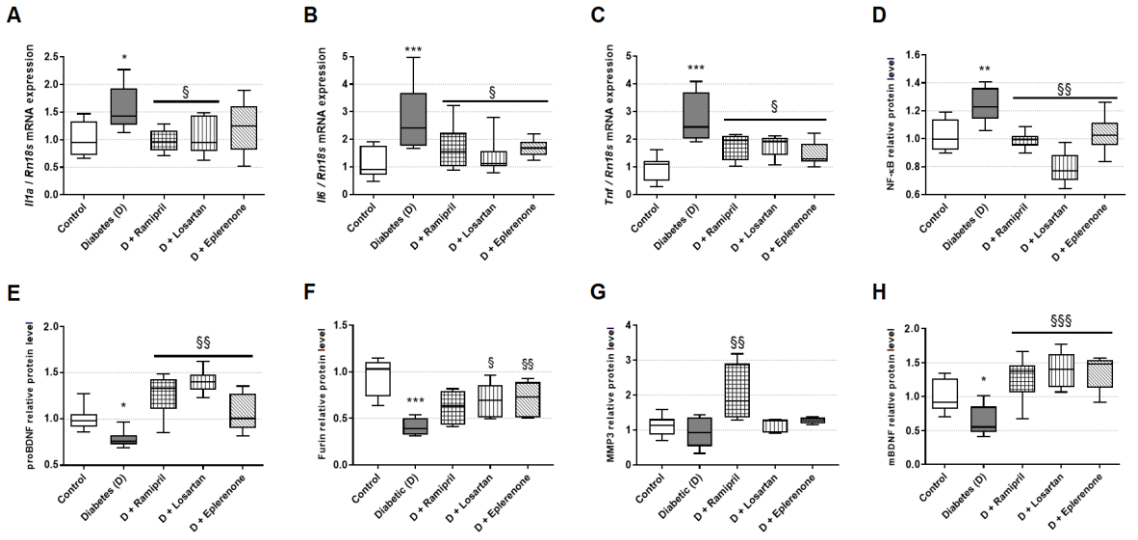


Figure 27. Proinflammatory responses and brain-derived natriuretic factor (BDNF) production in hippocampi of control, diabetic, and renin-angiotensin-aldosterone system inhibitor-treated rats (186)
(A-D) Hippocampal inflammatory cytokine and transcription factor levels: mRNA expression of (A) interleukin-1α (Il1a), (B) interleukin-6 (Il6) and (C) tumor necrosis factor (Tnf), and (D) protein level of nuclear factor kappa B (NFκB)
(E-H) Protein level of hippocampal BDNF isoforms: (E) precursor BDNF (proBDNF), and (H) mature BDNF (mBDNF); and its cleavage enzymes: (F) furin, and (G) matrix metalloproteinase 3 (MMP3)
(Values are presented as means ± 95% confidence intervals; n = 7 rats/group; one-way ANOVA followed by Bonferroni's multiple-comparison post hoc test; *p<0.05, **p<0.01, ***p<0.001 *vs*. Control; §p<0.05, §§p<0.01, §§§p<0.001 *vs*. Diabetes)

4.2.7. RAASi restore hippocampal BDNF production

BDNF signaling pathway plays a pivotal role in the pathophysiology of comorbid depression. Hippocampal synthesis of the precursor and the mature isoforms of BDNF decreased in diabetic rats. These adverse changes were fully reversed by RAASi treatment. (*Figure 27.* E, H). Serine protease enzymes are responsible for BDNF cleavage. We found that losartan and eplerenone increased the intracellular enzyme furin, while ramipril elevated the extracellular MMP3. (*Figure 27.* F, G)

5. Discussion

The prevalence of DM is increasing at an alarming rate; therefore, the risk of developing diabetic complications is becoming a concern for more and more people. DKD is one of the most common and detrimental microvascular consequences of DM, associated with CVD and comorbid depression. Significant progress has been made in the therapeutic management of DM-associated multiorgan complications over the past decade; however, the current treatment strategies are still not efficient enough for disease prevention. The pharmacological modulation of major regulatory pathways involved in DM pathophysiology, such as fibrosis or inflammation, may be a promising strategy.

International guidelines list RAASi as the gold-standard therapy in diabetic patients with micro- and macrovascular complications, although their exact protective mechanism besides their antihypertensive effect is not fully explored yet. Studies indicate that various RAASi decrease functional and structural multiorgan damage in DM. However, it is still not exactly clarified whether their protective properties are associated with or restricted to their blood pressure-regulating effects, and the exact molecular pathways involved need to be explored. Furthermore, even though the aldosterone escape phenomenon has been reported in DKD patients treated with ACEi or ARB (187, 188), aldosterone antagonists are still mainly used only as diuretics to treat hypertension. Therefore, further evidence is needed for their monotherapeutic use.

During my doctoral work, our primary goal was to evaluate the protective effects of different RAASi monotherapy against DM-induced complications in the kidney, heart and brain.

Previously, glomerulosclerosis was considered the primary site of fibrosis in DKD, but increasing evidence shows that hyperglycaemia-induced tubulointerstitial lesions also have a causative role.

First, we investigated cell cultures of proximal tubular cells and renal fibroblasts to independently assess the direct effects of glucose, hyperosmolality and profibrotic growth factors and the protective role of RAASi.

Our data not only confirmed the results of previous studies showing that TGFβ and CTGF expression are higher in HG-treated HK-2 cells (189-191), but also showed the

same effect of hyperglycaemia on PDGF expression. Furthermore, by applying the exact isosmotic mannitol dosage as in literature (182-195), we proved that the elevation of TGFβ and PDGF mRNA expression can be considered as a direct effect of glucotoxicity. At the same time, CTGF production could result from the complex impact of hyperglycaemia and hyperosmolarity.

To investigate the effects of RAASi, we used dosages that we previously proved are non-toxic to HK-2 cells. We demonstrated that monotherapy with various RAASi suppresses the production of PDGF and CTGF. Some of these effects were also shown in the literature: 10 μM losartan attenuated TGFβ-1 expression and epithelial-mesenchymal differentiation in hyperglycaemic (30 mM) NRK-52E cells (196), while 10 μM eplerenone completely blocked epithelial-mesenchymal transition in HK-2 cells. (197) Furthermore, ramipril and eplerenone decreased PDGF in experimental hepatic and myocardial fibrosis. (198, 199) These results suggest that RAASi can directly suppress the hyperglycaemia-induced profibrotic response not only in renal HK-2 cells, but also in other organs.

We were privileged to participate in the preclinical testing process of non-invasive biomarkers like FBN-C, TUM, PRO-C4, PRO-C3 and C3M, markers of renal fibrosis newly developed by Nordic Bioscience. The decreased fibronectin turnover in HG-treated HK-2 cells was restored by RAASi treatment, suggesting that RAASi interfere with the dysregulated remodelling of the interstitial ECM, which characterises fibrosis.

Hyperglycaemia also leads to excessive O-GlcNAcylation of various proteins, which negatively alters several critical cellular processes, including phosphorylation or NO production. The relevance of O-GlcNAcylation has already been emphasised in the diabetic heart by demonstrating its association with increased atherosclerosis and vascular inflammation. (200, 201) Still, the role of O-GlcNAc modification has yet to be fully explored in diabetic kidneys. We showed that hyperglycaemia induces O-GlcNAcylation in HK-2 cells and demonstrated that RAASi could ameliorate the detrimental effects of chronic accumulation of O-GlcNAc-modified proteins.

We investigated time-dependent effects of HG-induced O-GlcNAcylation in HK-2 cells. Twenty-four and 48 hours of HG treatment elevated the protein O-GlcNAcylation. Meanwhile, OGT, the enzyme responsible for adding the GlcNAc molecule, was only

transiently increased after 24 hours; by 48 hours, it returned to the basal level. These data suggest that renal OGT can decrease in a compensatory manner in the event of longer hyperglycaemia, to protect tubular cells against over-O-GlcNAcylation. A similar phenomenon was observed in the red blood cells of human diabetic patients. (202)

In contrast, the OGA, which removes O-GlcNAc modifications, appeared more susceptible to cellular O-GlcNAc levels and less sensitive to nutritional changes. OGA decreased below the control level in HG-treated cells after 24 hours; however, following this transient decline, OGA quickly increased, indicating a compensatory adaptive response against increased O-GlcNAcylation. This aligns with a previous study, which found that the OGA level is higher in leukocytes of diabetic individuals, suggesting that cells are trying to attenuate higher O-GlcNAcylation. (203)

It has already been shown that activated RAAS and hexosamine biosynthesis pathways play a significant role in the development of DKD. Literature data suggest a bilateral activation of O-GlcNAcylation and intrarenal RAAS. Ang II increases O-GlcNAc modification in mesangial cells. (204) This is also true the other way around: elevated O-GlcNAcylation can stimulate Ang expression. (164) This generates a vicious circle contributing to direct glucotoxic effects. In our *in vitro* studies, RAASi did not influence HG-induced protein O-GlcNAcylation or OGT expression, but minimized hyperglycaemic complications by increasing OGA. This is in line with the literature, where OGA overexpression ameliorates ECD. (205)

Evidence suggests that profibrotic factors such as PDGF and CTGF are key determinants in developing ECM deposition and epithelial-mesenchymal transition, leading to renal fibrosis. (55, 206) First, we confirmed that NRK-49F cells express PDGFR-β, which is essential for the action of both. As profibrotic factors produced by proximal tubules, PDGF and CTGF directly influence renal fibroblast morphology, proliferation, migration, and ECM production. Our next step was to investigate each process individually. To our knowledge, we were the first in the literature to do so.

We chose the dose of PDGF and CTGF based on their proliferating capacity on NRK-49F cells. PDGF and CTGF (10 ng/mL) treatment induced the NRK-49F fibroblasts and

increased PCNA and Ki67 levels. RAASi decreased PDGF-induced PCNA and Ki67 expression, but did not affect CTGF-induced changes.

PDGF and CTGF also promoted morphological alterations and re-arrangement of the actin cytoskeleton in NRK-49F cells, a transformation similar to that described in human fibroblasts after PDGF (207) and in podocytes following CTGF treatment. (208)

Our final *in vitro* experiment demonstrated that all the RAASi effectively reduced αSMA production induced by profibrotic growth factors and inhibited myofibroblast differentiation. This suggests that renal fibroblasts may represent one of the primary targets of RAASi in preventing fibrotic transformation.

Next, *in vivo* experiments were conducted to evaluate the various complex mechanisms involved in multiorgan complications associated with DKD. The blood pressure-lowering ability of RAASi is well known. It is also well documented that they are renoprotective (209), reduce CV oxidative stress (210), and improve depression scores in DM (211) independently of their antihypertensive effects. The renal antifibrotic potential of the ARB losartan (212) and the ACEi ramipril (213) has already been explored in murine models. Furthermore, RAASi have been shown to reduce renal fibrosis in DM (214, 215), cardiac fibrosis in renal failure (216), and neuroinflammation in psychiatric disorders. (217) However, the complexity of the exact shared mechanisms is still not fully clarified.

To test whether these multiorgan-protective characteristics of RAASi are restricted to their blood pressure-regulating effect, we chose lower treatment doses to avoid blood pressure changes. Similarly to our previous studies, DM did not induce hypertension, which is a typical feature in this STZ diabetes model in Wistar rats. This typical feature is probably a specific characteristic of DM-induced neuropathy in this strain, damaging equally the sympathetic and parasympathetic innervation of the kidneys and heart. (218)

RAASi treatment did not affect blood pressure either, confirming that we used non-depressor doses and that this model is appropriate for testing independent effects.

Our research group conducted extensive studies on the multiorgan, pleiotropic protection of RAASi, which goes beyond decreasing blood pressure and is related to other factors. (169, 219) We also observed that RAASi successfully improves severe functional and structural renal and cardiac damage in diabetic Wistar rats.

In our model, higher blood glucose levels were associated with worse renal function and lower creatinine clearance. DM-induced renal fibrosis was visible in Sirius Red-stained kidney sections, but it was also detectable non-invasively from urine. To the best of our knowledge, we were the first to use these newly developed ECM remodelling biomarkers to test the efficacy of an antifibrotic treatment. The increased TUM, PRO-C3, and C3M levels in diabetic rats confirm that this assay can be used as a new tool in diagnosis and disease monitoring. Since then, our collaborators at Nordic Bioscience have proven the diagnostic relevance of these markers in several kidney pathologies. PRO-C3 and C3M have been used to assess disease progression in patients with IgA nephropathy and ANCA-vasculitis. (220, 221) Recently, PRO-C3 helped stratification after an episode of AKI by identifying patients at higher risk of kidney disease progression. (222) While very promising, further research is needed to get regulatory approval for their clinical use.

As we proceeded to further investigations, we encountered the problem that renal and cardiac fibrosis have focal characteristics; the exact localisation of the injury is mostly not visually predictable, particularly in the case of small samples obtained from rats. (223, 224) Due to this focal nature of fibrotic organ injuries, conventional sample preparation methods can hardly produce reproducible false-positive or false-negative results.

After confirming our assumption on Masson's trichrome-stained sections, we modified our tissue preparation method, introducing lyophilization as the first step of sample processing. During lyophilization the tissue can easily be pulverized. (225) The portion of a homogenous powder represents the whole sample more than segmented parts from frozen tissues. By these, our PCR and Western blot measurements of profibrotic RNA and protein levels from frozen samples generated significantly higher scatter and more outlier data points than freeze-dried, pulverized ones.

We also demonstrated that the mRNA or protein content of the samples does not degrade significantly over time, nor when stored at room temperature. We also proved that lyophilization does not affect posttranslational modifications either, so samples can also be used for detecting phosphorylated proteins. (184) These findings have been

described in detail in the PhD thesis of my colleague, Tamas Lakat, with whom we performed these methodological developments. (226)

Micro- and macrovascular complications play a pivotal role in all types of diabetic organ damage; therefore, investigating CV injury is essential to comprehend multiorgan consequences. (227) The results from the FIDELIO-DKD and FIGARO-DKD randomized controlled trials demonstrated that the non-steroid MR antagonist finerenone reduced the risk of major CV events and HF in patients with CKD and T2DM. (228) In younger T1DM patients, the prevention of atherosclerosis is even more crucial to reduce the risk of CVD later in life, as it decreases their life expectancy at a dramatic rate. (229) The abdominal aorta is one of the places where initial changes in vascular structure occur in DM (230), and increased IMT is an early marker of atherosclerosis. In our study, RAASi improved CV function by preventing arterial wall thickening and fibrotic tissue accumulation in the media layer, without lowering blood pressure.

Atherosclerotic plaque formation increases vascular stiffness, which PWV can measure. This method is commonly used to monitor arterial stiffness in patients with T1DM. (88, 231) As a new finding, we established the non-invasive measurement of PWV that can also be conducted in small experimental animals, such as mice and rats, in addition to humans, which will facilitate future longitudinal studies. Furthermore, various studies have confirmed that normal-dose RAASi can prevent increases in PWV (232) and IMT (233), although our findings regarding blood pressure independence are novel.

Cardiac biomarkers are crucial in assessing heart function and strongly predict ventricular dysfunction and CV outcomes. Cardiac troponin, a component of the contractile apparatus in myocardial cells, is accepted as the standard biomarker for diagnosing and predicting myocardial injury. Additionally, Troponin I levels are often elevated in CVD patients with DM. (234)

BNP is primarily produced in the atria and ventricles in response to volume expansion and pressure overload. Its level is increased in T2DM rat models and diabetic patients. (235, 236) Some studies have shown that a standard dosage of RAASi can reduce troponin and BNP levels in rats with myocardial infarction. (237, 238) In our experimental setup, increases in troponin I and BNP confirmed myocardial injury and

left ventricular distress even without blood pressure changes in T1DM. Interestingly, all RAASi successfully prevented cardiac damage, even in low doses.

In renal disease with cardiac complications, klotho has gained attention recently as a sensitive and specific biomarker. As part of a unique endocrine system, it regulates the inactivation of oxidative stress, inflammation, and fibrotic pathways in the heart and kidneys. (239, 240) In T1DM patients, low serum klotho level is associated with a larger carotid artery IMT. Thus, it may be considered an early predictor of atherosclerosis. (241) Studies also report that klotho levels are lower in T2DM patients with CVD, supporting its protective role in cardiorenal disease. (242) In CKD patients, klotho was permanently decreased while FGF23 was elevated. (92) Furthermore multinomial regression analysis revealed that low klotho and higher FGF23 levels are linked to a greater risk of CV hospitalization as independent variables in T2DM patients. (243) Haplodeficiency of klotho gene was reported to increase collagen content in the media layer of mice aorta, suggesting that klotho could be closely related to vascular fibrotic changes, that can be blocked by eplerenone treatment. (244)

In line with these data, we also measured lower serum klotho and higher myocardial FGF23 levels in diabetic rats. All RAASi decreased FGF23, while only eplerenone could reverse the decline in serum klotho and reduce the medial fibrosis of the aorta. The sole efficacy of eplerenone might be explained by aldosterone directly downregulating klotho gene transcription by histone deacetylation (245); therefore, various RAASi affect the klotho-FGF23 axis differentially. Our results confirmed the potential of klotho and FGF23 as early and sensitive markers of cardiac dysfunction in the T1DM rat model and for monitoring responsiveness to low-dose RAASi treatment. We identified klotho as a possible novel target by which eplerenone could decrease fibrotic tissue accumulation in the media layer of the aorta.

Cardiac fibrosis is a final pathway through which HF develops. Myocardial fibrosis in patients with DM may occur independently of hypertension or coronary atherosclerosis. (246) It culminates in hyperglycemia-induced Ang II and aldosterone activity, promoting deposition of ECM proteins, leading to increased myocardial stiffness and impaired cardiac function. (247) Previous studies showed that Ang II induce fibroblasts' collagen production, (158) and regular and high-dose RAASi can reduce oxidative

stress of the blood vessel wall and ameliorate the formation of atherosclerotic plaque and cardiac fibrosis in a rat model of T1DM. (248, 249)

Based on our results showing that low-dose RAASi have antifibrotic effects in T1DM kidneys, it seemed plausible that they might prevent fibrosis in the diabetic heart even at low doses, independent of blood pressure changes. We demonstrated that myocardial injury, accumulation of fibrotic connective tissue, and the increase of αSMA protein (indicative of activation and differentiation of myocardial fibroblasts) were alleviated following low-dose RAASi treatment, supporting their inherent antifibrotic potential. We investigated the possible molecular mechanisms and found that the elevated expressions of PDGF and CTGF were reduced in all RAASi-treated rats.

It is widely accepted that chronic inflammation is a possible trigger for DCM. (250) IL-6, a common pro-inflammatory factor, causes persistent myocardial damage and cardiac dysfunction, while also modulating the interstitial matrix of the heart towards hypertrophy through natriuretic peptide production. (251) Increased serum IL-6 levels in T2DM patients were associated with left ventricular dysfunction. (252) In our experiments, the elevation of IL-6 and NFκB in the left ventricle also indicated the activation of inflammatory pathways in T1DM. We also demonstrated that hyperglycaemia-induced cardiac inflammation was diminished by losartan treatment. Interestingly, other RAASi drugs were less effective than losartan in preventing cardiac inflammation, at least in this model. Together with recent literature (237, 253), our findings support the anti-inflammatory role of losartan as a possible mechanism of action in preventing CVD.

VEGF plays a vital role in DM-induced cardioprotection and recovery of myocardial tissue. (254) Interestingly, VEGF production was protective in acute ischaemic or early diabetic conditions in murine kidneys. (255, 256) Still, in the heart under chronic hyperglycaemic conditions, VEGF increase detrimental effects in mice (257) and human T2DM patients as well. (258) We also found that VEGF protein level was elevated in T1DM rats, while it decreased over RAASi treatment.

HSP72 is a significant component of the endogenous defence, which may protect against tissue damage by acting as a molecular chaperone, facilitating the refolding of denatured proteins and the maintenance of structural integrity. (259) A substantial role

for HSP72 in DM has emerged as it was recently found to be elevated in T2DM. (260) In line with this, our results showed the elevation of HSP72 in the heart of T1DM rats, and RAASi treatment ameliorated this response. Although TNF is also part of the proinflammatory pathway, it was decreased in diabetic heart tissue in our experimental setup. This could be explained by its downregulation by HSP72, which has already been shown in the literature. (261, 262)

To further explore the role of inflammatory damage in DM-induced multiorgan complications, we investigated neuroinflammation in the brain related to DM-associated depression. Overactivation of local brain RAAS has been implicated in the pathophysiology of DM and certain neuropsychiatric disorders associated with neuroinflammation. (263, 264) Cerebral Ang II and aldosterone have been demonstrated to induce the release of proinflammatory cytokines. (265) In our diabetic rat model, we also found the activation of NFκB-mediated inflammatory response with elevated hippocampal IL-1, IL-6 and TNF levels. We showed that all RAASi mitigated the neuroinflammation. Based on these pathological findings, it is not that surprising that RAASi-treated hypertensive and T1DM patients have reduced depressive symptoms and require lower antidepressant dosages. (266, 267)

Proinflammatory cytokines have a stimulatory effect on the hypothalamic-pituitary-adrenal axis and contribute to changes in neuronal network activity, the release of neurotransmitters, neuronal growth and survival. (268, 269) IL-1 triggers the decline in cognitive function, worsens depression, and reduces BDNF expression in the hippocampus. (270, 271) Previously, we showed that ARB treatment mitigated the elevated neuroinflammation through NFκB, IL-1, and IL-6 in the brain of diabetic rats. (219) Our current results indicate that all RAASi can effectively reverse neuroinflammation and DM-induced reduction in BDNF levels. BDNF is synthesized as proBDNF, and then the N-terminal region is proteolytically cleaved by intracellular furin or extracellular MMPs to produce mBDNF. (272) We found that different RAASi act on different proteolytic cleavage pathways. Ramipril elevated extracellular MMP3, while the intracellular enzyme furin was increased by losartan and eplerenone. These results reveal potential pathways through which RAASi treatment can alleviate the detrimental neuronal effects of DM and comorbid depression.

6. Conclusions

1. RAASi treatment mitigates glucose-induced O-GlcNAcylation and profibrotic growth factor changes, and restores the novel non-invasive biomarker of fibronectin turnover in human proximal tubular epithelial cells. Furthermore, in rat kidney fibroblast cells RAASi decrease proliferative and fibrotic morphological changes.

2. Monotherapeutic treatment with low-dose RAASi is renoprotective and antifibrotic in T1DM rat kidneys. Novel urinary biomarkers can also trace improvement.

3. Lyophilized sample processing effectively reduces data scatter while investigating focal fibrotic changes in rat kidney and heart. This is confirmed by lower variances of αSMA mRNA and protein levels in the lyophilized, homogenized samples than within the conventionally processed frozen samples.

4. As part of CV protection, RAASi attenuate vascular stiffness, intimal thickness, and medial fibrosis in diabetic rat aortas, and decrease cardiac tissue damage, fibrotic protein accumulation and profibrotic factor increment without changing the blood pressure. Furthermore, low-dose RAASi treatment can also interfere with hypoxic and inflammatory pathways in diabetic hearts.

5. In DM-associated depression, RAASi can alleviate neuroinflammation via the NFκB inflammatory pathway and alter BDNF transformation by restoring cleavage enzymes.

7. Summary

DKD is one of the most common and detrimental consequences of DM, associated with CVD and comorbid depression. International guidelines list RAASi as the gold-standard therapy, although it is still unclear whether their protective properties are associated with or restricted to their antihypertensive effect. The exact molecular pathways have not yet been fully explored in T1DM.

Our primary goal was to explore the underlying mechanisms of the pleiotropic effect of monotherapeutic nondepressor-dose RAASi as protection against T1DM-induced renal, cardiac, and cerebral complications with special focus on fibrosis and inflammation.

Our previous experiments resulted in highly scattered data due to focal fibrosis, so we experimented with lyophilization during sample processing. We confirmed that using powdered homogenized samples improves the precision of measurements.

Firstly, *in vitro* experiments revealed that monotherapeutic RAASi have antifibrotic properties, mitigate O-GlcNAcylation in hyperglycaemic proximal tubular cells, and inhibit the growth factor-induced activation of fibroblast cells.

We also showed *in vivo* that monotherapeutic non-depressor dose RAASi treatment is renoprotective and antifibrotic in T1DM rats, and can also be monitored non-invasively by urinary biomarkers. Moreover, as part of CVD protection, RAASi attenuated arterial wall thickening, arterial stiffness increment, cardiac tissue damage and fibrosis without blood pressure changes. Furthermore, low-dose RAASi treatment also interfered with inflammatory pathways in the diabetic heart and brain. RAASi alleviated neuroinflammation and altered BDNF transformation by restoring cleavage enzymes in the hippocampi.

Our results provide evidence for the antifibrotic and anti-inflammatory properties of a non-depressor dose of RAASi, offering a possible novel therapeutic indication for them in T1DM-associated renal and cardiac complications. Our findings also support the link between DM and depression, where monotherapeutic RAASi can target neuroinflammation and the BDNF pathway. Furthermore we also emphasize that lyophilisation can be a superior sample processing method for higher reproducibility in case of focal lesions such as fibrosis.

8. References

1. White P, Wewer Albrechtsen N, Campbell J. Islet hormones at the intersection of glucose and amino acid metabolism. Nature Reviews Endocrinology. 2025.

2. IDF Diabetes Atlas Brussels, Belgium: International Diabetes Federation; 2025 11th edn.:[Available from: https://www.diabetesatlas.org.]

3. Type 1 Diabetes Index Brussels, Belgium: International Diabetes Federation; 2024 [Available from: https://dashboard.t1dindex.org/global-impact.]

4. Jermendy G, Kiss Z, Rokszin G, Abonyi-Tóth Z, Lengyel C, Kempler P, Wittmann I. Changing Patterns of Antihyperglycaemic Treatment among Patients with Type 2 Diabetes in Hungary between 2015 and 2020- Nationwide Data from a Register-Based Analysis. Medicina (Kaunas). 2022;58(10).

5. November 14-e a diabétesz világnapja Infographic: Hungarian Central Statistical Office; 2020 [Available from: https://www.ksh.hu/infografika/2020/infografika_diabetesz.pdf.]

6. T1DIndex Factsheet for Hungary in 2024 [Internet]. Type1 Diabetes Index. 2024. [Available from: https://t1dindex.shinyapps.io/dashboard/.]

7. Kempler Péter PZ, Kiss Zoltán, Wittmann István, Abonyi-Tóth Zsolt, Rokszin György, Jermendy György. A 2-es típusú diabetes előfordulása és költségterheinek alakulása Magyarországon 2001–2014 között – az Országos Egészségbiztosítási Pénztár adatbázis-elemzésének eredményei. Diabetologia Hungarica. 2016;XXIV.(3.):177-188.

8. Cukorbetegség Világnapja - November 14.: Nemzeti Egészségbiztosítási Alapkezelő; 2020 [Available from: https://www.neak.gov.hu/sajtoszoba/kozlemenyek_eu_napok/egeszsegugyi_vilagnapok_cukorbetegseg.]

9. ElSayed NA, Aleppo G, Aroda VR, Bannuru RR, Brown FM, Bruemmer D, Collins BS, Hilliard ME, Isaacs D, Johnson EL, Kahan S, Khunti K, Leon J, Lyons SK, Perry ML, Prahalad P, Pratley RE, Seley JJ, Stanton RC, Gabbay RA. 2. Classification and Diagnosis of Diabetes: Standards of Care in Diabetes-2023. Diabetes Care. 2023;46(Suppl 1):S19-S40.

10. Committee ADAPP. 2. Diagnosis and Classification of Diabetes: Standards of Care in Diabetes—2024. Diabetes Care. 2023;47(Supplement_1):S20-S42.

11. Petersmann A, Müller-Wieland D, Müller UA, Landgraf R, Nauck M, Freckmann G, Heinemann L, Schleicher E. Definition, Classification and Diagnosis of Diabetes Mellitus. Exp Clin Endocrinol Diabetes. 2019;127(S 01):S1-S7.

12. Bedros J, Jermendy Gy, Gaal Zs, Gero L, Hidvégi T, Kempler P, Lengyel Cs, Várkonyi T, Winkler G, Wittmann I. Egészségügyi szakmai irányelv : A diabetes mellitus kórismézéséről, a cukorbetegek antihyperglykaemiás kezeléséről és gondozásáról felnőttkorban. Diabetologia Hungarica. 2023;31. 331-444.

13. Shah AS, Zeitler PS, Wong J, Pena AS, Wicklow B, Arslanian S, Chang N, Fu J, Dabadghao P, Pinhas-Hamiel O, Urakami T, Craig ME. ISPAD Clinical Practice Consensus Guidelines 2022: Type 2 diabetes in children and adolescents. Pediatr Diabetes. 2022;23(7):872-902.

14. Li Z, Cheng Y, Wang D, Chen H, Chen H, Ming WK, Wang Z. Incidence Rate of Type 2 Diabetes Mellitus after Gestational Diabetes Mellitus: A Systematic Review and Meta-Analysis of 170,139 Women. J Diabetes Res. 2020;2020:3076463.

15. Quattrin T, Mastrandrea LD, Walker LSK. Type 1 diabetes. Lancet. 2023;401(10394):2149-2162.

16. Graham D Ogle FW, Gabriel A Gregory, Jayanthi Maniam. Type 1 diabetes estimates in children and adults Brussels, Belgium: International Diabetes Federation; 2022 [Available from: https://www.diabetesatlas.org.]

17. Redondo MJ, Gignoux CR, Dabelea D, Hagopian WA, Onengut-Gumuscu S, Oram RA, Rich SS. Type 1 diabetes in diverse ancestries and the use of genetic risk scores. Lancet Diabetes Endocrinol. 2022;10(8):597-608.

18. Rewers M, Ludvigsson J. Environmental risk factors for type 1 diabetes. The Lancet. 2016;387(10035):2340-2348.

19. Jakobsen O, Szereday L. The "Three Amigos" lurking behind type 1 diabetes: Hygiene, gut microbiota and viruses. Acta Microbiol Immunol Hung.2018;65(4):421-438.

20. Esposito S, Toni G, Tascini G, Santi E, Berioli MG, Principi N. Environmental Factors Associated With Type 1 Diabetes. Front Endocrinol (Lausanne). 2019;10:592.

21. Zorena K, Michalska M, Kurpas M, Jaskulak M, Murawska A, Rostami S. Environmental Factors and the Risk of Developing Type 1 Diabetes-Old Disease and New Data. Biology (Basel). 2022;11(4).

22. Skyler JS, Bakris GL, Bonifacio E, Darsow T, Eckel RH, Groop L, Groop PH, Handelsman Y, Insel RA, Mathieu C, McElvaine AT, Palmer JP, Pugliese A, Schatz

DA, Sosenko JM, Wilding JP, Ratner RE. Differentiation of Diabetes by Pathophysiology, Natural History, and Prognosis. Diabetes. 2017;66(2):241-255.

23. Gloyn AL, Drucker DJ. Precision medicine in the management of type 2 diabetes. Lancet Diabetes Endocrinol. 2018;6(11):891-900.

24. Chen S, Persson M, Wang R, Dalman C, Lee BK, Karlsson H, Gardner RM. Random capillary glucose levels throughout pregnancy, obstetric and neonatal outcomes, and long-term neurodevelopmental conditions in children: a group-based trajectory analysis. BMC Med. 2023;21(1):260.

25. Hattersley AT, Greeley SAW, Polak M, Rubio-Cabezas O, Njølstad PR, Mlynarski W, Castano L, Carlsson A, Raile K, Chi DV, Ellard S, Craig ME. ISPAD Clinical Practice Consensus Guidelines 2018: Diagnosing and managing monogenic diabetes in children and adolescents. Pediatric Diabetes. 2018;19(S27):47-63.

26. The Rare and Atypical Diabetes Network (RADIANT) Study: Design and Early Results. Diabetes Care. 2023;46(6):1265-1270.

27. Committee ADAPP. 2. Diagnosis and Classification of Diabetes: Standards of Care in Diabetes—2025. Diabetes Care. 2024;48(Supplement_1):S27-S49.

28. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol. 2018;14(2):88-98.

29. Yun JS, Ko SH. Current trends in cardiovascular disease epidemiology and cardiovascular risk management in type 2 diabetes. Metabolism. 2021;123:154838.

30. Faselis C, Katsimardou A, Imprialos K, Deligkaris P, Kallistratos M, Dimitriadis K. Microvascular Complications of Type 2 Diabetes Mellitus. Curr Vasc Pharmacol. 2020;18(2):117-124.

31. Wittmann I, Ladányi E, K, Balla J, Barna I, Deák Gy, Haris Á, Kovács T, Kulcsár I, Molnár GA, Remport Á, Reusz Gy, Tislér A, Újhelyi L, Wagner L, Zakar G. A Belügyminisztérium egészségügyi szakmai irányelve a felnőttkori idült vesebetegség diagnózisáról és kezeléséről. Egészségügyi Közlöny. 2025;75 (7):875-944.

32. de Boer IH, Khunti K, Sadusky T, Tuttle KR, Neumiller JJ, Rhee CM, Rosas SE, Rossing P, Bakris G. Diabetes management in chronic kidney disease: a consensus report by the American Diabetes Association (ADA) and Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int. 2022;102(5):974-989.

33. Li H, Lu W, Wang A, Jiang H, Lyu J. Changing epidemiology of chronic kidney disease as a result of type 2 diabetes mellitus from 1990 to 2017: Estimates from Global Burden of Disease 2017. J Diabetes Investig. 2021;12(3):346-356.

34. Perkins BA, Bebu I, de Boer IH, Molitch M, Tamborlane W, Lorenzi G, Herman W, White NH, Pop-Busui R, Paterson AD, Orchard T, Cowie C, Lachin JM. Risk Factors for Kidney Disease in Type 1 Diabetes. Diabetes Care. 2019;42(5):883-890.

35. Trikkalinou A, Papazafiropoulou AK, Melidonis A. Type 2 diabetes and quality of life. World J Diabetes. 2017;8(4):120-129.

36. Committee ADAPP 11. Chronic Kidney Disease and Risk Management: Standards of Care in Diabetes 2025. Diabetes Care. 2024;48(Supplement1):S239-S251.

37. Sagoo MK, Gnudi L. Diabetic Nephropathy: An Overview. Methods Mol Biol. 2020;2067:3-7.

38. Cai D, Hou B, Xie S.Amino acid analysis as a method of discovering biomarkers for diagnosis of diabetes and its complications.Amino Acids.2023;55(5):563-578

39. Ni WJ, Tang LQ, Wei W. Research progress in signalling pathway in diabetic nephropathy. Diabetes Metab Res Rev. 2015;31(3):221-233.

40. Peterson SB, Hart GW. New insights: A role for O-GlcNAcylation in diabetic complications. Crit Rev Biochem Mol Biol. 2016;51(3):150-161.

41. Qi B, Chen Y, Chai S, Lu X, Kang L. O-linked β-N-acetylglucosamine (O-GlcNAc) modification: Emerging pathogenesis and a therapeutic target of diabetic nephropathy. Diabet Med. 2025;42(2):e15436.

42. Agarwal R. Pathogenesis of Diabetic Nephropathy Arlington (VA): American Diabetes Association; 2021 [Available from: https://www.ncbi.nlm.nih.gov/books/NBK571720/ doi: 10.2337/db20211-2.]

43. Wu T, Ding L, Andoh V, Zhang J, Chen L. The Mechanism of Hyperglycemia-Induced Renal Cell Injury in Diabetic Nephropathy Disease: An Update. Life. 2023;13(2):539.

44. Haneda M, Utsunomiya K, Koya D, Babazono T, Moriya T, Makino H, Kimura K, Suzuki Y, Wada T, Ogawa S, Inaba M, Kanno Y, Shigematsu T, Masakane I, Tsuchiya K, Honda K, Ichikawa K, Shide K. A new classification of Diabetic Nephropathy 2014: a report from the Joint Committee on Diabetic Nephropathy. Clin Exp Nephrol. 2015;19(1):1-5.

45. Yang F, Chung AC, Huang XR, Lan HY. Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3. Hypertension. 2009;54(4):877-884.

46. Makino K, Makino T, Stawski L, Mantero JC, Lafyatis R, Simms R, Trojanowska M. Blockade of PDGF Receptors by Crenolanib Has Therapeutic Effect in Patient Fibroblasts and in Preclinical Models of Systemic Sclerosis. J Invest Dermatol. 2017;137(8):1671-1681.

47. Ihn H. Pathogenesis of fibrosis: role of TGF-β and CTGF. Current Opinion in Rheumatology. 2002;14(6):681-685.

48. Donovan J, Shiwen X, Norman J, Abraham D. Platelet-derived growth factor alpha and beta receptors have overlapping functional activities towards fibroblasts. Fibrogenesis & Tissue Repair. 2013;6:10.

49. Kolset SO, Reinholt FP, Jenssen T. Diabetic nephropathy and extracellular matrix. J Histochem Cytochem. 2012;60(12):976-986.

50. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. Nat Rev Nephrol. 2016;12(6):325-338.

51. Lichtman M, Otero-Vinas M, Falanga V. Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. Wound Repair Regen. 2016;24(2):215-222.

52. Garud MS, Kulkarni YA. Hyperglycemia to nephropathy via transforming growth factor beta. Curr Diabetes Rev. 2014;10(3):182-189.

53. Chen Y, Zou H, Lu H, Xiang H, Chen S. Research progress of endothelial-mesenchymal transition in diabetic kidney disease. J Cell Mol Med.2022;26(12):3313-3322.

54. Ma C, Chegini N. Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-beta1. Mol Hum Reprod. 1999;5(10):950-954.

55. Ostendorf T, Eitner F, Floege J. The PDGF family in renal fibrosis. Pediatr Nephrol. 2012;27(7):1041-1050.

56. Floege J, Eitner F, Alpers CE. A new look at platelet-derived growth factor in renal disease. J Am Soc Nephrol. 2008;19(1):12-23.

57. Lipson KE, Wong C, Teng Y, Spong S. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. Fibrogenesis Tissue Repair. 2012;5(Suppl 1):S24.

58. Kuppe C, Ibrahim MM, Kranz J, Zhang X, Ziegler S, Perales-Patón J, Jansen J, Reimer KC, Smith JR, Dobie R, Wilson-Kanamori JR, Halder M, Xu Y, Kabgani N, Kaesler N, Klaus M, Gernhold L, Puelles VG, Huber TB, Boor P, Menzel S, Hoogenboezem RM, Bindels EMJ, Steffens J, Floege J, Schneider RK, Saez-Rodriguez J, Henderson NC, Kramann R. Decoding myofibroblast origins in human kidney fibrosis. Nature. 2021;589(7841):281-286.

59. Yuan Q, Tan RJ, Liu Y. Myofibroblast in Kidney Fibrosis: Origin, Activation, and Regulation. Adv Exp Med Biol. 2019;1165:253-283.

60. Genovese F, Manresa AA, Leeming DJ, Karsdal MA, Boor P. The extracellular matrix in the kidney: a source of novel non-invasive biomarkers of kidney fibrosis? Fibrogenesis Tissue Repair. 2014;7(1):4.

61. Genovese F, Boor P, Papasotiriou M, Leeming DJ, Karsdal MA, Floege J. Turnover of type III collagen reflects disease severity and is associated with progression and microinflammation in patients with IgA nephropathy. Nephrol Dial Transplant. 2016;31(3):472-479.

62. Nielsen SH, Willumsen N, Brix S, Sun S, Manon-Jensen T, Karsdal M, Genovese F. Tumstatin, a Matrikine Derived from Collagen Type IVα3, is Elevated in Serum from Patients with Non-Small Cell Lung Cancer. Transl Oncol.2018;11(2):528-534.

63. Solis-Herrera C, Triplitt CL, Lynch JL. Nephropathy in youth and young adults with type 2 diabetes. Curr Diab Rep. 2014;14(2):456.

64. ElSayed NA, Aleppo G, Aroda VR, Bannuru RR, Brown FM, Bruemmer D, Collins BS, Hilliard ME, Isaacs D, Johnson EL, Kahan S, Khunti K, Leon J, Lyons SK, Perry ML, Prahalad P, Pratley RE, Seley JJ, Stanton RC, Gabbay RA. 11. Chronic Kidney Disease and Risk Management: Standards of Care in Diabetes—2023. Diabetes Care. 2022;46(Supplement_1):S191-S202.

65. ElSayed NA, Aleppo G, Aroda VR, Bannuru RR, Brown FM, Bruemmer D, Collins BS, Hilliard ME, Isaacs D, Johnson EL, Kahan S, Khunti K, Leon J, Lyons SK, Perry ML, Prahalad P, Pratley RE, Seley JJ, Stanton RC, Gabbay RA. 6. Glycemic

Targets: Standards of Care in Diabetes 2023. Diabetes Care. 2022;46(Supplement_1):S97-S110.

66. Committee ADAPP. 11. Chronic Kidney Disease and Risk Management: Standards of Care in Diabetes 2024. Diabetes Care. 2023;47(Supplement_1):S219-S230

67. Kjaergaard KD, Peters CD, Jespersen B, Tietze IN, Madsen JK, Pedersen BB, Novosel MK, Laursen KS, Bibby BM, Strandhave C, Jensen JD. Angiotensin Blockade and Progressive Loss of Kidney Function in Hemodialysis Patients: A Randomized Controlled Trial. American Journal of Kidney Diseases. 2014;64(6):892-901.

68. Fried LF, Emanuele N, Zhang JH, Brophy M, Conner TA, Duckworth W, Leehey DJ, McCullough PA, O'Connor T, Palevsky PM, Reilly RF, Seliger SL, Warren SR, Watnick S, Peduzzi P, Guarino P. Combined angiotensin inhibition for the treatment of diabetic nephropathy. N Engl J Med. 2013;369(20):1892-1903.

69. Dandamudi S, Slusser J, Mahoney DW, Redfield MM, Rodeheffer RJ, Chen HH. The Prevalence of Diabetic Cardiomyopathy: A Population-Based Study in Olmsted County, Minnesota. Journal of Cardiac Failure. 2014;20(5):304-309.

70. Lacolley P, Regnault V, Laurent S. Mechanisms of Arterial Stiffening. Arteriosclerosis, Thrombosis, and Vascular Biology. 2020;40(5):1055-1062.

71. Low Wang CC, Hess CN, Hiatt WR, Goldfine AB. Clinical Update: Cardiovascular Disease in Diabetes Mellitus: Atherosclerotic Cardiovascular Disease and Heart Failure in Type 2 Diabetes Mellitus - Mechanisms, Management, and Clinical Considerations. Circulation. 2016;133(24):2459-2502.

72. Lind M, Bounias I, Olsson M, Gudbjörnsdottir S, Svensson AM, Rosengren A. Glycaemic control and incidence of heart failure in 20,985 patients with type 1 diabetes: an observational study. Lancet. 2011;378(9786):140-146.

73. Rangaswami J, Bhalla V, Blair JEA, Chang TI, Costa S, Lentine KL, Lerma EV, Mezue K, Molitch M, Mullens W, Ronco C, Tang WHW, McCullough PA. Cardiorenal Syndrome: Classification, Pathophysiology, Diagnosis, and Treatment Strategies: A Scientific Statement From the American Heart Association. Circulation. 2019;139(16):e840-e878.

74. Jia G, Hill MA, Sowers JR. Diabetic Cardiomyopathy: An Update of Mechanisms Contributing to This Clinical Entity. Circ Res. 2018;122(4):624-638.

75. Bloom SI, Islam MT, Lesniewski LA, Donato AJ. Mechanisms and consequences of endothelial cell senescence. Nat Rev Cardiol. 2023;20(1):38-51.

76. Lei S, Li H, Xu J, Liu Y, Gao X, Wang J, Ng KF, Lau WB, Ma XL, Rodrigues B, Irwin MG, Xia Z. Hyperglycemia-induced protein kinase C β2 activation induces diastolic cardiac dysfunction in diabetic rats by impairing caveolin-3 expression and Akt/eNOS signaling. Diabetes. 2013;62(7):2318-2328.

77. Clausen P, Jacobsen P, Rossing K, Jensen JS, Parving HH, Feldt-Rasmussen B. Plasma concentrations of VCAM-1 and ICAM-1 are elevated in patients with Type 1 diabetes mellitus with microalbuminuria and overt nephropathy. Diabet Med. 2000;17(9):644-649.

78. Liu X, Luo F, Pan K, Wu W, Chen H. High glucose upregulates connective tissue growth factor expression in human vascular smooth muscle cells. BMC Cell Biol. 2007;8:1.

79. Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac fibrosis: The fibroblast awakens. Circulation Research. 2016;118(6):1021-1040.

80. Medrano-Bosch M, Simón-Codina B, Jiménez W, Edelman ER, Melgar-Lesmes P. Monocyte-endothelial cell interactions in vascular and tissue remodeling. Front Immunol. 2023;14:1196033.

81. Skilton MR, Celermajer DS, Cosmi E, Crispi F, Gidding SS, Raitakari OT, Urbina EM. Natural History of Atherosclerosis and Abdominal Aortic Intima-Media Thickness: Rationale, Evidence, and Best Practice for Detection of Atherosclerosis in the Young. J Clin Med. 2019;8(8).

82. Gow ML, Varley BJ, Nasir RF, Skilton MR, Craig ME. Aortic intima media thickness in children and adolescents with type 1 diabetes: A systematic review. Pediatric Diabetes. 2022;23(4):489-498.

83. Morrison KM, Dyal L, Conner W, Helden E, Newkirk L, Yusuf S, Lonn E. Cardiovascular risk factors and non-invasive assessment of subclinical atherosclerosis in youth. Atherosclerosis. 2010;208(2):501-505.

84. Kim JA, Jang HJ, Martinez-Lemus LA, Sowers JR. MTOR/p70S6 kinase activation by ANG II inhibits insulin-stimulated endothelial nitric oxide synthase and vasodilation. Am J Physiol Endocrinol Metab. 2012;302(2):E201-208.

85. Jia G, Habibi J, DeMarco VG, Martinez-Lemus LA, Ma L, Whaley-Connell AT, Aroor AR, Domeier TL, Zhu Y, Meininger GA, Mueller KB, Jaffe IZ, Sowers JR. Endothelial Mineralocorticoid Receptor Deletion Prevents Diet-Induced Cardiac Diastolic Dysfunction in Females. Hypertension. 2015;66(6):1159-1167.

86. Rosengren A, Dikaiou P. Cardiovascular outcomes in type 1 and type 2 diabetes. Diabetologia. 2023;66(3):425-437.

87. Simova I. Intima-media thickness: appropriate evaluation and proper measurement. J Cardiol Pract. 2015;13(21):1-14.

88. Tougaard NH, Theilade S, Winther SA, Tofte N, Ahluwalia TS, Hansen TW, Rossing P, Frimodt-Møller M. Carotid-Femoral Pulse Wave Velocity as a Risk Marker for Development of Complications in Type 1 Diabetes Mellitus. Journal of the American Heart Association. 2020;9(19):e017165.

89. Townsend RR, Wilkinson IB, Schiffrin EL, Avolio AP, Chirinos JA, Cockcroft JR, Heffernan KS, Lakatta EG, McEniery CM, Mitchell GF, Najjar SS, Nichols WW, Urbina EM, Weber T. Recommendations for Improving and Standardizing Vascular Research on Arterial Stiffness. Hypertension. 2015;66(3):698-722.

90. Wang Q, Ren D, Li Y, Xu G. Klotho attenuates diabetic nephropathy in db/db mice and ameliorates high glucose-induced injury of human renal glomerular endothelial cells. Cell Cycle. 2019;18(6-7):696-707.

91. Guo Y, Zhuang X, Huang Z, Zou J, Yang D, Hu X, Du Z, Wang L, Liao X. Klotho protects the heart from hyperglycemia-induced injury by inactivating ROS and NF-κB-mediated inflammation both in vitro and in vivo. Biochim Biophys Acta Mol Basis Dis. 2018;1864(1):238-251.

92. Navarro-García JA, González-Lafuente L, Fernández-Velasco M, Ruilope LM, Ruiz-Hurtado G. Fibroblast Growth Factor-23-Klotho Axis in Cardiorenal Syndrome: Mediators and Potential Therapeutic Targets. Frontiers in Physiology. 2021;12.

93. Committee ADAPP. 10. Cardiovascular Disease and Risk Management: Standards of Care in Diabetes 2024. Diabetes Care. 2023;47(Supplement_1):S179-S218.

94. Danne T, Garg S, Peters AL, Buse JB, Mathieu C, Pettus JH, Alexander CM, Battelino T, Ampudia-Blasco FJ, Bode BW, Cariou B, Close KL, Dandona P, Dutta S, Ferrannini E, Fourlanos S, Grunberger G, Heller SR, Henry RR, Kurian MJ, Kushner

JA, Oron T, Parkin CG, Pieber TR, Rodbard HW, Schatz D, Skyler JS, Tamborlane WV, Yokote K, Phillip M. International Consensus on Risk Management of Diabetic Ketoacidosis in Patients With Type 1 Diabetes Treated With Sodium-Glucose Cotransporter (SGLT) Inhibitors. Diabetes Care. 2019;42(6):1147-1154.

95. Huelsmann M, Neuhold S, Resl M, Strunk G, Brath H, Francesconi C, Adlbrecht C, Prager R, Luger A, Pacher R, Clodi M. PONTIAC (NT-proBNP selected prevention of cardiac events in a population of diabetic patients without a history of cardiac disease): a prospective randomized controlled trial. J Am Coll Cardiol. 2013;62(15):1365-1372.

96. Brandt-Jacobsen N, Lav Madsen P, Johansen M, Rasmussen J, Forman J, Holm M, Rye Jørgensen N, Faber J, Rossignol P, Schou M, Kistorp C. Mineralocorticoid Receptor Antagonist Improves Cardiac Structure in Type 2 Diabetes: Data From the MIRAD Trial. JACC: Heart Failure. 2021;9(8):550-558.

97. de Boer IH, Khunti K, Sadusky T, Tuttle KR, Neumiller JJ, Rhee CM, Rosas SE, Rossing P, Bakris G. Diabetes Management in Chronic Kidney Disease: A Consensus Report by the American Diabetes Association (ADA) and Kidney Disease: Improving Global Outcomes (KDIGO). Diabetes Care. 2022;45(12):3075-3090.

98. Yu MK, Weiss NS, Ding X, Katon WJ, Zhou XH, Young BA. Associations between depressive symptoms and incident ESRD in a diabetic cohort. Clin J Am Soc Nephrol. 2014;9(5):920-928.

99. Chen S, Zhang Q, Dai G, Hu J, Zhu C, Su L, Wu X. Association of depression with pre-diabetes, undiagnosed diabetes, and previously diagnosed diabetes: a meta-analysis. Endocrine. 2016;53(1):35-46.

100. Zalai D, Szeifert L, Novak M. Psychological distress and depression in patients with chronic kidney disease. Semin Dial. 2012;25(4):428-438.

101. Payne M, Steck S, George R, Steffens D. Fruit, vegetable, and antioxidant intakes are lower in older adults with depression. J Acad NutrDiet.2012;112:2022-2027.

102. Zhao JL, Jiang WT, Wang X, Cai ZD, Liu ZH, Liu GR. Exercise, brain plasticity, and depression. CNS Neurosci Ther. 2020;26(9):885-895.

103. Jing X, Chen J, Dong Y, Han D, Zhao H, Wang X, Gao F, Li C, Cui Z, Liu Y, Ma J. Related factors of quality of life of type 2 diabetes patients: a systematic review and meta-analysis. Health Qual Life Outcomes. 2018;16(1):189.

104. Ahola AJ, Harjutsalo V, Forsblom C, Pouwer F, Groop PH. Depression Is Associated With Progression of Diabetic Nephropathy in Type 1 Diabetes. Diabetes Care. 2021;44(1):174-180.
105. Moulton CD, Pickup JC, Ismail K. The link between depression and diabetes: the search for shared mechanisms. Lancet Diabetes Endocrinol. 2015;3(6):461-471.
106. Yu M, Zhang X, Lu F, Fang L. Depression and Risk for Diabetes: A Meta-Analysis. Can J Diabetes. 2015;39(4):266-272.
107. Anderson RJ, Freedland KE, Clouse RE, Lustman PJ. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes Care. 2001;24(6):1069-1078.
108. Ahola AJ, Harjutsalo V, Saraheimo M, Forsblom C, Groop PH. Purchase of antidepressant agents by patients with type 1 diabetes is associated with increased mortality rates in women but not in men. Diabetologia. 2012;55(1):73-79.
109. Alencar SBV, de Lima FM, Dias LDA, Dias VDA, Lessa AC, Bezerra JM, Apolinário JF, de Petribu KC. Depression and quality of life in older adults on hemodialysis. Braz J Psychiatry. 2020;42(2):195-200.
110. Traisathit P, Moolkham K, Maneeton N, Thongsak N, Maneeton B. Associated factors for depressive disorder in patients with end-stage renal disease treated with continuous ambulatory peritoneal dialysis. Ther Clin Risk Manag. 2019;15:541-548.
111. Novak M, Molnar MZ, Szeifert L, Kovacs AZ, Vamos EP, Zoller R, Keszei A, Mucsi I. Depressive symptoms and mortality in patients after kidney transplantation: a prospective prevalent cohort study. Psychosom Med. 2010;72(6):527-534.
112. Clinical descriptions and diagnostic requirements for ICD-11 mental, behavioural and neurodevelopmental disorders Geneva, Switzerland: World Health Organization;2024[Available from: www.who.int/publications/i/item/9789240077263]
113. Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. 1961;4:561-571.
114. Zung WW. A SELF-RATING DEPRESSION SCALE. Arch Gen Psychiatry. 1965;12:63-70.
115. Rafeyan R, Papakostas GI, Jackson WC, Trivedi MH. Inadequate Response to Treatment in Major Depressive Disorder: Augmentation and Adjunctive Strategies. J Clin Psychiatry. 2020;81(3).

116. Dodd S, Bauer M, Carvalho AF, Eyre H, Fava M, Kasper S, Kennedy SH, Khoo JP, Lopez Jaramillo C, Malhi GS, McIntyre RS, Mitchell PB, Castro AMP, Ratheesh A, Severus E, Suppes T, Trivedi MH, Thase ME, Yatham LN, Young AH, Berk M. A clinical approach to treatment resistance in depressed patients: What to do when the usual treatments don't work well enough? World J Biol Psychiatry. 2021;22(7):483-494.
117. Trivedi MH, Lin EH, Katon WJ. Consensus recommendations for improving adherence, self-management, and outcomes in patients with depression. CNS Spectr. 2007;12(8 Suppl 13):1-27.
118. van der Feltz-Cornelis C, Allen SF, Holt RIG, Roberts R, Nouwen A, Sartorius N. Treatment for comorbid depressive disorder or subthreshold depression in diabetes mellitus: Systematic review and meta-analysis. Brain Behav. 2021;11(2):e01981.
119. Fang T, Zhang Q, Wang Z, Liu JP. Bidirectional association between depression and diabetic nephropathy by meta-analysis. PLoS One. 2022;17(12):e0278489.
120. Ortiz R, Kluwe B, Odei JB, Echouffo Tcheugui JB, Sims M, Kalyani RR, Bertoni AG, Golden SH, Joseph JJ. The association of morning serum cortisol with glucose metabolism and diabetes: The Jackson Heart Study. Psychoneuroendocrinology. 2019;103:25-32.
121. Tang AL, Thomas SJ, Larkin T. Cortisol, oxytocin, and quality of life in major depressive disorder. Qual Life Res. 2019;28(11):2919-2928.
122. Bao A, Swaab D. Corticotropin-releasing hormone and arginine vasopressin in depression focus on the human postmortem hypothalamus. Vitam Horm.2010;82:339-365.
123. Erichsen JM, Fadel JR, Reagan LP. Peripheral versus central insulin and leptin resistance: Role in metabolic disorders, cognition, and neuropsychiatric diseases. Neuropharmacology. 2022;203:108877.
124. Manjarrez G, Herrera R, Leon M, Hernandez RJ. A low brain serotonergic neurotransmission in children with type 1 diabetes detected through the intensity dependence of auditory-evoked potentials. Diabetes Care. 2006;29(1):73-77.
125. Stuart MJ, Baune BT. Depression and type 2 diabetes: Inflammatory mechanisms of a psychoneuroendocrine co-morbidity. Neuroscience & Biobehavioral Reviews. 2012;36(1):658-676.

126. Li J, Huang M, Shen X. The association of oxidative stress and pro-inflammatory cytokines in diabetic patients with hyperglycemic crisis. J Diabetes Complications. 2014;28(5):662-666.
127. Harsanyi S, Kupcova I, Danisovic L, Klein M. Selected Biomarkers of Depression: What Are the Effects of Cytokines and Inflammation? Int J Mol Sci. 2022;24(1).
128. Beurel E, Toups M, Nemeroff CB. The Bidirectional Relationship of Depression and Inflammation: Double Trouble. Neuron. 2020;107(2):234-256.
129. Reddy P, Lent-Schochet D, Ramakrishnan N, McLaughlin M, Jialal I. Metabolic syndrome is an inflammatory disorder: A conspiracy between adipose tissue and phagocytes. Clin Chim Acta. 2019;496:35-44.
130. Wang H, He Y, Sun Z, Ren S, Liu M, Wang G, Yang J. Microglia in depression: an overview of microglia in the pathogenesis and treatment of depression. J Neuroinflammation. 2022;19(1):132.
131. Zhang YY, Wang L, Guo H, Han TT, Chang YH, Cui XC. Levetiracetam attenuates diabetes-associated cognitive impairment and microglia polarization by suppressing neuroinflammation. Front Pharmacol. 2023;14:1145819.
132. Korczak DJ, Pereira S, Koulajian K, Matejcek A, Giacca A. Type 1 diabetes mellitus and major depressive disorder: evidence for a biological link. Diabetologia. 2011;54(10):2483-2493.
133. Tong L, Prieto G, Kramár E, Smith E, Cribbs D, Lynch G, Cotman CW. Brain-derived neurotrophic factor-dependent synaptic plasticity is suppressed by interleukin-1β via p38 mitogen-activated protein kinase. Journal of Neuroscience. 2012;32(49):17714-17724.
134. Zhang JC, Yao W, Hashimoto K. Brain-derived Neurotrophic Factor (BDNF)-TrkB Signaling in Inflammation-related Depression and Potential Therapeutic Targets. Curr Neuropharmacol. 2016;14(7):721-731.
135. Hang P, Ge F, Li P, Liu J, Zhu H, Zhao J. The regulatory role of the BDNF/TrkB pathway in organ and tissue fibrosis. Histol Histopathol. 2021;36(11):1133-1143.
136. Rozanska O, Uruska A, Zozulinska-Ziolkiewicz D. Brain-Derived Neurotrophic Factor and Diabetes. Int J Mol Sci. 2020;21(3).

137. Koshimizu H, Kiyosue K, Hara T, Hazama S, Suzuki S, Uegaki K, Nagappan G, Zaitsev E, Hirokawa T, Tatsu Y, Ogura A, Lu B, Kojima M. Multiple functions of precursor BDNF to CNS neurons: negative regulation of neurite growth, spine formation and cell survival. Mol Brain. 2009;2:27.

138. Hennigan A, O'Callaghan RM, Kelly AM. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochem Soc Trans. 2007;35(Pt 2):424-427.

139. Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Front Mol Neurosci. 2010;3:1.

140. Neubauer B, Schrankl J, Steppan D, Neubauer K, Sequeira-Lopez ML, Pan L, Gomez RA, Coffman TM, Gross KW, Kurtz A, Wagner C. Angiotensin II Short-Loop Feedback: Is There a Role of Ang II for the Regulation of the Renin System In Vivo? Hypertension. 2018;71(6):1075-1082.

141. Ksiazek SH, Hu L, Andò S, Pirklbauer M, Säemann MD, Ruotolo C, Zaza G, La Manna G, De Nicola L, Mayer G, Provenzano M. Renin-Angiotensin-Aldosterone System: From History to Practice of a Secular Topic. Int J Mol Sci. 2024;25(7).

142. Mendoza-Torres E, Oyarzún A, Mondaca-Ruff D, Azocar A, Castro PF, Jalil JE, Chiong M, Lavandero S, Ocaranza MP. ACE2 and vasoactive peptides: novel players in cardiovascular/renal remodeling and hypertension. Ther Adv Cardiovasc Dis. 2015;9(4):217-237.

143. Taguchi S, Azushima K, Yamaji T, Suzuki T, Abe E, Tanaka S, Hirota K, Tsukamoto S, Morita R, Kobayashi R, Kinguchi S, Yamashita A, Wakui H, Tamura K. Angiotensin II type 1 receptor-associated protein deletion combined with angiotensin II stimulation accelerates the development of diabetic kidney disease in mice on a C57BL/6 strain. Hypertens Res. 2024;47(1):55-66.

144. Das S, Senapati P, Chen Z, Reddy MA, Ganguly R, Lanting L, Mandi V, Bansal A, Leung A, Zhang S, Jia Y, Wu X, Schones DE, Natarajan R. Regulation of angiotensin II actions by enhancers and super-enhancers in vascular smooth muscle cells. Nature Communications. 2017;8(1):1467.

145. Colin M, Delaitre C, Foulquier S, Dupuis F. The AT(1)/AT(2) Receptor Equilibrium Is a Cornerstone of the Regulation of the Renin Angiotensin System beyond the Cardiovascular System. Molecules. 2023;28(14).

146. Kamide K. Role of Renin-Angiotensin-Aldosterone System in Metabolic Syndrome and Obesity-related Hypertension. Curr Hypertens Rev. 2014.

147. Lovshin JA, Boulet G, Lytvyn Y, Lovblom LE, Bjornstad P, Farooqi MA, Lai V, Cham L, Tse J, Orszag A, Scarr D, Weisman A, Keenan HA, Brent MH, Paul N, Bril V, Perkins BA, Cherney DZ. Renin-angiotensin-aldosterone system activation in long-standing type 1 diabetes. JCI Insight. 2018;3(1).

148. Bader M, Ganten D. Update on tissue renin-angiotensin systems. J Mol Med (Berl). 2008;86(6):615-621.

149. Tain YL, Hsu CN. The Renin-Angiotensin System and Cardiovascular-Kidney-Metabolic Syndrome: Focus on Early-Life Programming. Int J Mol Sci. 2024;25(6).

150. Mascolo A, Sessa M, Scavone C, De Angelis A, Vitale C, Berrino L, Rossi F, Rosano G, Capuano A. New and old roles of the peripheral and brain renin-angiotensin-aldosterone system (RAAS): Focus on cardiovascular and neurological diseases. Int J Cardiol. 2017;227:734-742.

151. O'Connor AT, Clark MA. Astrocytes and the Renin Angiotensin System: Relevance in Disease Pathogenesis. Neurochem Res. 2018;43(7):1297-1307.

152. Urmila A, Rashmi P, Nilam G, Subhash B. Recent Advances in the Endogenous Brain Renin-Angiotensin System and Drugs Acting on It. J Renin Angiotensin Aldosterone Syst. 2021;2021:9293553.

153. Wang R, Fang D, Lin T, Liang W, Qiao H. Aldosterone activates ERK phosphorylation in the nucleus tractus solitarius. Neurosci Lett. 2023;803:137188.

154. Sherrod M, Davis DR, Zhou X, Cassell MD, Sigmund CD. Glial-specific ablation of angiotensinogen lowers arterial pressure in renin and angiotensinogen transgenic mice. Am J Physiol Regul Integr Comp Physiol. 2005;289(6):R1763-1769.

155. Grafe LA, Flanagan-Cato LM. Differential effects of mineralocorticoid and angiotensin II on incentive and mesolimbic activity. Horm Behav. 2016;79:28-36.

156. Zhou Z, Orchard SG, Nelson MR, Fravel MA, Ernst ME. Angiotensin Receptor Blockers and Cognition: a Scoping Review. Curr Hypertens Rep. 2024;26(1):1-19.

157. Rossi F, Mascolo A, Mollace V. The pathophysiological role of natriuretic peptide-RAAS cross talk in heart failure. Int J Cardiol. 2017;226:121-125.

158. Lijnen P, Petrov V. Role of intracardiac renin-angiotensin-aldosterone system in extracellular matrix remodeling.Methods Find Exp Clin Pharmacol.2003;25(7):541-564.

159. Böckmann I, Lischka J, Richter B, Deppe J, Rahn A, Fischer DC, Heineke J, Haffner D, Leifheit-Nestler M. FGF23-Mediated Activation of Local RAAS Promotes Cardiac Hypertrophy and Fibrosis. Int J Mol Sci. 2019;20(18).
160. Zhuo JL, Li XC. New insights and perspectives on intrarenal renin-angiotensin system: focus on intracrine/intracellular angiotensin II. Peptides. 2011;32(7):1551-1565.
161. Nishiyama A, Kobori H. Independent regulation of renin-angiotensin-aldosterone system in the kidney. Clin Exp Nephrol. 2018;22(6):1231-1239.
162. Reinhold SW, Krüger B, Barner C, Zoicas F, Kammerl MC, Hoffmann U, Bergler T, Banas B, Krämer BK. Nephron-specific expression of components of the renin-angiotensin-aldosterone system in the mouse kidney. J Renin Angiotensin Aldosterone Syst. 2012;13(1):46-55.
163. Zimpelmann J, Kumar D, Levine DZ, Wehbi G, Imig JD, Navar LG, Burns KD. Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat. Kidney Int. 2000;58(6):2320-2330.
164. Hsieh TJ, Fustier P, Zhang SL, Filep JG, Tang SS, Ingelfinger JR, Fantus IG, Hamet P, Chan JS. High glucose stimulates angiotensinogen gene expression and cell hypertrophy via activation of the hexosamine biosynthesis pathway in rat kidney proximal tubular cells. Endocrinology. 2003;144(10):4338-4349.
165. Rüster C, Wolf G. Angiotensin II as a morphogenic cytokine stimulating renal fibrogenesis. J Am Soc Nephrol. 2011;22(7):1189-1199.
166. Matavelli LC, Siragy HM. Reduction of aldosterone production improves renal oxidative stress and fibrosis in diabetic rats. J Cardiovasc Pharmacol. 2013;61(1):17-22.
167. Oroszlán M, Bieri M, Ligeti N, Farkas A, Meier B, Marti HP, Mohacsi P. Sirolimus and everolimus reduce albumin endocytosis in proximal tubule cells via an angiotensin II-dependent pathway. Transpl Immunol. 2010;23(3):125-132.
168. Wei J, Li Z, Ma C, Zhan F, Wu W, Han H, Huang Y, Li W, Chen D, Peng Y. Rho kinase pathway is likely responsible for the profibrotic actions of aldosterone in renal epithelial cells via inducing epithelial-mesenchymal transition and extracellular matrix excretion. Cell Biol Int. 2013;37(7):725-730.
169. Banki NF, Ver A, Wagner LJ, Vannay A, Degrell P, Prokai A, Gellai R, Lenart L, Szakal DN, Kenesei E, Rosta K, Reusz G, Szabo AJ, Tulassay T, Baylis C, Fekete A.

Aldosterone antagonists in monotherapy are protective against streptozotocin-induced diabetic nephropathy in rats. PLoS One. 2012;7(6):e39938.

170. Coppey LJ, Davidson EP, Rinehart TW, Gellett JS, Oltman CL, Lund DD, Yorek MA. ACE inhibitor or angiotensin II receptor antagonist attenuates diabetic neuropathy in streptozotocin-induced diabetic rats. Diabetes. 2006;55(2):341-348.

171. Failli P, Alfarano C, Franchi-Micheli S, Mannucci E, Cerbai E, Mugelli A, Raimondi L. Losartan counteracts the hyper-reactivity to angiotensin II and ROCK1 over-activation in aortas isolated from streptozotocin-injected diabetic rats. Cardiovasc Diabetol. 2009;8:32.

172. Hao L, Kanno Y, Fukushima R, Watanabe Y, Ishida Y, Suzuki H. Effects of eplerenone on heart and kidney in two-kidney, one-clip rats. Am J Nephrol. 2004;24(1):54-60.

173. Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE. Recommendations for blood pressure measurement in humans and experimental animals. Part 2: Blood pressure measurement in experimental animals: a statement for professionals from the subcommittee of professional and public education of the American Heart Association council on high blood pressure research. Hypertension. 2005;45(2):299-310.

174. Salvi P. Pulse Wave Velocity and Pulse Wave Analysis in Experimental Animals. Pulse Waves: How Vascular Hemodynamics Affects Blood Pressure. Cham: Springer International Publishing; 2017. p. 211-219.

175. Nielsen MJ, Nedergaard AF, Sun S, Veidal SS, Larsen L, Zheng Q, Suetta C, Henriksen K, Christiansen C, Karsdal MA, Leeming DJ. The neo-epitope specific PRO-C3 ELISA measures true formation of type III collagen associated with liver and muscle parameters. Am J Transl Res. 2013;5(3):303-315.

176. Bager C, Gudmann N, Willumsen N, Leeming D, Karsdal M, Bay-Jensen A, Høgdall E, Balslev I, He Y. Quantification of fibronectin as a method to assess ex vivo extracellular matrix remodeling. Biochem Biophys Res Commun. 2016;478(2):586-591.

177. Leeming DJ, Karsdal MA, Rasmussen LM, Scholze A, Tepel M. Association of systemic collagen type IV formation with survival among patients undergoing hemodialysis. PLoS One. 2013;8(8):e71050.

178. Yamate J, Kuribayashi M, Kuwamura M, Kotani T, Ogihara K. Differential immunoexpressions of cytoskeletons in renal epithelial and interstitial cells in rat and

canine fibrotic kidneys, and in kidney-related cell lines under fibrogenic stimuli. Experimental and Toxicologic Pathology. 2005;57(2):135-147.

179. Gosslau A, Dittrich W, Willig A, Jaros P. Cytological effects of platelet-derived growth factor on mitochondrial ultrastructure in fibroblasts. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2001;128(2):241-249.

180. Yang M, Huang H, Li J, Huang W, Wang H. Connective tissue growth factor increases matrix metalloproteinase-2 and suppresses tissue inhibitor of matrix metalloproteinase-2 production by cultured renal interstitial fibroblasts. Wound Repair Regen. 2007;15(6):817-824.

181. Yang M, Huang H, Li J, Li D, Wang H. Tyrosine phosphorylation of the LDL receptor-related protein (LRP) and activation of the ERK pathway are required for connective tissue growth factor to potentiate myofibroblast differentiation. FASEB J. 2004;18(15):1920-1921.

182. Koszegi S, Molnar A, Lenart L, Hodrea J, Balogh DB, Lakat T, Szkibinszkij E, Hosszu A, Sparding N, Genovese F, Wagner L, Vannay A, Szabo AJ, Fekete A. RAAS inhibitors directly reduce diabetes-induced renal fibrosis via growth factor inhibition. J Physiol. 2019;597(1):193-209.

183. Thomasova D, Anders HJ. Cell cycle control in the kidney. Nephrol Dial Transplant. 2015;30(10):1622-1630.

184. Molnar A, Lakat T, Hosszu A, Szebeni B, Balogh A, Orfi L, Szabo AJ, Fekete A, Hodrea J. Lyophilization and homogenization of biological samples improves reproducibility and reduces standard deviation in molecular biology techniques. Amino Acids. 2021;53(6):917-928.

185. Balogh DB, Molnar A, Degi A, Toth A, Lenart L, Saeed A, Barczi A, Szabo AJ, Wagner LJ, Reusz G, Fekete A. Cardioprotective and Antifibrotic Effects of Low-Dose Renin-Angiotensin-Aldosterone System Inhibitors in Type 1 Diabetic Rat Model. Int J Mol Sci. 2023;24(23)

186. Balogh DB, Molnar A, Hosszu A, Lakat T, Hodrea J, Szabo AJ, Lenart L, Fekete A. Antidepressant effect in diabetes-associated depression: A novel potential of RAAS inhibition. Psychoneuroendocrinology. 2020;118:104705

187. Nakagawa T. Diabetic nephropathy: Aldosterone breakthrough in patients on an ACEI. Nat Rev Nephrol. 2010;6(4):194-196.

188. Moranne O, Bakris G, Fafin C, Favre G, Pradier C, Esnault VL. Determinants and changes associated with aldosterone breakthrough after angiotensin II receptor blockade in patients with type 2 diabetes with overt nephropathy. Clin J Am Soc Nephrol. 2013;8(10):1694-1701.
189. Lin HC, Paul CR, Kuo CH, Chang YH, Chen WS, Ho TJ, Day CH, Velmurugan BK, Tsai Y, Huang CY. Glycyrrhiza uralensis root extract ameliorates high glucose-induced renal proximal tubular fibrosis by attenuating tubular epithelial-myofibroblast transdifferentiation by targeting TGF-β1/Smad/Stat3 pathway. J Food Biochem. 2022;46(5):e14041.
190. Zou X, Wu M, Tu M, Tan X, Long Y, Xu Y, Li M. 4-octyl itaconate inhibits high glucose induced renal tubular epithelial cell fibrosis through TGF-β-ROS pathway. J Recept Signal Transduct Res. 2024:1-8.
191. Mesarosova L, Ochodnicky P, Leemans JC, Florquin S, Krenek P, Klimas J. High glucose induces HGF-independent activation of Met receptor in human renal tubular epithelium. J Recept Signal Transduct Res. 2017;37(6):535-542.
192. Xu P, Guan M, Bi J, Wang D, Zheng Z, Xue Y. High glucose down-regulates microRNA-181a-5p to increase pro-fibrotic gene expression by targeting early growth response factor 1 in HK-2 cells. Cellular Signalling. 2017;31:96-104.
193. Montero RM, Bhangal G, Pusey CD, Frankel AH, Tam FWK. CCL18 synergises with high concentrations of glucose in stimulating fibronectin production in human renal tubuloepithelial cells. BMC Nephrology. 2016;17(1):139.
194. Visavadiya NP, Li Y, Wang S. High Glucose Upregulates Upstream Stimulatory Factor 2 in Human Renal Proximal Tubular Cells through Angiotensin II-Dependent Activation of CREB. Nephron Experimental Nephrology. 2011;117(3):e62-e70.
195. Gou R, Chen J, Sheng S, Wang R, Fang Y, Yang Z, Wang L, Tang L. KIM-1 Mediates High Glucose-Induced Autophagy and Apoptosis in Renal Tubular Epithelial Cells. Cellular Physiology and Biochemistry. 2016;38(6):2479-2488.
196. Zhang W, Song S, Liu F, Liu Y, Zhang Y. Beta-casomorphin-7 prevents epithelial-mesenchymal transdifferentiation of NRK-52E cells at high glucose level: Involvement of AngII-TGF-beta1 pathway. Peptides. 2015;70:37-44.
197. Wei J, Li Z, Ma C, Zhan F, Wu W, Han H, Huang Y, Li W, Chen D, Peng Y. Rho kinase pathway is likely responsible for the profibrotic actions of aldosterone in

renal epithelial cells via inducing epithelial–mesenchymal transition and extracellular matrix excretion. Cell Biology International. 2013;37(7):725-730.

198. Li X, Meng Y, Yang XS, Mi LF, Cai SX. ACEI attenuates the progression of CCl4-induced rat hepatic fibrogenesis by inhibiting TGF-beta1, PDGF-BB, NF-kappaB and MMP-2,9. World J Gastroenterol. 2005;11(31):4807-4811.

199. Nishioka T, Suzuki M, Onishi K, Takakura N, Inada H, Yoshida T, Hiroe M, Imanaka-Yoshida K. Eplerenone attenuates myocardial fibrosis in the angiotensin II-induced hypertensive mouse: involvement of tenascin-C induced by aldosterone-mediated inflammation. J Cardiovasc Pharmacol. 2007;49(5):261-268.

200. Fülöp N, Marchase RB, Chatham JC. Role of protein O-linked N-acetyl-glucosamine in mediating cell function and survival in the cardiovascular system. Cardiovasc Res. 2007;73(2):288-297.

201. Marsh S, Dell'Italia L, Chatham J. Activation of the hexosamine biosynthesis pathway and protein O-GlcNAcylation modulate hypertrophic and cell signaling pathways in cardiomyocytes from diabetic mice. Amino Acids. 2011;40(3):819-828.

202. Park K, Saudek CD, Hart GW. Increased expression of beta-N-acetylglucosaminidase in erythrocytes from individuals with pre-diabetes and diabetes. Diabetes. 2010;59(7):1845-1850.

203. Springhorn C, Matsha TE, Erasmus RT, Essop MF. Exploring leukocyte O-GlcNAcylation as a novel diagnostic tool for the earlier detection of type 2 diabetes mellitus. J Clin Endocrinol Metab. 2012;97(12):4640-4649.

204. James L, Ingram A, Ly H, Thai K, Cai L, Scholey J. Angiotensin II activates the GFAT promoter in mesangial cells. Am J Physiol Renal Physiol.2001;281(1):F151-162.

205. Makino A, Dai A, Han Y, Youssef KD, Wang W, Donthamsetty R, Scott BT, Wang H, Dillmann WH. O-GlcNAcase overexpression reverses coronary endothelial cell dysfunction in type 1 diabetic mice.Am J Physiol Cell Physiol.2015;309:C593-599.

206. Shi-Wen X, Leask A, Abraham D. Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. Cytokine Growth Factor Rev. 2008;19(2):133-144.

207. Hedberg KM, Bengtsson T, Safiejko-Mroczka B, Bell PB, Lindroth M. PDGF and neomycin induce similar changes in the actin cytoskeleton in human fibroblasts. Cell Motil Cytoskeleton. 1993;24(2):139-149.

208. Fuchshofer R, Ullmann S, Zeilbeck LF, Baumann M, Junglas B, Tamm ER. Connective tissue growth factor modulates podocyte actin cytoskeleton and extracellular matrix synthesis and is induced in podocytes upon injury. Histochemistry and Cell Biology. 2011;136(3):301.

209. Izuhara Y, Nangaku M, Inagi R, Tominaga N, Aizawa T, Kurokawa K, van Ypersele de Strihou C, Miyata T. Renoprotective properties of angiotensin receptor blockers beyond blood pressure lowering. J Am Soc Nephrol. 2005;16(12):3631-3641.

210. Fiordaliso F, Cuccovillo I, Bianchi R, Bai A, Doni M, Salio M, De Angelis N, Ghezzi P, Latini R, Masson S. Cardiovascular oxidative stress is reduced by an ACE inhibitor in a rat model of streptozotocin-induced diabetes.Life Sci.2006;79(2):121-129.

211. Pavlatou MG, Mastorakos G, Lekakis I, Liatis S, Vamvakou G, Zoumakis E, Papassotiriou I, Rabavilas AD, Katsilambros N, Chrousos GP. Chronic administration of an angiotensin II receptor antagonist resets the hypothalamic–pituitary–adrenal (HPA) axis and improves the affect of patients with diabetes mellitus type 2: Preliminary results. Stress. 2008;11(1):62-72.

212. Xing L, Song EL, Jia XB, Ma J, Li B, Gao X. Nephroprotective effect of losartan in IgA model rat. J Int Med Res. 2019;47(10):5205-5215.

213. Seccia TM, Belloni AS, Guidolin D, Sticchi D, Nussdorfer GG, Pessina AC, Rossi GP. The renal antifibrotic effects of angiotensin-converting enzyme inhibition involve bradykinin B2 receptor activation in angiotensin II-dependent hypertension. J Hypertens. 2006;24(7):1419-1427.

214. Peng X, Su H, Liang D, Li J, Ting WJ, Liao SC, Huang CY. Ramipril and resveratrol co-treatment attenuates RhoA/ROCK pathway-regulated early-stage diabetic nephropathy-associated glomerulosclerosis in streptozotocin-induced diabetic rats. Environ Toxicol. 2019;34(7):861-868.

215. Han KH, Kang YS, Han SY, Jee YH, Lee MH, Han JY, Kim HK, Kim YS, Cha DR. Spironolactone ameliorates renal injury and connective tissue growth factor expression in type II diabetic rats. Kidney Int. 2006;70(1):111-120.

216. Törnig J, Amann K, Ritz E, Nichols C, Zeier M, Mall G. Arteriolar wall thickening, capillary rarefaction and interstitial fibrosis in the heart of rats with renal failure:the effects of ramipril, nifedipine and moxonidine. J Am Soc Nephrol. 1996;7(5):667-675.

217. Torika N, Asraf K, Roasso E, Danon A, Fleisher-Berkovich S. Angiotensin Converting Enzyme Inhibitors Ameliorate Brain Inflammation Associated with Microglial Activation: Possible Implications for Alzheimer's Disease. Journal of Neuroimmune Pharmacology. 2016;11(4):774-785.
218. De Angelis K, Irigoyen MC, Morris M. Diabetes and cardiovascular autonomic dysfunction: application of animal models. Auton Neurosci. 2009;145(1-2):3-10.
219. Lenart L, Balogh DB, Lenart N, Barczi A, Hosszu A, Farkas T, Hodrea J, Szabo AJ, Szigeti K, Denes A, Fekete A. Novel therapeutic potential of angiotensin receptor 1 blockade in a rat model of diabetes-associated depression parallels altered BDNF signalling. Diabetologia. 2019;62(8):1501-1513.
220. Sparding N, Neprasova M, Maixnerova D, Genovese F, Karsdal MA, Kollar M, Koprivova H, Hruskova Z, Tesar V. Unique Biomarkers of Collagen Type III Remodeling Reflect Different Information Regarding Pathological Kidney Tissue Alterations in Patients with IgA Nephropathy. Biomolecules. 2023;13(7).
221. Satrapova V, Sparding N, Genovese F, Karsdal MA, Bartonova L, Frausova D, Honsova E, Kollar M, Suchanek M, Koprivova H, Rysava R, Bednarova V, Tesar V, Hruskova Z. Biomarkers of fibrosis, kidney tissue injury and inflammation may predict severity and outcome of renal ANCA - associated vasculitis. Front Immunol. 2023;14:1122972.
222. Sparding N, Genovese F, Karsdal MA, Selby NM. Collagen type III formation but not degradation is associated with risk of kidney disease progression and mortality after acute kidney injury. Clin Kidney J. 2025;18(2):sfae413.
223. Schipke J, Brandenberger C, Rajces A, Manninger M, Alogna A, Post H, Mühlfeld C. Assessment of cardiac fibrosis: a morphometric method comparison for collagen quantification. J Appl Physiol (1985). 2017;122(4):1019-1030.
224. Courtoy GE, Leclercq I, Froidure A, Schiano G, Morelle J, Devuyst O, Huaux F, Bouzin C. Digital Image Analysis of Picrosirius Red Staining: A Robust Method for Multi-Organ Fibrosis Quantification and Characterization. Biomolecules. 2020;10(11).
225. Román B, González-Verdejo CI, Peña F, Nadal S, Gómez P. Evaluation of different pulverisation methods for RNA extraction in squash fruit: lyophilisation, cryogenic mill and mortar grinding. Phytochem Anal. 2012;23(6):622-626.

226. Lakat T. Lyophilization: a novel sample preprocessing method to improve the quantitative evaluation of tissue fibrosis 2023.

227. Beckman JA, Creager MA. Vascular Complications of Diabetes. Circulation Research. 2016;118(11):1771-1785.

228. Filippatos G, Anker SD, Agarwal R, Ruilope LM, Rossing P, Bakris GL, Tasto C, Joseph A, Kolkhof P, Lage A, Pitt B; FIGARO-DKD Investigators. Finerenone Reduces Risk of Incident Heart Failure in Patients With Chronic Kidney Disease and Type 2 Diabetes: Analyses From the FIGARO-DKD Trial. Circulation. 2022;145(6):437-447.

229. Franco O, Steyerberg E, Hu F, Mackenbach J, Nusselder W. Associations of Diabetes Mellitus With Total Life Expectancy and Life Expectancy With and Without Cardiovascular Disease. Archives of Internal Medicine. 2007;167(11):1145-1151.

230. Ameer O, Salman I, Alwadi A, Ouban A, Abu-Owaimer F, AlSharari S, Bukhari IA. Regional functional and structural abnormalities within the aorta as a potential driver of vascular disease in metabolic syndrome. Experimental Physiology. 2021;106(3):771-788.

231. Shah AS, Wadwa RP, Dabelea D, Hamman RF, D'Agostino Jr R, Marcovina S, Daniels SR, Dolan LM, Fino NF, Urbina EM. Arterial stiffness in adolescents and young adults with and without type 1 diabetes: the SEARCH CVD study. Pediatric Diabetes. 2015;16(5):367-374.

232. Masuda S, Tamura K, Wakui H, Kanaoka T, Ohsawa M, Maeda A, Dejima T, Yanagi M, Azuma K, Umemura S. Effects of angiotensin II type 1 receptor blocker on ambulatory blood pressure variability in hypertensive patients with overt diabetic nephropathy. Hypertens Res. 2009;32(11):950-955.

233. Sonoda M, Aoyagi T, Takenaka K, Uno K, Nagai R. A One-Year Study of the Antiatherosclerotic Effect of the Angiotensin-II Receptor Blocker Losartan in Hypertensive Patients. Comparison With Angiotensin-Converting Enzyme Inhibitors. International Heart Journal. 2008;49(1):95-103.

234. Segre CAW, Hueb W, Garcia RMR, Rezende PC, Favarato D, Strunz CMC, Sprandel Mda C, Roggério A, Carvalho AL, Maranhão RC, Ramires JA, Kalil Filho R. Troponin in diabetic patients with and without chronic coronary artery disease. BMC Cardiovascular Disorders. 2015;15(1):72.

235. Wolsk E, Claggett B, Pfeffer MA, Diaz R, Dickstein K, Gerstein HC, Lawson FC, Lewis EF, Maggioni AP, McMurray JJV, Probstfield JL, Riddle MC, Solomon SD, Tardif JC, Køber L. Role of B-Type Natriuretic Peptide and N-Terminal Prohormone BNP as Predictors of Cardiovascular Morbidity and Mortality in Patients With a Recent Coronary Event and Type 2 Diabetes Mellitus. Journal of the American Heart Association. 2017;6(6):e004743.

236. Poret JM, Battle C, Mouton AJ, Gaudet DA, Souza-Smith F, Gardner JD, Braymer HD, Harrison-Bernard L, Primeaux SD. The prevalence of cardio-metabolic risk factors is differentially elevated in obesity-prone Osborne-Mendel and obesity-resistant S5B/Pl rats. Life Sciences. 2019;223:95-101.

237. Al-Kouh A, Babiker F, Al-Bader M. Renin-Angiotensin System Antagonism Protects the Diabetic Heart from Ischemia/Reperfusion Injury in Variable Hyperglycemia Duration Settings by a Glucose Transporter Type 4-Mediated Pathway. Pharmaceuticals (Basel). 2023;16(2).

238. Bayir Y, Cadirci E, Suleyman H, Halici Z, Keles MS. Effects of Lacidipine, Ramipril and Valsartan on Serum BNP Levels in Acute and Chronic Periods Following Isoproterenol-Induced Myocardial Infarction in Rats. Eurasian J Med.2009;41(1):44-48.

239. Li X, Li Z, Li B, Zhu X, Lai X. Klotho improves diabetic cardiomyopathy by suppressing the NLRP3 inflammasome pathway. Life Sci. 2019;234:116773.

240. Fu Y, Cao J, Wei X, Ge Y, Su Z, Yu D. Klotho alleviates contrast-induced acute kidney injury by suppressing oxidative stress, inflammation, and NF-KappaB/NLRP3-mediated pyroptosis. Int Immunopharmacol. 2023;118:110105.

241. Keles N, Dogan B, Kalcik M, Caliskan M, Keles NN, Aksu F, Bulut M, Kostek O, Isbilen B, Yilmaz Y, Oguz A. Is serum Klotho protective against atherosclerosis in patients with type 1 diabetes mellitus? J Diabetes Complications. 2016;30(1):126-132.

242. Lin Y, Sun Z. In Vivo Pancreatic β-Cell–Specific Expression of Antiaging Gene Klotho: A Novel Approach for Preserving β-Cells in Type 2 Diabetes. Diabetes. 2014;64(4):1444-1458.

243. Silva AP, Mendes F, Carias E, Gonçalves RB, Fragoso A, Dias C, Tavares N, Café HM, Santos N, Rato F, Leão Neves P, Almeida E. Plasmatic Klotho and FGF23 Levels as Biomarkers of CKD-Associated Cardiac Disease in Type 2 Diabetic Patients. Int J Mol Sci. 2019;20(7).

244. Chen K, Zhou X, Sun Z. Haplodeficiency of Klotho Gene Causes Arterial Stiffening via Upregulation of Scleraxis Expression and Induction of Autophagy. Hypertension. 2015;66(5):1006-1013.
245. Lai L, Cheng P, Yan M, Gu Y, Xue J. Aldosterone induces renal fibrosis by promoting HDAC1 expression, deacetylating H3K9 and inhibiting klotho transcription. Mol Med Rep. 2019;19(3):1803-1808.
246. Armstrong AC, Ambale-Venkatesh B, Turkbey E, Donekal S, Chamera E, Backlund J-Y, Cleary P, Lachin J, Bluemke DA, Lima JA. Association of Cardiovascular Risk Factors and Myocardial Fibrosis With Early Cardiac Dysfunction in Type 1 Diabetes: The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study. Diabetes Care. 2016;40(3):405-411.
247. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. Pharmacol Rev. 2000;52(1):11-34.
248. Liu W, Gong W, He M, Liu Y, Yang Y, Wang M, Wu M, Guo S, Yu Y, Wang X, Sun F, Li Y, Zhou L, Qin S, Zhang Z. Spironolactone protects against diabetic cardiomyopathy in streptozotocin-induced diabetic rats. Journal of diabetes research.2018.
249. Wang L, Li J, Li D. Losartan reduces myocardial interstitial fibrosis in diabetic cardiomyopathy rats by inhibiting JAK/STAT signaling pathway. Int J Clin Exp Pathol. 2015;8(1):466-473.
250. Mann DL. Innate immunity and the failing heart: the cytokine hypothesis revisited. Circ Res. 2015;116(7):1254-1268.
251. Kanda T, Takahashi T. Interleukin-6 and cardiovascular diseases. Jpn Heart J. 2004;45(2):183-193.
252. Dinh W, Füth R, Nickl W, Krahn T, Ellinghaus P, Scheffold T, Bansemir L, Bufe A, Barroso MC, Lankisch M. Elevated plasma levels of TNF-alpha and interleukin-6 in patients with diastolic dysfunction and glucose metabolism disorders. Cardiovasc Diabetol. 2009;8:58.
253. Liu Q, Dong S, Zhou X, Zhao Y, Dong B, Shen J, Yang K, Li L, Zhu D. Effects of Long-Term Intervention with Losartan, Aspirin and Atorvastatin on Vascular Remodeling in Juvenile Spontaneously Hypertensive Rats. Molecules. 2023;28(4).

254. Braile M, Marcella S, Cristinziano L, Galdiero MR, Modestino L, Ferrara AL, Varricchi G, Marone G, Loffredo S. VEGF-A in Cardiomyocytes and Heart Diseases. Int J Mol Sci. 2020;21(15).

255. Zhang Y, Nakano D, Guan Y, Hitomi H, Uemura A, Masaki T, Kobara H, Sugaya T, Nishiyama A. A sodium-glucose cotransporter 2 inhibitor attenuates renal capillary injury and fibrosis by a vascular endothelial growth factor–dependent pathway after renal injury in mice. Kidney International. 2018;94(3):524-535.

256. Sivaskandarajah GA, Jeansson M, Maezawa Y, Eremina V, Baelde HJ, Quaggin SE. Vegfa protects the glomerular microvasculature in diabetes. Diabetes. 2012;61(11):2958-2966.

257. Karpanen T, Bry M, Ollila HM, Seppänen-Laakso T, Liimatta E, Leskinen H, Kivelä R, Helkamaa T, Merentie M, Jeltsch M, Paavonen K, Andersson LC, Mervaala E, Hassinen IE, Ylä-Herttuala S, Oresic M, Alitalo K. Overexpression of vascular endothelial growth factor-B in mouse heart alters cardiac lipid metabolism and induces myocardial hypertrophy. Circ Res. 2008;103(9):1018-1026.

258. Sasso FC, Torella D, Carbonara O, Ellison GM, Torella M, Scardone M, Marra C, Nasti R, Marfella R, Cozzolino D, Indolfi C, Cotrufo M, Torella R, Salvatore T. Increased Vascular Endothelial Growth Factor Expression But Impaired Vascular Endothelial Growth Factor Receptor Signaling in the Myocardium of Type 2 Diabetic Patients With Chronic Coronary Heart Disease. Journal of the American College of Cardiology. 2005;46(5):827-834.

259. Daugaard M, Rohde M, Jäättelä M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Letters. 2007;581(19):3702-3710.

260. de Lemos Muller CH, Schroeder HT, Rodrigues-Krause J, Krause M. Extra and intra cellular HSP70 levels in adults with and without metabolic disorders: a systematic review and meta-analysis. Cell Stress Chaperones. 2023;28(6):761-771.

261. Tang D, Kang R, Xiao W, Wang H, Calderwood SK, Xiao X. The Anti-inflammatory Effects of Heat Shock Protein 72 Involve Inhibition of High-Mobility-Group Box 1 Release and Proinflammatory Function in Macrophages1. The Journal of Immunology. 2007;179(2):1236-1244.

262. Ferat-Osorio E, Sánchez-Anaya A, Gutiérrez-Mendoza M, Boscó-Gárate I, Wong-Baeza I, Pastelin-Palacios R, Pedraza-Alva G, Bonifaz LC, Cortés-Reynosa P, Pérez-Salazar E, Arriaga-Pizano L, López-Macías C, Rosenstein Y, Isibasi A. Heat shock protein 70 down-regulates the production of toll-like receptor-induced pro-inflammatory cytokines by a heat shock factor-1/constitutive heat shock element-binding factor-dependent mechanism. Journal of Inflammation. 2014;11(1):19.

263. Vargas R, Rincón J, Pedreañez A, Viera N, Hernández-Fonseca JP, Peña C, Mosquera J. Role of angiotensin II in the brain inflammatory events during experimental diabetes in rats. Brain research. 2012;1453:64-76.

264. Gebre A, Altaye B, Atey T, Tuem K, Berhe D. Targeting renin–angiotensin system against Alzheimer's disease. Frontiers in pharmacology. 2018;9:440.

265. Dinh QN, Young MJ, Evans MA, Drummond GR, Sobey CG, Chrissobolis S. Aldosterone-induced oxidative stress and inflammation in the brain are mediated by the endothelial cell mineralocorticoid receptor. Brain Res. 2016;1637:146-153.

266. Nasr SJ, Crayton JW, Agarwal B, Wendt B, Kora R. Lower frequency of antidepressant use in patients on renin-angiotensin-aldosterone system modifying medications. Cell Mol Neurobiol. 2011;31(4):615-618.

267. Ahola AJ, Harjutsalo V, Forsblom C, Groop PH. Renin-angiotensin-aldosterone-blockade is associated with decreased use of antidepressant therapy in patients with type 1 diabetes and diabetic nephropathy. Acta Diabetol. 2014;51(4):529-533.

268. Silverman MN, Pearce BD, Biron CA, Miller AH. Immune modulation of the hypothalamic-pituitary-adrenal axis during viral infection.ViralImmunol.2005;18:41-78.

269. Song C. The effect of thymectomy and IL-1 on memory: implications for the relationship between immunity and depression. BrainBehavImmun.2002;16(5):557-568.

270. Felger JC, Lotrich FE. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience. 2013;246:199-229.

271. Zhang J-c, Wu J, Fujita Y, Yao W, Ren Q, Yang C, Li SX, Shirayama Y, Hashimoto K. Antidepressant Effects of TrkB Ligands on Depression-Like Behavior and Dendritic Changes in Mice After Inflammation. International Journal of Neuropsychopharmacology. 2015;18(4).

272. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nature Reviews Neuroscience. 2005;6(8):603-614.

9. Bibliography of the candidate's publications

Research articles related to the theme of the PhD thesis

Gellai R, Hodrea J, Lenart L, Hosszu A, Koszegi S, Balogh D, Ver A, Banki NF, Fulop N, **Molnar A**, Wagner L, Vannay A, Szabo AJ, Fekete A. Role of O-linked N-acetylglucosamine modification in diabetic nephropathy. Am J Physiol Renal Physiol. 2016;311(6):F1172-f1181. **IF=3.611**

Koszegi S[*], **Molnar A**[*], Lenart L, Hodrea J, Balogh DB, Lakat T, Szkibinszkij E, Hosszu A, Sparding N, Genovese F, Wagner L, Vannay A, Szabo AJ, Fekete A. RAAS inhibitors directly reduce diabetes-induced renal fibrosis via growth factor inhibition. J Physiol. 2019;597(1):193-209. **IF=4.547**

Balogh DB, **Molnar A**, Hosszu A, Lakat T, Hodrea J, Szabo AJ, Lenart L, Fekete A. Antidepressant effect in diabetes-associated depression: A novel potential of RAAS inhibition. Psychoneuroendocrinology. 2020;118:104705. **IF=4.9**

Molnar A[*], Lakat T[*], Hosszu A, Szebeni B, Balogh A, Orfi L, Szabo AJ, Fekete A, Hodrea J. Lyophilization and homogenization of biological samples improves reproducibility and reduces standard deviation in molecular biology techniques. Amino Acids. 2021;53(6):917-928. **IF=3,789**

Balogh DB[*], **Molnar A**[*], Degi A, Toth A, Lenart L, Saeed A, Barczi A, Szabo AJ, Wagner LJ, Reusz G, Fekete A. Cardioprotective and Antifibrotic Effects of Low-Dose Renin-Angiotensin-Aldosterone System Inhibitors in Type 1 Diabetic Rat Model. Int J Mol Sci. 2023;24(23). **IF=4.9**

Other publications

Hodrea J, Saeed A, **Molnar A**, Fintha A, Barczi A, Wagner LJ, Szabo AJ, Fekete A, Balogh DB. SGLT2 inhibitor dapagliflozin prevents atherosclerotic and cardiac complications in experimental type 1 diabetes. PLoS One. 2022;17(2):e0263285. **IF=3.7**

10. Acknowledgements

I want to express my deep sense of gratitude to my supervisor, Professor Andrea Fekete, for her invaluable patience and time for shaping my research approach. Her tireless dedication and guidance are vital to our research group. I am grateful for the fantastic opportunities and continuous support I received throughout and after my PhD study, and even before as a student researcher.

I wish to express my thankfulness to Professor Attila Szabó for the privilege to carry out my PhD research within the framework provided by the Research Laboratory and further work as a physician at the Pediatric Centre, Semmelweis University.

I am also profoundly grateful to the post-docs of our group, Dora Balogh, Judit Hodrea, Lilla Lenart, and Adam Hosszu, for polishing my research skills. Without their valuable experience and insightful suggestions, which helped me throughout the experiments, it would not have been possible to conduct this research. I learned a lot from their critical advice while improving my writing skills as well.

I am sincerely thankful to all current and previous members of the MTA-SE Lendület "Momentum" Diabetes Research Group for the motivating attitude, selfless support and professional help. Special thanks to Tamas Lakat for all the collaboration and his immense help in figure design. I am grateful to Renata Gellai, Sandor Koszegi, Edgar Szkibinszkij and Adrienn Barczi for their teaching and assistance in the beginning of my studies. I would like to thank our Danish collaborators Nadja Sparding and Federica Genovese for this fruitful international experience. I need to gratefully mention Laszlo Wagner for his clinically relevant comments, Adar Saeed for her help with the rat heart experiments, and Maria Bernath for helping with the *in vitro* experiments and laboratory work. I was privileged to have the helpful insights of the following colleagues as well: Adam Vannay, Beata Szebeni, Apor Veres-Szekely, Domonkos Pap, Istvan Takacs. I am also extremely grateful to Akos Toth, Eszter Levai and Csenge Pajtok for creating a friendly atmosphere.

Above all special thanks to my whole family for their enormous support and love I received throughout these years. Without their constant help and endless encouragement this work could not have been completed. I am blessed to have you all.