PLEIOTROPIC EFFECTS OF SGLT2 INHIBITORS IN DIABETES: FOCUSING ON THE KIDNEY

Introduction:

Diabetic kidney disease (DKD) remained the leading cause of chronic kidney disease, while cardiovascular complications are the main reason of death in DM patients worldwide. In DM renin-angiotensin-aldosterone system (**RAAS**) **inhibitors are the gold-standard therapy in diabetic patients for long with renal impairment or hypertension**. However, the activation of RAAS is still only one part in the puzzle of the complex; the direct toxic vascular and parenchymal effect of hyperglycaemia, is certainly major primary factor contributing to the renal, and cardiovascular damage. Previously we published that **hyperglycaemia induces protein O-linked N-Acetylglucosamine modification (O-GlcNAcylation),** contributing to the progression of diabetic complications.

Sodium-glucose co-transporter 2 inhibitors (SGLT2i) (gliflozins) are a novel and very promising class of drugs in the treatment of DM; that lowers blood glucose level by increasing urinary glucose excretion. More importantly gliflozins provide additional beneficial reductions in DM-associated all-cause mortality. **Dapagliflozine (DAPA) is a prominent member of SGLT2i** that has already been used worldwide. Gliflozins open new avenues for oral therapies in patients with T2DM, however it is still an open question whether **SGLT2i might have renoprotective and cardioprotective properties in T1DM**. It has to be also clarified whether these pleiotropic effects are direct consequences of SGLT inhibition or are a result of the improvement in hyperglycaemia. Our proposal aimed to address these questions.

Here we investigated the pleiotropic effects of the SGLT2 inhibitor DAPA in monotherapy or as add-on treatment to the angiotensin II receptor antagonist losartan (LOS) in a model of streptozotocin (STZ) induced T1DM.

Specific aims were:

1. ***to evaluate the effect of DAPA on glucose metabolism in a T1DM rat model***
2. ***to assess the effect of SGLT2 inhibition on the evolution of DKD***
3. ***to compare the aforementioned effects of DAPA monotherapy with a combination therapy of DAPA+ LOS***
4. ***to investigate the possible cardioprotective effect of DAPA***
5. ***to discover the underlying molecular mechanisms of these pleiotropic effects in vitro and in vivo***

Results:

1. *To evaluate the effect of DAPA on glucose metabolism in a T1DM rat model*

As expected, DAPA improved metabolic features of STZ-induced T1DM, such as impaired weight gain, high levels of blood glucose, fructosamine, and serum lipids. Blood glucose level reached a decline of 41% by the second wk and by the end of the experiment, it was 47% lower in DAPA versus the diabetic group. No severe hypoglycaemic episode was detected (*Table 1*).

Metabolic parameters	Control (C)	Diabetic (D)	D+DAPA
Body weight (g)	442±35.4	256±29.9***	340±35.0§§
Glucose (mmol/L)	6.42±0.58	33.3±1.06***	16.2±5.29***§§§
Fructosamine (µmol/L)	143±3.74	277±12.3***	198±34.9§§§
Total cholesterol (mmol/L)	1.96±0.15	2.82±0.30***	1.89±0.40§§§
Triglycerides (mmol/L)	1.24±0.51	3.12±1.17**	1.00±0.51§§§
LDL-C (mmol/L)	0.44±0.15	0.84±0.11***	0.48±0.13§§§
GOT (U/L)	127±19.6	382±164***	187±24.1§§
GPT (U/L)	43.0±8.39	181±82.1***	80.1±16.1§§
Glucosuria	UN	346±47.1***	491±94.2***§§

Table 1. C, Control; D, diabetic; D+DAPA, dapagliflozin-treated diabetic; LDL-C, low-density lipoprotein cholesterol; GOT, serum glutamate-oxaloacetate transaminase; GPT, serum glutamate-pyruvate transaminase;*p* values indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n = 6 /group). **p<0.01 *vs*. Control, ***p<0.001 *vs.* Control, §§p<0.01 *vs.* Diabetic, §§§p<0.001 *vs*. Diabetic UN: undetectable

Aim: 2.-3. To assess the effect of SGLT2 inhibition on the evolution of DKD and to compare the effects of monotherapy with a combination therapy of DAPA+LOS

Protein levels of SGLT2 and GLUT2 were upregulated in diabetic kidneys. This was not unexpected in light of multiple studies showing increased renal SGLT2 expression in various diabetic rodent models. DAPA treatment minimized protein levels of both glucose transporters to control levels (*Fig.1*.)

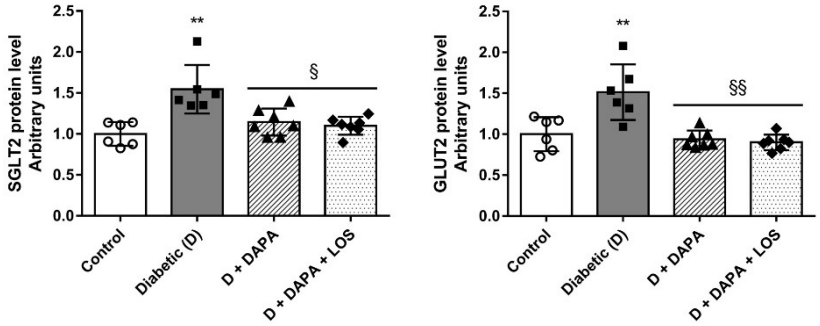

Figure 1. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test or by Kruskal-Wallis test with Dunn correction (n=6 in control and diabetic and n=7 in treatment groups). **p<0.01 vs. Control, §p<0.05 vs. Diabetic, §§p<0.01 vs. Diabetic

Development of DKD was confirmed by the decline of renal function after 6 weeks of diabetes. Creatinine clearance decreased, while serum creatinine, BUN and albumin excretion elevated in diabetic rats. DAPA markedly improved creatinine clearance, serum creatinine, BUN and albumin excretion (*Fig 2 A-D*).

Creatinine and BUN are suboptimal markers of renal injury, therefore various specific biomarkers of renal damage (KIM-1, NGAL, Cystatin-C, AST) were also determined in the serum, urine and some in the kidney. Urinary excretion of KIM-1 and NGAL were elevated in the diabetic group vs. controls, while DAPA decreased their levels by more than 50% indicating milder tubular damage. In parallel, *Havcr1* (KIM-1) and *Lcn2* (NGAL) mRNA expressions were increased in the diabetic kidney as well (*data not shown*). Furthermore, creatinine clearance correlated with urinary KIM-1 and urinary NGAL. LOS had no further protective effect (*Fig 2 E-H)*.

Histological changes were consistent with functional deterioration. PAS-stained sections revealed massive hypertrophy, mesangial matrix expansion and basal membrane thickening in the glomeruli of diabetic rats. DAPA minimized mesangial matrix expansion and ameliorated structural damage as reflected by smaller PAS positive glomerular areas (*Fig 2 I)*.

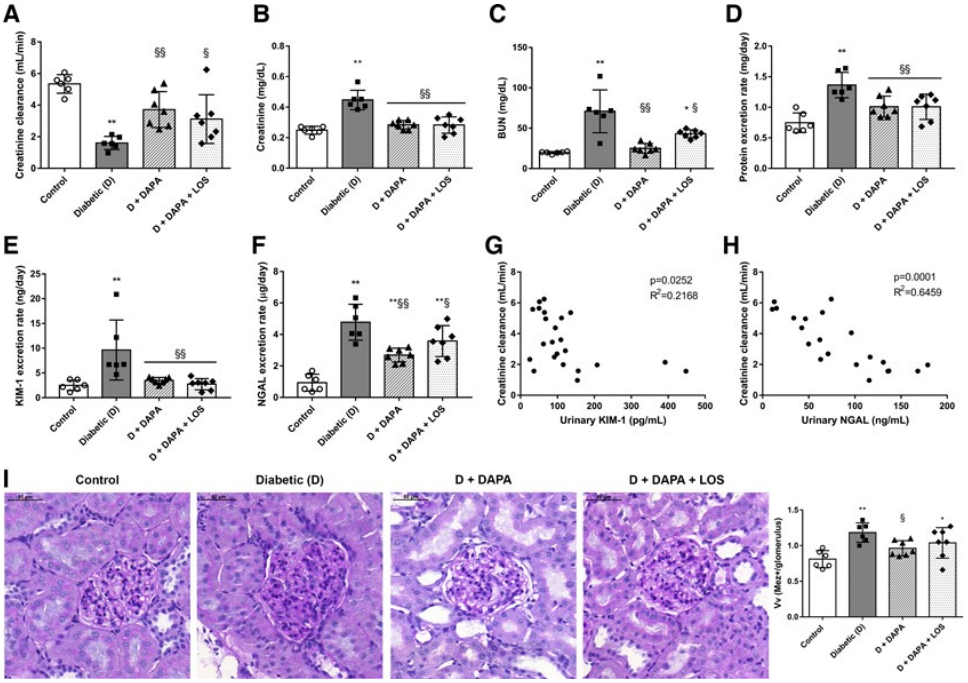

Figure 2. (A) Creatinine clearance, (B) serum creatinine, (C) blood urea nitrogen (BUN) and (D) albumin excretion and (E) urinary levels of kidney injury molecule-1 (KIM-1), (F) urinary levels of neutrophil gelatinase-associated lipocalin (NGAL). (E,F) Scatter plot illustrating the correlation between creatinine clearance and KIM-1 or NGAL, resp. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n=6 in control and diabetic and n=7 in treatment groups). *p<0.05 *vs.* Control, **p<0.01 *vs.* Control, §p<0.05 *vs.* Diabetic, §§p<0.01 *vs.* Diabetic

Our collaborator partner, Nordic Bioscience develops novel urinary biomarkers of ECM remodeling, which are promising in early diagnosis and prognosis of renal fibrosis and might replace the invasive renal biopsy. In our experiment, collagen III formation (rPRO-C3), MMP-9-mediated degradation of type III collagen (uC3M) and type IV collagen (TUM) were elevated in diabetic rats. DAPA treatment decreased rPRO-C3 and TUM levels, while uC3M remained unchanged (*Fig. 3)*.

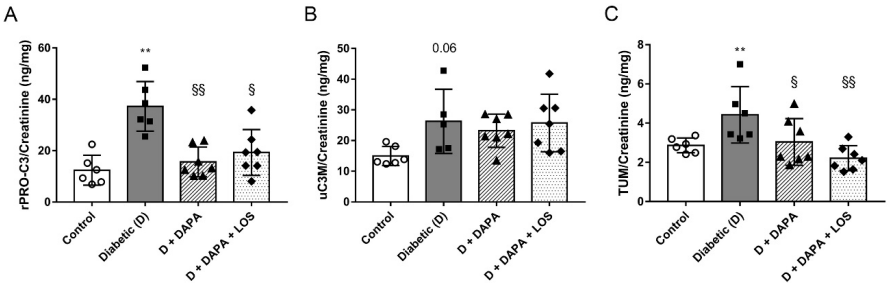

Figure 3. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n=6 in control and diabetic and n=7 in treatment groups). **p<0.01 *vs.* Control, §p<0.05 *vs.* Diabetic, §§p<0.01 *vs.* Diabetic

Weak collagen staining was detected in glomeruli and around blood vessels in control kidneys. Extensive fibrotic tissue accumulation was observed in diabetic kidneys, as shown by collagen deposition in the interstitium. DM-induced collagen deposition was lower in the DAPA group compared with the diabetic group, whereas DAPA + LOS treatment had no further effect. Considerable fibronectin-positive staining was detected in the glomeruli and to a lesser extent in the tubulointerstitium of diabetic kidneys, which was attenuated by both treatments. In parallel renal fibronectin gene expression decreased by 50% in DAPA-treated rats. The DAPA + LOS combination had no additional effect on the decrement *(Fig.4)*.

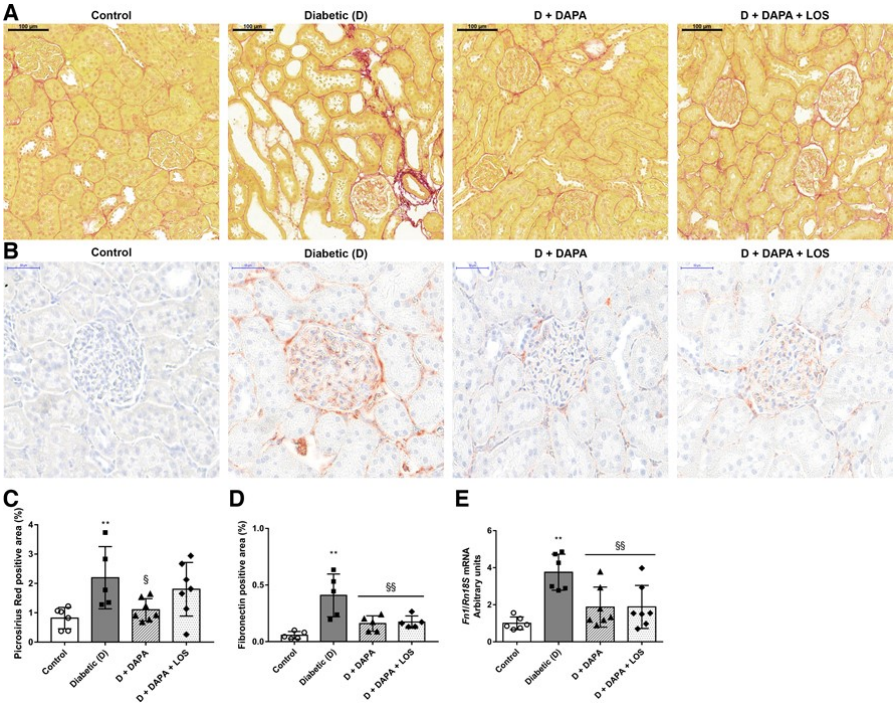

Figure 4. *A*: Representative Sirius red-stained kidney sections of control, diabetic (D), dapagliflozin (D + DAPA)-, and DAPA + losartan (D + DAPA + LOS)-treated diabetic rats. Original magnification: ×200. Scale bar = 100 µm. B: representative fibronectin-stained kidney sections of control, D, D + DAPA, and D + DAPA + LOS rats. Original magnification: ×400. Scale bar = 50 µm. C: percentage of the picrosirius red-positive stained area. D: percentage of the fibronectin-positive stained area. E: renal mRNA expression of fibronectin 1 (Fn1) was normalized to 18S ribosomal RNA (Rn18S) mRNA expression. Bars indicate means ± SD, and data were analyzed by one-way ANOVA with a Holm-Sidak multiple-comparisons test (n = 6 in control and diabetic groups and n = 7 in treatment groups). **P < 0.01 vs. the control group; §P < 0.05 vs. the diabetic group; §§P < 0.01 vs. the diabetic group.

Aim 4: To investigate the possible cardioprotective antifibrotic effects of DAPA

The proof of cardiovascular safety for new glucose-lowering therapies has been required by US FDA since 2008. SGLT2i are showing reduction in the relative risk of heart failure in T2DM, and recently in non-diabetic heart failure patients suggesting that cardioprotection does not only relate to their antihyperglycemic action. Despite the encouraging results, the underlying molecular mechanisms remain incompletely understood. Furthermore, the literary data is scarce about the effect of SGLT2i in T1DM.

After 6 weeks of DM, MAP remained unaltered in all groups. In line with the literature STZ-induced DM was associated with reduction in heart rate, which was reversed by DAPA treatment.

Cardiac hypertrophy developed in diabetic rats as indicated by increased heart to body weight ratio. DAPA treatment prevented cardiac hypertrophy (*Table 2*). Since addition of LOS has no further positive effect data are not further shown in this summary.

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

Table 2. B Values indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n = 6/group). ***p<0.001 *vs.* Control,

Aortic intima media thickening (IMT) is an early marker of atherosclerosis. Changes in aortic IMT occur at early stages of T1DM and these become more severe with the duration of the disease. Histological examination of aortic IMT showed prominent wavy internal elastic lamina in control rats. Aorta of diabetic rats showed intimal thickening, irregularity and diffused elastic membranes, which was prevented by DAPA. In parallel we showed that increased serum level of myocardial hypertrophy marker ANP, as well as the biomarker of chronic left ventricular dysfuntion BNP was diminished by DAPA. mRNA expressions of *Nppa* (ANP) and *Nppb* (BNP) measured in the left ventricle were also less elevated in DAPA- treated group (*Fig.5*).

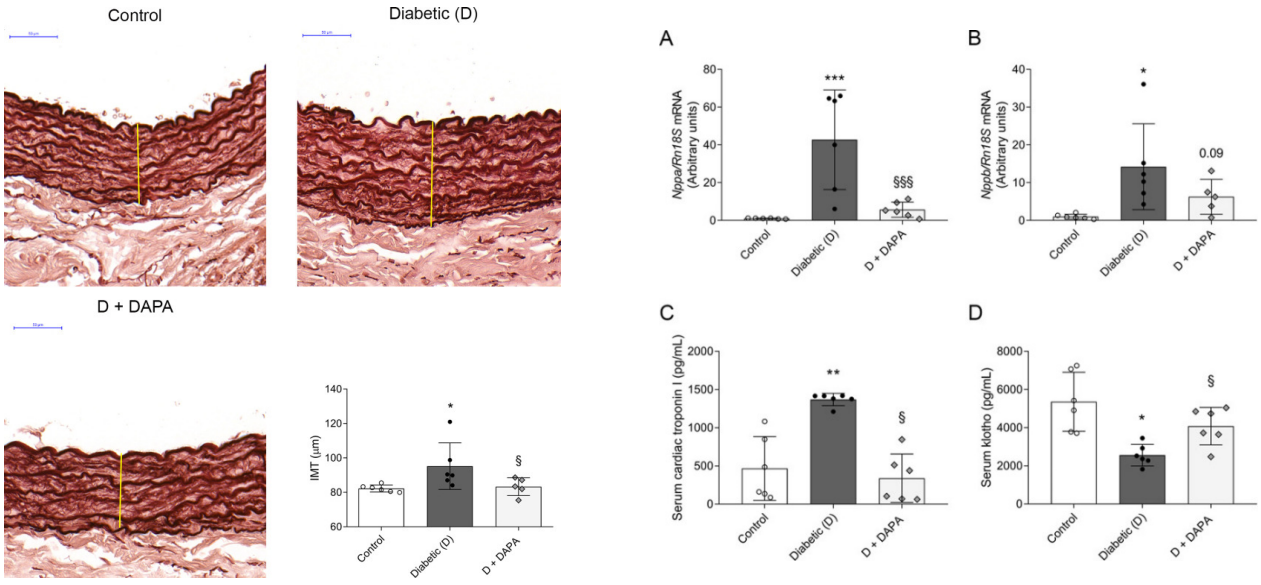

Figure 5. Left Panel: Representative orcein stained aorta sections and quantitative evaluation of intima-media thickness. Original magnification, x200. Scale bar, 50 μm. Elastic fibers are stained brown after orcein staining. They are visualized as either thin fibers or elastic lamella. Right panel: mRNA expression of (A) atrial natriuretic peptide (*Nppa*) and (B) B-type natriuretic peptide (*Nppb*). mRNA expressions were normalized to *Rn18S* mRNA expression. (C) Serum levels of cardiac troponin I. (D) Serum levels of klotho. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test or Kruskal-Wallis with Dunn comparison test (n = 6/group). **p<0.01 *vs*. Control, ***p<0.001 *vs*. Control, §p<0.05 *vs*. D §§§p<0.001 *vs*. Diabetic.

DM is associated with induction of pro-fibrotic genes and interstitial cardiac fibrosis. Diabetic rats had an increased left ventricular mRNA expression of profibrotic markers. DAPA treatment diminished the elevation of *Tgfb1*, *Ctgf* and *Serpine1*. In parallel, myofibroblast marker α-SMA protein levels were upregulated in the diabetic group, which was lowered by DAPA. DM-induced higher fibronectin (*Fn)* expression was suspended by DAPA indicating milder myocardial remodeling and fibrogenesis. Diabetes-induced intramyocardial collagen deposition was reduced almost to control levels in the DAPA-treated group confirming its protective role in decreasing cardiac fibrosis (*Fig 6)*.

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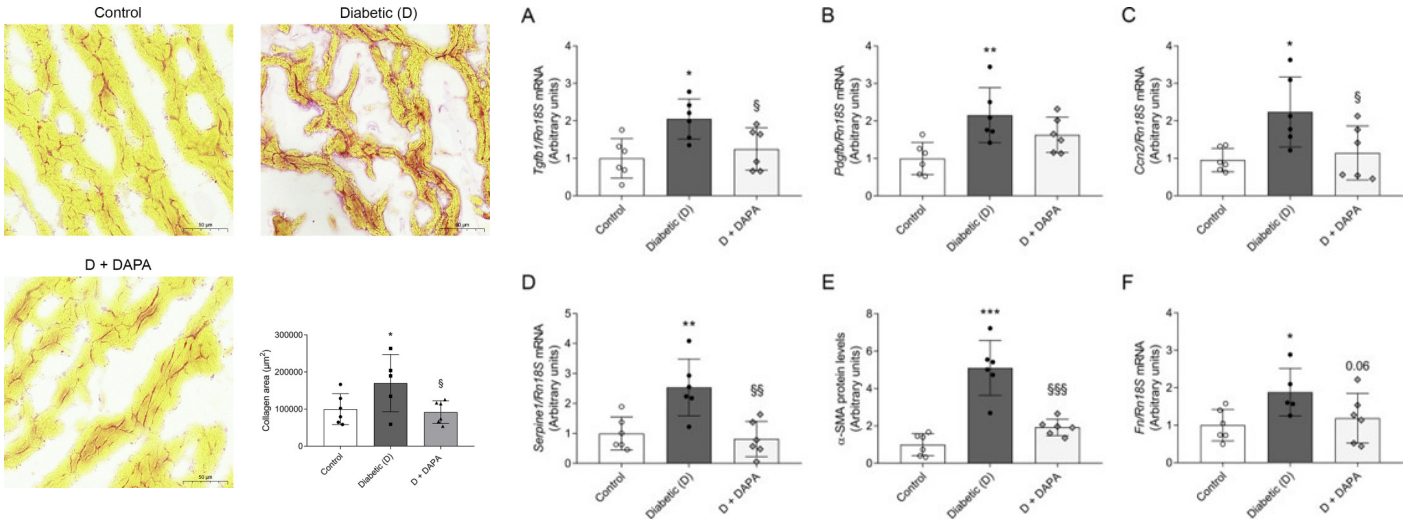

Figure 6. Left panel: Representative picrosirius red stained heart sections and quantitative evaluation of fibrosis. The red-stained area was measured based on the red colour intensity. The myocardial field of measurement was manually selected with the caution of avoiding the endocardium and perivascular connective tissue. Original magnification, x400. Scale bar, 50 μm **Right panel**:.mRNA expression of (A) transforming growth factor β (Tgfb1), (B) platelet-derived growth factor (Pdgfb), and (C) connective tissue growth factor (Ccn2) of control, diabetic and dapagliflozin treated diabetic rats (D+DAPA). (D) mRNA expression of Serpine1 (also known as plasminogen activator inhibitor-1). (E) Protein level of α-smooth muscle actin (α-SMA). Proteins were normalized to total protein Ponceau S staining as loading control. (F) mRNA expression of fibronectin (Fn). mRNA expressions were normalized to Rn18S mRNA expression. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n = 6/group) *p<0.05 vs. Control, **p<0.01 vs. Control, ***p<0.001 vs. Control, §p<0.05 vs. Diabetic, §§p<0.01 vs. Diabetic, §§§p<0.001 vs. Diabetic.

Aim 5: to discover the underlying molecular mechanisms of these pleiotropic effects in vitro and in vivo

Several various pathogenic pathways have been implicated in the pathogenesis of diabetes induced fibrosis. In our studies we investigated three main mechanisms, like (1) chronic inflammation, (2) hypoxia and (3) increased O-GlcNAcylation. Due to limited space only part of our results will be discussed here.

Inflammation: Pro-inflammatory cytokines (e.g. TNF-α, IL-1β) cause cardiac myocyte hypertrophy, contractile dysfunction, left ventricule dilatation and modulate the interstitial matrix of the heart. Increased serum TNF-α and IL-6 levels of diabetic patients are associated with diastolic dysfunction. In our experiment, elevation of *Il1b*, *Il6*, *Tnf* and *Ccl2* in the left ventricle indicate the activation of inflammatory signaling pathways, similarly to other T1DM models. We showed that DM-induced cardiac inflammation is diminished by DAPA supporting the anti-inflammatory role of SGLT2i as a possible mechanism of action in the prevention of heart failure (*Fig 7*).

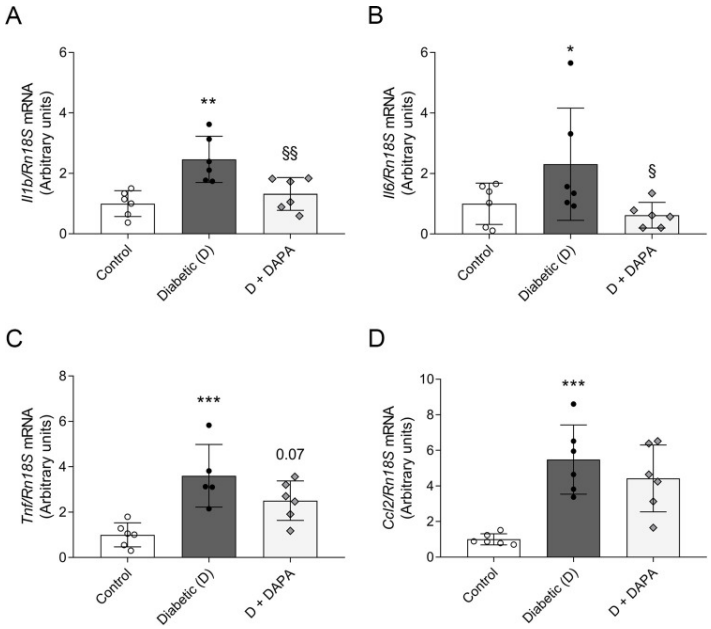

Figure 7. mRNA expression of (A) interleukin-1β (*Il1b*), (B) interleukin-6 (*Il6*), (C) tumor necrosis factor (*Tnf*), and (D) monocyte chemoattractant protein 1 (*Ccl2*) of control, diabetic and dapagliflozin treated diabetic rats (D+DAPA). mRNA expressions were normalized to *Rn18S* mRNA expression. Bars indicate means±SDs and data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (n = 6/group) *p<0.05 *vs.* Control, **p<0.01 *vs.* Control, ***p<0.001 *vs.* Control, §p<0.05 *vs.* Diabetic, §§p<0.01 *vs.* Diabetic.§p<0.05 vs. Diabetic, §§p<0.01 vs. Diabetic.

Hypoxia: Hypoxia is a major driver of disease progression in DM leading to fibrosis of the target organs. A hypoxic chamber was used to investigate the effect of DAPA independent of its antihyperglycemic property. Hypoxic injury was confirmed by increased HIF-1α mRNA expression and protein level. DAPA suspended HIF-1α elevation, indicating a milder hypoxic injury. Moreover, the treatment prevented HIF-1α translocation to the nucleus, thereby confirming abolished HIF-1α upregulation. In various cell types, hypoxia increases CTGF and PDGFB expression in a HIF-1α-dependent way. Here, we showed that DAPA decreases hypoxia-induced CTGF and PDGF production, suggesting that its antifibrotic effects might be in direct connection with diminished hypoxia (*Fig. 8*).

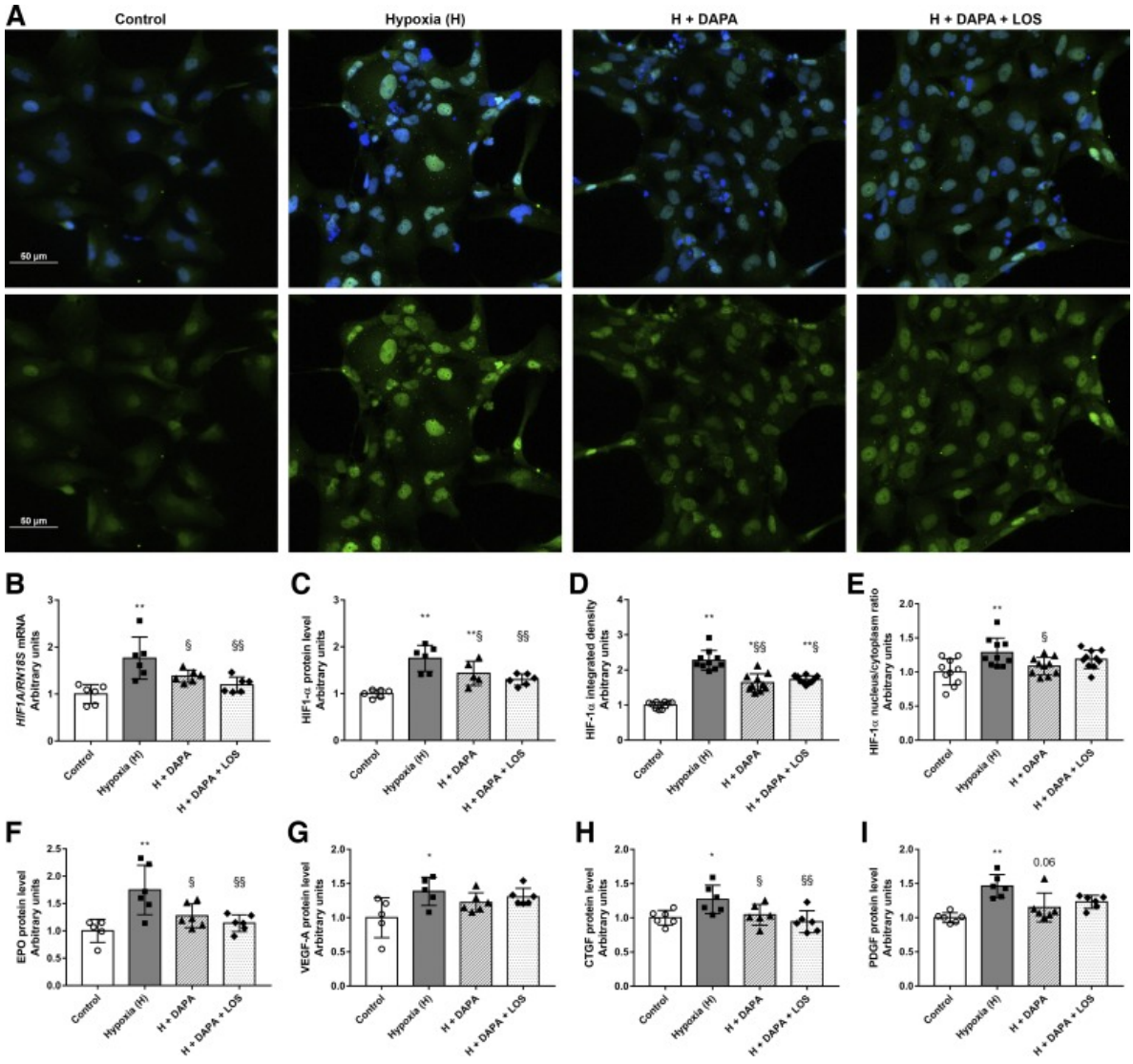

Figure 8. Dapagliflozin (DAPA) treatment minimizes tubular hypoxia. *A*: hypoxia-inducible factor-1α (HIF-1α) immunocytochemistry (green, HIF-1α; blue, nucleus; ×20 objective; scale bar = 50 µm). *B–I*: HIF1A mRNA expression (*B*), HIF-1α protein level (*C*), HIF-1α integrated density (*D*), HIF-1α nucleus-to-cytosol ratio (*E*), and protein levels of erythropoietin (EPO; *F*), VEGF-A (*G*), connective tissue growth factor (CTGF; *H*), and platelet-derived growth factor (PDGF; *I*) in human proximal tubular cells (HK-2 cells). HIF1A was normalized to 18S ribosomal RNA (RN18S) mRNA expression. Proteins were normalized to total protein Ponceau S staining as a loading control. Proximal tubular cells were treated with DAPA or DAPA + losartan (LOS) for 22 h before 2 h hypoxia (1% O_2). Bars indicate means ± SD, and data were analyzed by one-way ANOVA with a Holm-Sidak multiple-comparisons test or Kruskal-Wallis with Dunn comparison test (n = 5–6 per group). *P < 0.05 vs. the control group; **P < 0.01 vs. the control group; §P < 0.05 vs. hypoxia; §§P < 0.01 vs. hypoxia.

***O*-GlcNAcylation**: We have previously shown that, in diabetic rats protein *O*-GlcNAcylation is icreased. Because DAPA blocks glucose uptake in proximal tubular cells, we hypothesized that DAPA modifies protein *O*-GlcNAcylation, thus affecting fibrotic processes. DAPA minimized elevated protein *O*-GlcNAcylation and reduced OGT levels in HK-2 cells and in the kidney as well. However, OGA, which is responsible for removing *O*-GlcNAc residue, remained unchanged, suggesting that DAPA inhibits the addition of *O*-GlcNAc rather than facilitates its removal. In parallel, whereas all profibrotic growth factors were upregulated under hyperglycemic conditions, only CTGF was reduced by DAPA. Surprisingly, TGFB1 and PDGFB were not affected (*Fig. 9*).

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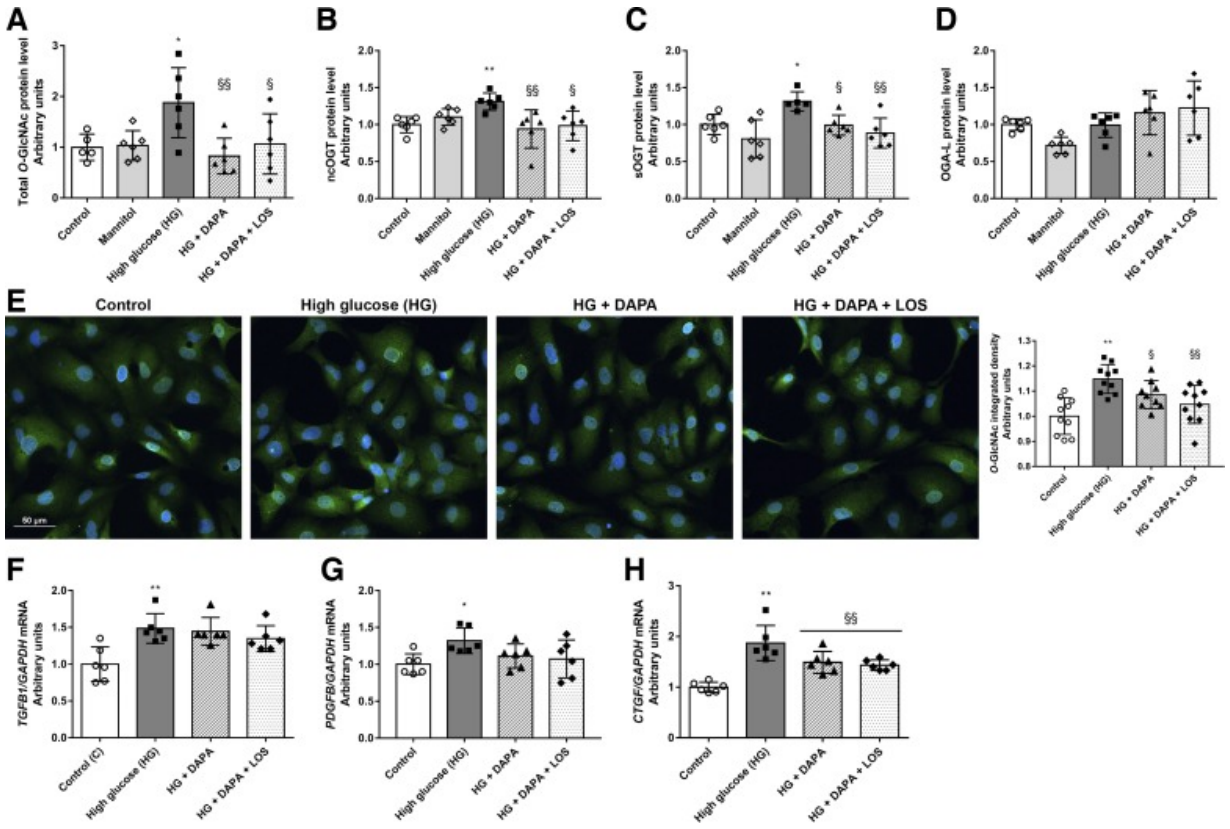

Figure 9. DAPA reduces hyperglycemia-induced protein *O*-GlcNAcylation in human proximal tubular cells. Proximal tubular cells were incubated with normal glucose (5.5 mM), high mannitol (35 mM), or high glucose (HG; 35 mM) for 24 h. *A–D*: total protein levels of *O*-GlcNAcylation, nucleocytoplasmic *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase (ncOGT), small *O*-GlcNAc transferase (sOGT), and *O*-GlcNAcase (OGA-L). Proteins were normalized to total protein Ponceau S staining as a loading control. *E*: representative immunocytochemistry staining and integrated density of *O*-GlcNAc (green, *O*-GlcNAc; blue, nucleus; ×20 objective; scale bar = 50 µm). *F–H*: mRNA expression of transforming growth factor β (TGFB1), platelet-derived growth factor-B (PDGFB), and connective tissue growth factor (CTGF), which were normalized to GAPDH mRNA expression. Bars indicate means ± SD, and data were analyzed by one-way ANOVA with a Holm-Sidak multiple-comparisons test or Kruskal-Wallis with Dunn comparison test (n = 5–6 per group). *$P < 0.05$ vs. the control group; **$P < 0.01$ vs. the control group; §$P < 0.05$ vs. HG; §§$P < 0.01$ vs. HG.

SUMMARY

Our results are the first experimental evidence for the antifibrotic effect of DAPA under hyperglycemic, hypoxic and chronic inflammative conditions that occur simultaneously in diabetes. These findings provide novel data supporting the link between glucose toxicity, tubular hypoxia, and fibrosis, a vicious trio that seems to be targeted by DAPA. All of these mechanisms are important parts in the puzzle of the complex system behind the organo-protective effect of SGLT2i both in the kidney and in the heart.

We presented our results at several international and national congresses and published the results in 7 peer-reviewed D1-Q1 journals. Parts of the data have been included in two PhD thesis successfully defended recently, and one PhD thesis will be submitted this year. Furthermore, a successful MTA DsC dissertation has also been defended based on the data. Colleagues won several prizes with the publications (Best nephrology paper of the year in 2019 and 2021 by the Hungarian Renal Association), best oral poster presentation at the ERA-EDTA Meeting in 2020 and 2021, best TDK presentation (2x) and OTDK first prize.

We broadened the technical and infrastructural background of our Institute and opened new collaborations with international research groups (Nadja Sparding, Biomedical Sciences, Faculty of Health and Medical Science, University of Copenhagen) and Pharma companies (Federica Genovese, Nordic Bioscience); as well as with national imaging core facilities (Balázs Besztercei, Institute of Clinical Experimental Research and Attila Fintha Deparment of Pathology).

Altogether we can conclude that with our novel experimental results and other achievements we successufully completed the grant aims in the given timeframe and increased the international competitiveness and visibility of our research group.