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# METABOLIC CARDIO- AND RENO-PROTECTIVE EFFECTS OF EMPAGLIFLOZIN IN A PREDIABETIC RAT MODEL

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The mechanisms behind the cardiovascular and renal benefits of empagliflozin is not fully understood. The positive impact of the medication on cardiovascular mortality can not be solely attributed to its antidiabetic effect, with a metabolic mechanism possibly involved. To investigate the metabolic effects of empagliflozin treatment (10 mg/kg/day for 6 weeks), we used an adult male rat model with serious vascular complications associated with metabolic syndrome and prediabetes. Impaired glucose tolerance, severe albuminuria and impaired insulin sensitivity were induced by intragastric administration of methylglyoxal and high sucrose diet feeding for four months. Although empagliflozin decreased body weight, non-fasting glucose and insulin, glucagon levels remained unchanged. In addition, empagliflozin increased adiponectin levels (+40%; $p < 0.01$) and improved skeletal muscle insulin sensitivity. Increased non-esterified fatty acids (NEFA) in empagliflozin-treated rats is understood to generate ketone bodies. Empagliflozin increased β-hydroxybutyrate levels in serum (+66%; $p < 0.05$) and the myocardium (30%; $p < 0.01$), suggesting its possible involvement as an alternative substrate for metabolism. Empagliflozin switched substrate utilisation in the myocardium, diverting glucose oxidation to fatty acid oxidation. Representing another favorable effect, empagliflozin also contributed to decreased uric acid plasma levels (–19%; $p < 0.05$). In the kidney cortex, empagliflozin improved oxidative and dicarbonyl stress parameters and increased gene expression of β-hydroxybutyrate dehydrogenase, an enzyme involved in ketone body utilisation. In addition, empagliflozin decreased microalbuminuria (–27%; $p < 0.01$) and urinary neutrophil gelatinase-associated lipocalin (NGAL) excretion (–29%; $p < 0.01$). Our results reveal the important systemic metabolic effect of empagliflozin on alterations in substrate utilisation and on increased ketone body use in prediabetic rats. Improved oxidative and dicarbonyl stress and decreased uric acid are also possibly involved in the cardio- and reno-protective effects of empagliflozin.

Key words: *empagliflozin, prediabetes, ketone body, insulin sensitivity, oxidative stress, neutrophil gelatinase-associated lipocalin, methylglyoxal, adiponectin, superoxide dismutase, glutathione peroxidase*

## INTRODUCTION

Drugs with antidiabetic effects have potential cardiovascular effect - harmful, beneficial, or neutral (1). According to the outcomes of recent large-scale randomised clinical trials, antihyperglycaemic drugs with positive cardiovascular benefits, such as glucagon-like peptide-1 (GLP-1) receptor agonists and, primarily, sodium-glucose cotransporter (SGLT)2 inhibitors, are the most effective. Recent clinical trials (2) including the EMPA-REG OUTCOME trial, the first clinical study of empagliflozin administration (3, 4), demonstrate that treatment with SGLT2 inhibitors significantly reduces cardiovascular events, including heart failure hospitalisation, in diabetic patients. After empagliflozin treatment in diabetic patients with established macrovascular complications, the primary major adverse cardiac events were attenuated by 14%, cardiovascular mortality by 38% and all-cause mortality by 32%. In the EMPA-REG OUTCOME trial, renal benefit was also confirmed by a 45% reduction in chronic renal disease progression (5).

Since the publication of the outcomes of the EMPA-REG trial, subsequent clinical and experimental studies have focused on elucidating other potential mechanisms, given that the antidiabetic effect cannot solely explain the cardiovascular and renal benefits of empagliflozin. There is a growing consensus that heamodynamic and systemic metabolic effects may play a role, dispelling the belief that glycaemic effects, weight gain and reduced blood pressure are exclusively involved. Although various mechanisms has been suggested, including osmotic diuresis, natriuresis and a shift in fuel metabolism associated with improvements in cardiac metabolism and bioenergetics (6), the exact mechanism is not completely understood.

Animal studies using rodent diabetic models have shown that SGLT2i may improve endothelial function (7), reduce myocardial fibrosis (8) and enhance systolic and diastolic

function (9). In addition, increased circulating levels of ketone bodies after empagliflozin treatment have led to the hypothesis that increased ketone body utilisation in the heart may improve cardiac efficiency, thus explaining the cardioprotective effect of empagliflozin (6, 10). However, data on real tissue levels and the metabolism of ketone bodies in relation to cardiovascular function are lacking, with no direct evidence for myocardial ketone body utilisation. The EMPA-REG study notably lacked any analysis of ketone body concentrations (3, 4). The effects of empagliflozin in non-diabetic subjects are not yet known, while studies of prediabetic animal models are scarce and inconsistent (11, 12).

To contribute to clarifying the mechanism, we investigated the metabolic effects of empagliflozin in relation to myocardial and renal metabolism using a prediabetic rat model.

This prediabetic model results from the combination of two accepted approaches in an experimental metabolic disturbances modelling area: methylglyoxal and high sucrose diet administration. In experimental studies, methylglyoxal is used to induce vascular complications, impair glucose tolerance and activate inflammatory process (13, 14) comparable to prediabetes and insulin resistance states. Based on previous experiences, high sucrose diet used in this study indicates that such a diet facilitates the development of metabolic disturbances associated with prediabetes in both human and animal models (15, 16). In this study we analysed on parameters of lipid metabolism, insulin sensitivity, oxidative and dicarbonyl stress, urinary markers and first of all ketone bodies utilization after empagliflozin treatment in MG- and high sucrose diet-induced animal model. To date, no study has been performed to monitor and compare the effect of empagliflozin on ketone body metabolism in heart and kidney in prediabetic model and beyond its hypoglycemic effect.

## MATERIALS AND METHODS

### *Animals and study design*

All of the experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), which is in compliance with European Community Council recommendations (86/609/ECC) for the use of laboratory animals, and approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine.

Adult male Wistar rats at the age of 3 months (purchased from Charles River, Germany) were fed a standard chow diet (23% protein, 43% starch, 7% fat, 5% fibre, 1% vitamin and mineral mixture; Bonagro, Czech Republic). To induce prediabetic condition, rats were administrated methylglyoxal (0.5 mg/kg b.w.) intragastrically three times a week for 4 months and fed at the same time a high sucrose diet (70 kcal % as sucrose, 20 kcal % as protein, 10 kcal % as fat). This intervention resulted in impaired glucose tolerance, the onset of albuminuria, and in chronic vascular complications with histologically verified progressive nephropathy, potentiating most of the symptoms associated with metabolic syndrome and a prediabetic state. After 4 months, both animal groups were treated with empagliflozin, (Jardiance, Boehringer Ingelheim, Germany) at a dose of 10 mg/kg/day for 6 weeks, mixed into standard diet. By the end of the experiment, the animals had reached 8.5 months of age. The design of the study is shown in *Fig. 1*.

Rats were held in temperature- (22ºC) and humidity-controlled conditions under a 12-h/12-h light/dark cycle with free access to food and water. At the beginning of the study, body weight, serum glucose and triglycerides were measured, with rats randomly divided into experimental groups (n = 6). Urine samples were collected for 16 hours when the animals were housed in metabolic cages during which time urine was effectively separates into tubes. At the end of the experiment, rats were sacrificed by decapitation after a light anesthetization (zoletil 5 mg/kg b.w.) in a postprandial state. Aliquots of serum and tissue samples were collected and stored at –80ºC for further analysis.

### *Biochemical analysis*

Serum levels of triglycerides, glucose, non-esterified fatty acids (NEFA), and total and HDL cholesterol were measured using commercially available kits (Erba Lachema, Czech Republic; Roche Diagnostics, Germany). For serum creatinine and uric acid determination, enzymatic spectrophotometrical methods were used (available kits from Roche Diagnostics, Germany). Serum insulin, glucagon, leptin, adiponectin, monocyte chemoattractant protein-1 (MCP-1) and carboxymethyl lysine (CML) concentrations were determined using Rat ELISA Kits (Mercodia AB, Sweden; MyBioSource, USA; eBioscience-Bender, Austria). Urinary neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) were also determined using Rat ELISA Kits (MyBiosource, USA). Serum and tissue β-hydroxybutyrate (BHB) concentrations were measured using a colorimetric assay kit (Sigma, USA).

For the oral glucose tolerance test (OGTT), blood glucose was determined after a glucose load (300 mg/100g b.w.) administered intragastrically following overnight fasting. Blood was drawn from the tail before the glucose load at 0 min and then at 30, 60 and 120 min thereafter.

To determine triglycerides and cholesterol in tissues, samples were extracted in a chloroform/methanol mixture. The resulting pellet was dissolved in isopropyl alcohol, after which triglyceride content was determined by enzymatic assay (Erba-

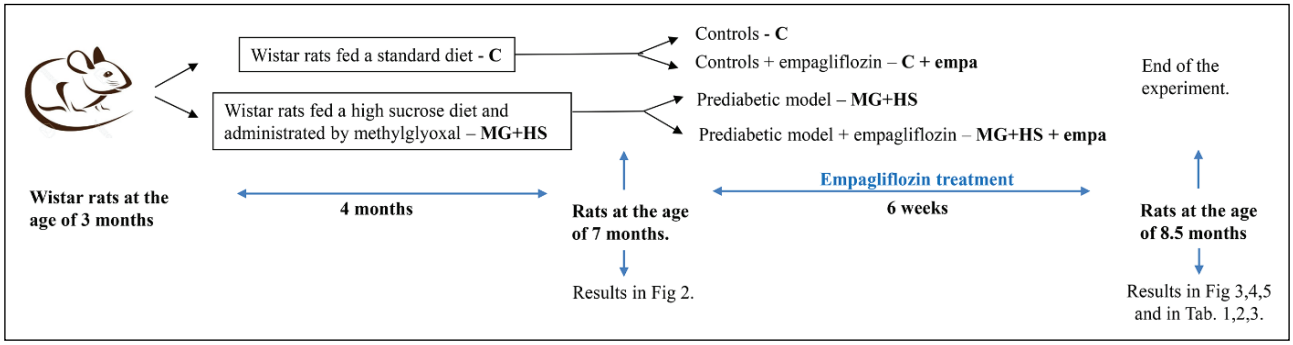


*Fig. 1.* Design of the study.

Lachema, Czech Republic). For determination of hepatic glycogen, liver tissues were hydrolysed and precipitated by ethanol; aliquots were measured using a glucose oxidase assay (Erba-Lachema, Czech Republic). As a marker of skeletal muscle insulin sensitivity, basal and insulin-stimulated glycogen synthesis were determined *ex vivo* in isolated skeletal muscle by measuring the incorporation of $^{14}$C-U glucose into glycogen, as described previously (17). Glucose and palmitate oxidation in the myocardium was measured *ex vivo* in heart tissue sections based on incorporation of $^{14}$C-U glucose and $^{14}$C-palmitate into $CO_2$, respectively. Basal and adrenaline-stimulated lipolysis in the epididymal adipose tissue was measured *ex vivo* according to the release of NEFA (17).

The level of albumin in urine was analysed using the high-performance liquid chromatography (HPLC) method with UV-VIS detection according to Contois *et al.* (18) and adjusted for creatinine concentration.

*Oxidative and dicarbonyl stress parameters*

Levels of reduced (GSH) and oxidised (GSSG) forms of glutathione were determined using the HPLC method with fluorescence detection according to the HPLC diagnostic kit (Chromsystems, Germany). Activity of antioxidant enzymes, superoxide dismutase (SOD) and glutahione peroxidase (GPx) was analysed using Cayman Chemicals assay kits (Ann Arbor, MI, USA). Lipoperoxidation products were assessed based on levels of thiobarbituric acid-reactive substances (TBARS) by assaying the reaction with thiobarbituric acid. Methylglyoxal concentrations were determined using the HPLC method with fluorescence detection after derivatisation with 1,2-diamino-benzene (19).

*Relative mRNA expression*

Total RNA were isolated from tissues using RNA Blue (Top-Bio, Czech Republic). Reverse transcription and quantitative real-time PCR analysis was performed using the TaqMan RNA-to-$C_T$ 1-Step Kit and the TaqMan Gene Expression Assay (Applied Biosystems, USA) and carried out on the ViiA™ 7 Real Time PCR System (Applied Biosystems, USA). Relative expression was determined after normalisation against β-actin and *Hprt1* as an internal reference and calculated using the $2^{-\Delta\Delta Ct}$ method; results were run in triplicate. TaqMan primers assay ID for *Bdh1* - Rn00588855_m1, for *Nrf2* - Rn00582415_m1, *Actb* - Rn00667869_m1 and *Hprt1* - Rn01527840_m1.

*Histological evaluation*

Middle-plane sagittal kidney slices were fixed in formaldehyde for 48 hours and processed into paraffin blocks using standard techniques. Three-µm-thick sections were stained using routine haematoxylin and eosin and evaluated by a histopathologist. The samples were not blinded for histopathologist.

*Statistical analysis*

In our study, one-way ANOVA was applied to analyze the parameters describing the prediabetic strain damage before empagliflozin treatment. Two-way ANOVA was performed to examine the effect of empagliflozin treatment in two different strains of rats, as well as to find out any treatment and strain interactions to determine whether the empagliflozin treatment have different effect in prediabetic model (MG and HS induced rats) or control rats. For detailed comparsions, Bonferroni *post-hoc* ($p < 0.05$) evaulation of significant differences between these groups was applied. All results are expressed as mean ± SD. Statistical analysis was performed using BMDP Statistical Software.

## RESULTS

Methylglyoxal and high-sucrose diet administration effect

After 4 months of methylglyoxal and high-sucrose diet administration, preceding empagliflozin administration, high sucrose- and methylglyoxal-induced rats (prediabetic model) exhibited impaired glucose tolerance and severe albuminuria (*Fig. 2*) compared to non-induced animals. There were no differences in body weight (608 ± 19 versus 604 ± 18 g), serum fasting glucose (4.89 ± 0.15 versus 4.85 ± 0.16 mmol/l) or triglyceride concentrations (2.58 ± 0.54 versus 2.01 ± 0.26 mmol/l) between both experimental groups of rats before empagliflozin treatment. Methylglyoxal and high-sucrose-diet administration also had no effect on food or water intake.

At the end of the experiments, prediabetic rats exhibited markedly increased adiposity, weight of left ventricle, insulinaemia, NEFA and ectopic triglyceride accumulation in the

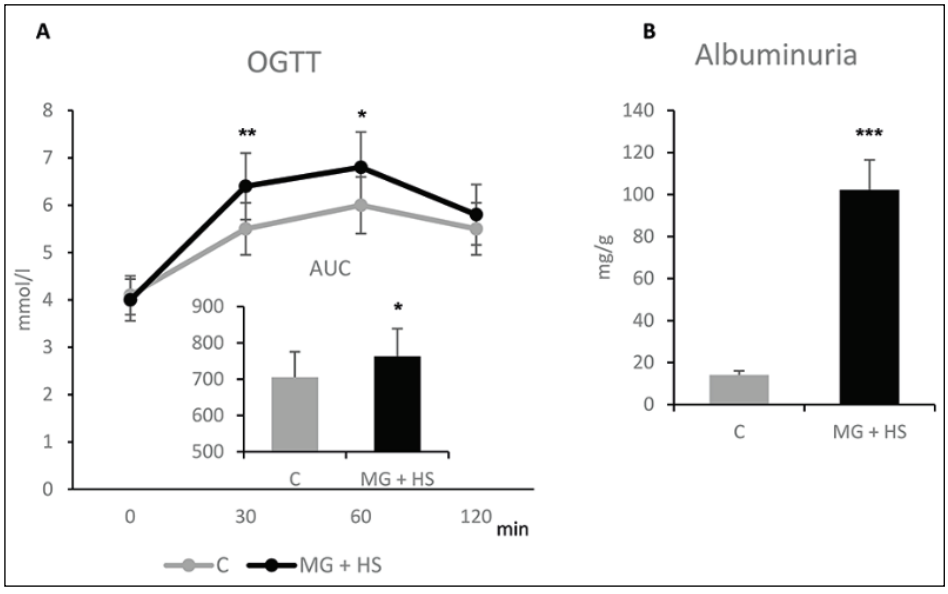


*Fig. 2.* Effect of methylglyoxal and high-sucrose-diet administration on glucose tolerance and albuminuria in a rat model of prediabetes and controls before empagliflozin treatment.
Data are expressed as mean ± SD (n = 6) and analysed by One-way ANOVA; * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$; OGTT, oral glucose tolerance test.
C, group of control rats; MG+HS, methylglyoxal- and high-sucrose diet-induced model of prediabetes.

*Table 1.* Basal metabolic characteristics and parameters of glucose metabolism and insulin sensitivity after empagliflozin treatment in a rat model of prediabetes (MG + HS) and control rats (C).

| | C | C + empa | MG+HS | MG+HS + empa | P1 | P2 | P Inter-action |
|---|---|---|---|---|---|---|---|
| **Body weight** (g) | 684 ± 58 | 634 ± 46 | 710 ± 51 | 651 ± 58 | n.s. | < 0.05 | n.s. |
| **Epididymal adipose tissue weight** (mg/g) | 1.71 ± 0.57 | 1.53 ± 0.58 | 2.35 ± 0.27 | 2.28 ± 0.48 | < 0.01 | n.s. | n.s. |
| **Weight of left ventricle** (g/100g b.w.) | 0.12 ± 0.01 | 0.11 ± 0.05 | 0.16 ± 0.05 | 0.12 ± 0.01* | < 0.05 | < 0.05 | < 0.05 |
| **Non-fasting glucose** (mmol/l) | 6.4 ± 0.6 | 6.3 ± 0.4 | 7.0 ± 0.3 | 6.4 ± 0.4* | n.s. | < 0.05 | n.s. |
| **Insulin** (nmol/l) | 0.334 ± 0.134 | 0.217 ± 0.153 | 0.586 ± 0.135 | 0.400 ± 0.106* | < 0.01 | < 0.01 | n.s. |
| **Glucagon** (ng/ml) | 0.172 ± 0.034 | 0.194 ± 0.036 | 0.197 ± 0.081 | 0.217 ± 0.037 | n.s. | n.s. | n.s. |
| **Insulin/glucagon ratio** | 2.18 ± 0.59 | 1.24 ± 0.76* | 2.51 ± 0.51 | 1.87 ± 0.49 | < 0.05 | < 0.01 | n.s. |
| **β-hydroxybutyrate** (μmol/l) | 0.479 ± 0.160 | 0.535 ± 0.113 | 0.441 ± 0.122 | 0.734 ± 0.217* | n.s. | < 0.05 | n.s. |
| **Serum uric acid** (mmol/l) | 106.5 ± 13.1 | 83.6 ± 10.3* | 97.5 ± 11.8 | 79.4 ± 10.7* | n.s. | < 0.05 | n.s. |
| **Serum creatinine** (mmol/l) | 19.0 ± 2.6 | 19.0 ± 1.5 | 18.0 ± 1.4 | 19.0 ± 2.8 | n.s. | n.s. | n.s. |
| **CML** (ng/ml) | 9.12 ± 1.68 | 10.52 ± 2.48 | 12.04 ± 0.95 | 11.37 ± 1.93 | < 0.05 | n.s. | n.s. |
| **MCP-1** (pmol/l) | 0.852 ± 0.139 | 0.782 ± 0.191 | 0.817 ± 0.117 | 0.829 ± 0.277 | n.s. | n.s. | n.s. |
| **Leptin** (μg/ml) | 12.52 ± 5.20 | 8.89 ± 3.81 | 33.10 ± 5.88 | 21.16 ± 4.91* | < 0.01 | < 0.05 | < 0.05 |

Values are given as mean ± SD; n = 6 for each group; P interaction - probability reflecting the effect of empagliflozin treatment x strain interaction (protective effects of empagliflozin against aggravation due to prediabetic state); P1, probability reflecting the effect of the strain analysed by Two-way ANOVA; P2, probability reflecting the effect of empagliflozin treatment analysed by Two-way ANOVA; for multiple comparsion between groups, the Bonferroni *post-hoc* test was applied; * indicates $p < 0.05$ significance of empagliflozin treatment versus non-treated rats of control group or MG + HS group. CML, carboxymethyl lysine; MCP-1, monocyte chemoattractant protein.

*Table 2.* Serum, tissue lipids and glycogen after empagliflozin treatment in a rat model of prediabetes (MG+HS) and control rats (C).

| | C | C + empa | MG+HS | MG+HS + empa | P1 | P2 | P interaction |
|---|---|---|---|---|---|---|---|
| **TAG in serum** (mmol/l) | 1.93 ± 0.83 | 2.40 ± 0.91 | 2.60 ± 0.57 | 4.20 ± 1.28* | < 0.05 | < 0.05 | n.s. |
| **Cholesterol in serum** (mmol/l) | 2.70 ± 0.48 | 2.80 ± 0.77 | 2.10 ± 0.24 | 2.44 ± 0.58 | n.s. | n.s. | n.s. |
| **HDL-C** (mmol/l) | 1.84 ± 0.31 | 1.86 ± 0.45 | 1.36 ± 0.34 | 1.33 ± 0.38 | < 0.05 | n.s. | n.s. |
| **NEFA** (mmol/l) | 0.28 ± 0.06 | 0.37 ± 0.11 | 0.36 ± 0.06 | 0.47 ± 0.18 | < 0.05 | < 0.01 | n.s. |
| **Hepatic TAG** (μmol/g) | 9.37 ± 2.80 | 11.35 ± 2.63 | 22.95 ± 7.02 | 28.24 ± 10.87 | < 0.001 | n.s. | n.s. |
| **Hepatic glycogen** (μmol/g) | 315.3 ± 34.5 | 213.6 ± 25.0** | 279.3 ± 24.2 | 264.6 ± 43.0 | n.s. | n.s. | < 0.01 |
| **TAG in muscle** (μmol/g) | 4.81 ± 1.43 | 5.08 ± 1.56 | 7.39 ± 1.15 | 5.57 ± 1.57* | n.s. | < 0.05 | < 0.05 |
| **TAG in the myocardium** (μmol/g) | 4.02 ± 1.77 | 3.92 ± 1.40 | 4.15 ± 1.92 | 4.22 ± 1.77 | n.s. | n.s. | n.s. |
| **TAG in the kidneys** (μmol/g) | 5.00 ± 1.04 | 3.01 ± 0.78* | 5.65 ± 1.75 | 5.37 ± 0.98 | n.s. | n.s. | < 0.05 |

Values are given as mean ± SD; n = 6 for each group; P interaction, probability reflecting the effect of empagliflozin treatment x strain interaction (protective effects of empagliflozin against aggravation due to prediabetic state); P1, probability reflecting the effect of the strain analysed by Two-way ANOVA; P2, probability reflecting the effect of empagliflozin treatment analysed by Two-way ANOVA; for multiple comparsion between groups, the Bonferroni *post-hoc* test was applied; * indicates $p < 0.05$; ** indicates $p < 0.01$ significance of empagliflozin treatment versus non-treated rats of control group or MG+HS group; TAG, triacylglycerol; NEFA, non-esterified fatty acid.

liver and muscles compared to controls (*Table 1* and *2*). Methylglyoxal and high-sucrose diet administration also impaired insulin sensitivity in skeletal muscles (*Fig. 3*). Methylglyoxal-derived AGE carboxymethyl lysine (CML) in serum and methylglyoxal concentrations in the kidney cortex significantly increased in prediabetic rats (*Table 1* and *Fig. 5*). Methylglyoxal and high-sucrose diet administration worsened oxidative stress parameters in the myocardium and in the kidney cortex (*Table 3*), while levels of glutathione markedly reduced and lipoperoxidation TBARS products increased.

*Empagliflozin treatment effect on basal metabolic parameters, glucose metabolism and insulin sensitivity*

As shown in *Table 1*, empagliflozin treatment in prediabetic rats decreased body weight (–9%), serum non-fasting glucose and insulin levels; however, glucagon levels and weight of epididymal adipose tissue remained unchanged. In addition, empagliflozin increased adiponectin concentrations and improved skeletal muscle insulin sensitivity and decreased adrenaline-stimulated lipolysis (*Fig. 3*).

*Empagliflozin treatment effect on lipids, ketone bodies and substrate utilisation*

Empagliflozin administration slightly increased serum triglycerides, but total- and HDL-cholesterol were not affected (*Table 2*). Increased NEFA in empagliflozin-treated rats can be used to generate ketone bodies. Decreased insulin/glucagon ratio and reduced hepatic glycogen can create suitable conditions for ketone body production. Serum levels of β-hydroxybutyrate (BHB) increased after empagliflozin administration, indicating its possible involvement in the positive effect of empagliflozin as an alternative substrate for metabolism. In prediabetic rats, BHB concentrations increased markedly in the myocardium and slightly in the kidney cortex after empagliflozin administration (*Fig. 4* and *5*). However, gene expression of the β-hydroxybutyrate dehydrogenase (BDH1) enzyme, which is involved in ketone body utilisation, increased significantly in the renal cortex but decreased in the myocardium after empagliflozin treatment. Empagliflozin treatment altered substrate utilisation in the myocardium, with empagliflozin-treated rats exhibiting decreased glucose oxidation and increased palmitate oxidation compared to controls (*Fig. 4* and *5*).

*Empagliflozin treatment effect on markers of renal function*

As shown in *Table 1* and *Fig. 5*, empagliflozin-treated rats exhibited reduced levels of albuminuria and the renal tubular marker NGAL. Empagliflozin administration had no effect on levels of KIM-1 in urine, or on serum creatinine levels. While it did contribute to decreasing serum uric acid levels (*Table 1*), there were no changes in urinary uric acid excretion after empagliflozin administration.

As expected, glucose urinary excretion was considerably higher after empagliflozin administration (in control rats: 48.8 ± 7.2 versus 0.6 ± 0.2 mmol/g creatinine, $p < 0.001$; in prediabetic rats: 42.2 ± 6.1 versus 1.1 ± 0.4 mmol/g creatinine, $p < 0.001$).

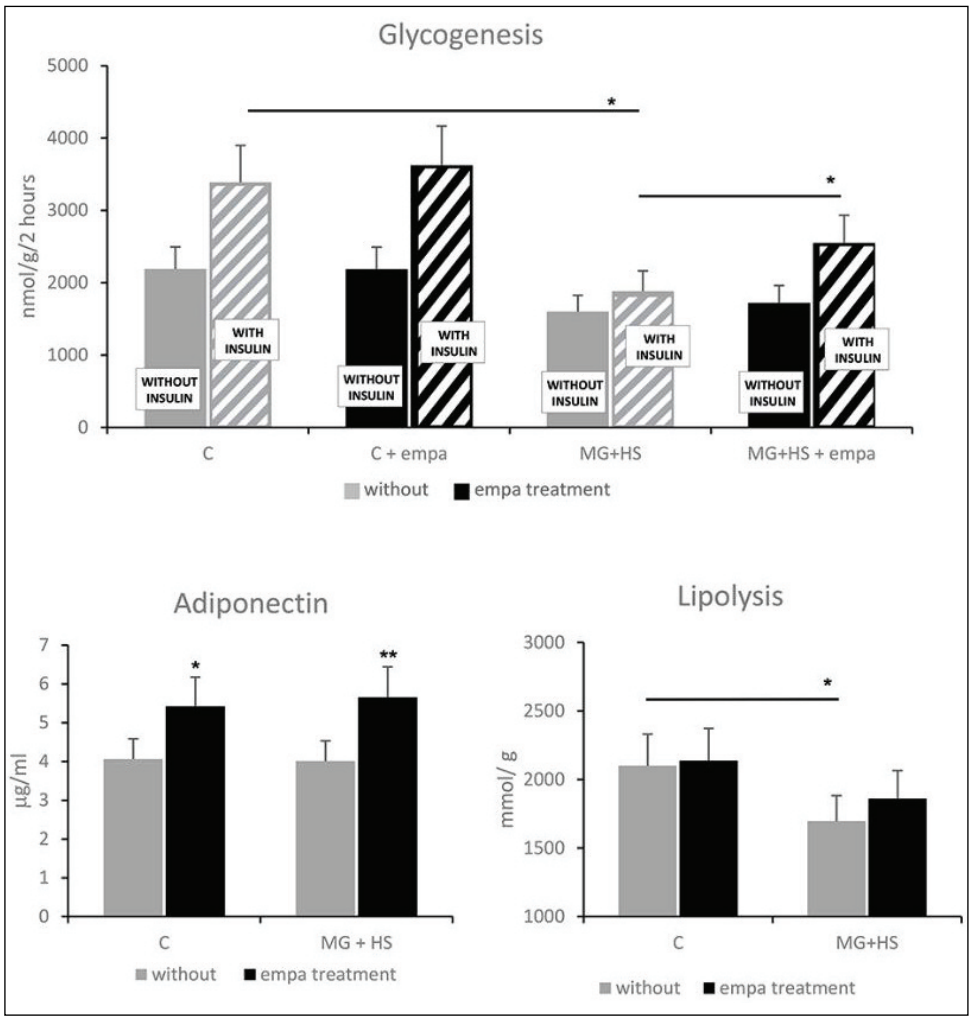


*Fig. 3.* Effect of empagliflozin on insulin sensitivity parameters - muscle glycogenesis (without and with insulin stimulation), adiponectin levels and adipose tissue lipolysis - in a rat model of prediabetes and control rats.
Data are expressed as mean ± SD (n = 6) and analysed by Two-way ANOVA; * denotes $p < 0.05$; ** denotes $p < 0.01$; C, group of control rats; MG+HS, methylglyoxal- and high-sucrose diet-induced model of prediabetes.

*Empagliflozin treatment effect on oxidative and dicarbonyl stress*

In the kidney cortex, empagliflozin markedly improved oxidative and dicarbonyl stress parameters, resulting in reduced methylglyoxal levels (*Fig. 5*) and elevated levels of glutathione and antioxidant SOD and GPx enzyme activity (*Table 3*) compared to untreated rats. These alterations in oxidative and dicarbonyl stress parameters in the kidney cortex were associated with the trend of an increase in relative mRNA expression of transcriptional factor Nrf2 which controls antioxidant and lipogenic genes (in control rats: 1.008 ± 0.136 versus 0.912 ± 0.102, n.s.; in prediabetic rats: 1.087 ± 0.198 versus 0.882 ± 0.058, n.s.). Oxidative stress parameters also slightly improved in the myocardium, reflected in elevated SOD activity and levels of the reduced form of glutathione in empagliflozin-treated animals (*Table 3*). Lipoperoxidation products in the form of TBARS significantly decreased in the kidney cortex as well as in the myocardium after empagliflozin administration.

*Empagliflozin treatment effect on inflammation*

Although serum leptin levels decreased in empagliflozin-treated rats, there were no changes in pro-inflammatory MCP-1 levels after empagliflozin administration (*Table 1*).

Signs of reduced inflammatory infiltrates and renal fibrosis in empagliflozin-treated rats were verified by histological-based observations (*Fig. 5*).

## DISCUSSION

Unlike the EMPA-REG OUTCOME trial, the first study to demonstrate the cardiovascular and renal benefits of empagliflozin treatment in diabetic patients, in our study cardiovascular risk reduced irrespective of its impact on glycaemia. The mechanism behind the cardio- and nephro-protective effects of empagliflozin are not be directly related to its hypoglycaemic impact. In an effort to help to clarify the empagliflozin effect not only on renal damage but also on aggravated glucose and lipid metabolism, an experimentally induced animal model of prediabetes with chronic vascular complications and renal function impairment in the absence of hyperglycaemia was used. In addition to signs of insulin resistance, including hyperinsulinemia, increased NEFA and impaired glucose tolerance, animals exhibited left ventricle hypertrophy and severe albuminuria. Methylglyoxal, a highly reactive dicarbonyl compound, has been implicated as a detrimental influence in the aetiology of cardiovascular and renal complications (20). According to our previous study (14) and work by other authors (21, 22), methylglyoxal administration causes endothelial/vascular dysfunctions and atherosclerosis, activates oxidative stress, and leads to structural and functional alterations in the kidneys. The prediabetic rats used in the present study exhibited methylglyoxal accumulation in the kidneys and elevated serum levels of CML, a methylglyoxal-derived AGE. To potentiate insulin resistance, metabolic syndrome and prediabetes complications, and not just

*Table 3.* Oxidative stress markers in the myocardium and kidney cortex after empagliflozin treatment in a rat model of prediabetes (MG+HS) and control rats (C).

| **Myocardium** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | C | **C + empa** | **MG+HS** | **MG+HS + empa** | **P1** | **P2** | **P interaction** |
| **SOD** (U/mg protein) | 0.091 ± 0.009 | 0.109 ± 0.013* | 0.085 ± 0.008 | 0.103 ± 0.008* | n.s. | < 0.01 | n.s. |
| **GPx** (μM NADPH/min/mg protein) | 163 ± 19 | 176 ± 30 | 144 ± 31 | 184 ± 48 | n.s. | n.s. | n.s. |
| **GSH** (μmol/g protein) | 21.24 ± 1.58 | 22.55 ± 4.16 | 14.94 ± 1.66 | 19.71 ± 1.15** | < 0.02 | < 0.05 | n.s. |
| **GSSG** (μmol/g protein) | 1.80 ± 0.11 | 1.65 ± 0.29 | 1.53 ± 0.23 | 1.46 ± 0.44 | < 0.05 | n.s. | n.s. |
| **TBARS** (nmol/mg protein) | 0.59 ± 0.14 | 0.66 ± 0.06 | 0.73 ± 0.05 | 0.55 ± 0.07* | < 0.05 | n.s. | < 0.05 |
| **Kidney cortex** | | | | | | | |
| | **C** | **C + empa** | **MG+HS** | **MG+HS + empa** | **P1** | **P2** | **P interaction** |
| **SOD** (U/mg protein) | 0.061 ± 0.015 | 0.89 ± 0.012* | 0.056 ± 0.012 | 0.078 ± 0.016* | n.s. | < 0.01 | n.s. |
| **GPx** (μM NADPH/min/mg protein) | 204 ± 39 | 250 ± 30* | 183 ± 30 | 215 ± 29 | n.s. | < 0.01 | n.s. |
| **GSH** (μmol/g protein) | 24.18 ± 3.91 | 29.53 ± 5.51* | 20.19 ± 3.81 | 27.26 ± 2.45** | < 0.02 | < 0.001 | n.s. |
| **GSSG** (μmol/g protein) | 1.19 ± 0.34 | 1.52 ± 0.06 | 1.95 ± 0.57 | 1.46 ± 0.38* | < 0.05 | n.s. | < 0.01 |
| **TBARS** (nmol/mg protein) | 0.63 ± 0.14 | 0.48 ± 0.11* | 0.87 ± 0.08 | 0.72 ± 0.12* | < 0.01 | < 0.05 | n.s. |

Values are given as mean ± SD; n = 6 for each group; P interaction, probability reflecting the effect of empagliflozin treatment x strain interaction (protective effects of empagliflozin against aggravation due to prediabetic state); P1, probability reflecting the effect of the strain analysed by Two-way ANOVA; P2, probability reflecting the effect of empagliflozin treatment analysed by Two-way ANOVA; for multiple comparsion between groups, the Bonferroni *post-hoc* test was applied; * indicates $p < 0.05$; ** indicates $p < 0.01$ significance of empagliflozin treatment versus non-treated rats of control group or MG+HS group; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced form of glutathione; GSSG, oxidised form of glutathione; TBARS, thiobarbituric acid-reactive substance.

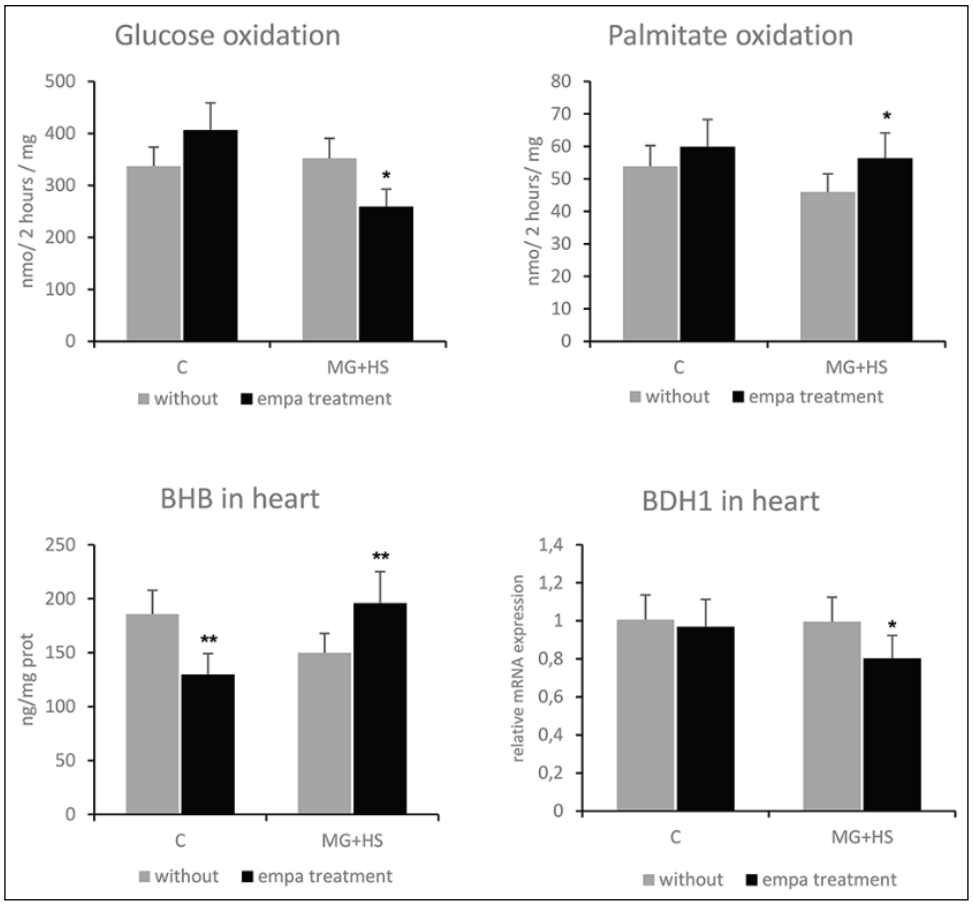


*Fig. 4.* Effect of empagliflozin treatment on the myocardium - glucose and palmitate oxidation, BHB concentration and BDH1 gene expression - in a rat model of prediabetes and control rats after empagliflozin administration.
Data are expressed as mean ± SD (n = 6) and analysed by two-way ANOVA; * denotes $p < 0.05$; ** denotes $p < 0.01$. C, group of control rats; MG+HS, methylglyoxal- and high-sucrose diet-induced model of prediabetes; BHB, β-hydroxybutyrate; BDH1, β-hydroxybutyrate dehydrogenase.

methylglyoxal-induced vascular damage, animals were fed a high-sucrose diet (23).

As expected, empagliflozin treatment decreased body weight associated with urinary glucose loss of energetic substrates. A slightly beneficial effect on body weight is known from animal and human studies (24). Other types of white adipose tissue are likely to be involved in weight loss after empagliflozin treatment, so there was no significant reduction in epididymal adipose tissue only tended to decrease. Weight loss can also contribute to the improvement of insulin sensitivity in skeletal muscle after empagliflozin administration. Although adipose tissue insulin sensitivity was not affected, empagliflozin positively influenced adipocytokine secretion. While elevated levels of adiponectin can improve insulin sensitivity, this adipocytokine also exhibits anti-inflammatory and antiatherogenic properties (25, 26) understood to be involved in the cardiovascular benefit of empagliflozin. Elevated leptin levels have been associated with the progression of insulin resistance and the promotion of inflammation and atherosclerosis (27). Leptin may have a pathophysiological role in sodium regulation as well as in cardiac inflammation and fibrosis (28). Although positive alterations in adiponectin and leptin plasma levels have been observed in other empagliflozin studies (29, 30), it is unclear whether empagliflozin action directly impacts on adipose tissue function or whether it is associated with visceral fat loss.

Surprisingly, in this study, empagliflozin slightly increased serum triglyceride and hepatic triglyceride accumulation. Most, but not all, clinical trials have shown that administration of SGLT2i increases both HDL-C and LDL-C and decreases serum triglycerides (31, 32). In the EMPA-REG OUTCOME trial, empagliflozin had no effect on serum triglycerides, with the reported cardiovascular benefit occurring despite an increase in LDL-C (5). Improving dyslipidaemia is therefore not likely to be a principal mechanism in the cardioprotective effect of empagliflozin. In fact, empagliflozin altered circulating lipid profiles, increased lipolysis, delayed the clearence of triglyceride-rich lipoproteins and promoted a trend for NEFA utilisation (33-35), which can slightly increase triglyceride levels.

Representing an important finding of our study, empagliflozin had a systemic effect on alterations in substrate utilisation, with glucose oxidation diverted in preference for fatty acid oxidation in the myocardium after empagliflozin administration. Elevated NEFA caused by increased lipolysis in tandem with a reduction in the insulin/glucagon ratio following empagliflozin treatment created suitable conditions for ketone body generation as an alternative substrate for metabolism. According to the "thrifty substrate" hypothesis (6, 10), it is assumed that BHB may act as a superfuel for the diabetic or failing heart while ameliorating myocardial energy metabolism and effectiveness. Ketone body utilisation decreased oxygen consumption and acetylCoA accumulation in mitochondria, thus more effectively producing ATP, a preferred energy substrate for cardiomyocytes. Although most of the clinical (4, 36-38) and animal (39, 40) studies of empagliflozin have confirmed elevated circulating levels of BHB in the blood, only a few animal studies have investigated the effect of empagliflozin on ketone body metabolism in the myocardium. In the present study, we observed elevated levels of BHB in the myocardium in empagliflozin-treated prediabetic rats, but decreased levels in empagliflozin-treated control rats. In a study with diabetic obese rats with heart faliure, Abdurrachim *et al.* (40) reported that empagliflozin reduced myocardial ketone utilisation despite increasing circulating levels of BHB. In our study, gene

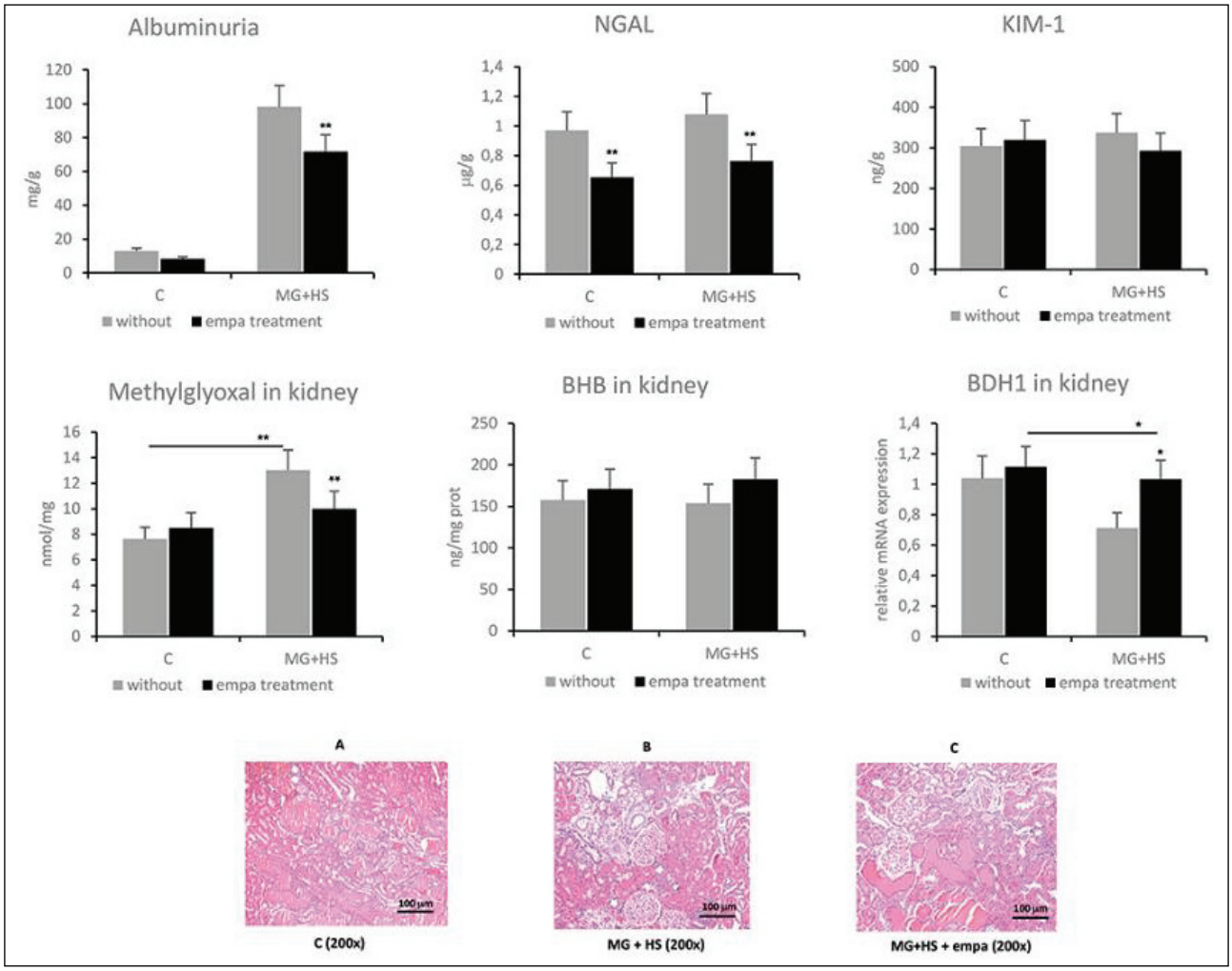


*Fig. 5.* Effect of empagliflozin treatment on the kidneys and urine renal markers in a rat model of prediabetes and control rats; BHB, β-hydroxybutyrate; BDH1, β-hydroxybutyrate dehydrogenase 1.
(A): View of the kidney with miniature groups of interstitial round-cellular inflammatory cells. (B): View of the kidney with chronic progressive nephropathy. Longitudinally sectioned kidney with several glomeruli (50>), and 40% of glomeruli with mesangial amount increasing with several leukocytes and occasional fibrous deposits with slightly thickened capillary wall. A part of glomeruli (<15%) are disintegrated with a thickened parietal layer of the Bowman‘s capsule, including the presence individual (<1%) totally lytic glomeruli. In the contact, several groups of hyalinized renal tubules are visible. The process is accompanied by interstitial edema and limited interstitial round-cellular inflammation. (C): View of the kidney with chronic progressive nephropathy. Longitudinally sectioned kidney with several glomeruli (50>), and 20% of glomeruli with mesangial amount increasing with several leukocytes and occasional fibrous deposits with slightly thickened capillary wall, including thickened parietal layer of the Bowman's capsule. Surrounding individual groups of disintegrated tubules, including regenerating tubules are present. The process is accompanied by interstitial edema and limited interstitial round-cellular inflammation.
Data are expressed as mean ± SD (n = 6) and analysed by Two-way ANOVA; * denotes $p < 0.05$; ** denotes $p < 0.01$. C, group of control rats; MG+HS, model of prediabetic rats; MG+HS + empa, prediabetic rats after empagliflozin treatment.

expression of myocardial BDH1, an enzyme involved in BHB utilisation, also decreased in empagliflozin-treated rats. Another study of diabetic mice (4) reported that empagliflozin did not affect BDH1 protein content in the heart. Overall, animal studies have yet to unequivocally confirm that empagliflozin increases oxidation or preferably metabolises BHB in the myocardium. Nonetheless, elevated levels of BHB in the myocardium have been shown to provide cardioprotective effects due to the ketone's antioxidant and anti-arrhythmic properties (41). In our study, elevated BHB levels in the myocardium after empagliflozin treatment ameliorated oxidative stress markers. Moreover, ketone bodies have been shown to increase the mechanical efficiency of the failing heart (42), in turn improving cardiac function.

Another possible mechanism behind the cardioprotective effect of empagliflozin, independent of the ketone body theory, is associated with reduced serum levels of uric acid. According to most epidemiological studies, an association between elevated serum uric acid levels and cardiovascular diseases exists. Uric acid levels are an important predictor of prognosis in heart failure and increase risk for cardiovascular mortality (43, 44). Although in serum uric acid acts as an antioxidant, when presents in cytoplasm in the cells, uric acid can converts into a prooxidant agent and promotes oxidative stress and through this mechanism can participate in the pathophysiology of cardiovascular diseases. Except oxidative stress, several other mechanisms has been suggested which elevated uric acid exerts deleterious effects on cardiovascular health including reduced

availability of nitric oxide and endothelial dysfunction, promotion of local and systemic inflammation or proliferation of vascular smooth muscle cells (45). It was hypothesized that the reduction in serum uric acid levels by SGLT2 inhibitors might be attritable to the function of glucose transporter 9 or can associate with oxidative stress amelioration (46). Similar to our results, small uric acid after empagliflozin reductions in serum treatment were observed in the EMPA-REG OUTCOME trial (5). Also, a meta-analysis of 12 randomised clinical trials indicates that empagliflozin significantly reduces serum uric acid levels (47).

Alleviation of oxidative and dicarbonyl stress can play a key role in the beneficial effect of empagliflozin on the development of vascular complications (48). In this study, empagliflozin treatment markedly improved oxidative stress parameters in the kidney and reduced methylglyoxal renal levels. In a study of streptozotocin-induced diabetic rats, empagliflozin treatment also alleviated renal oxidative stress (49) while elevating the activity of SOD and GPx in the kidneys. Although it is widely acknowledged that oxidative stress plays an important role in the development of vascular disorders (50), methylglyoxal accumulation has been implicated in vascular dysfunction complications, especially at a microvascular level. Recently, we found that methylglyoxal activates oxidative and inflammatory pathways, inhibiting angiogenesis at a transcriptome level (14). In renal cells, methylglyoxal directly inhibits the electron respiratory chain and modifies protein structure, which may in turn affect their function. Decreased renal methylglyoxal after empagliflozin treatment can markedly improve microvascular complications in the kidneys. According to our previous results, metformin administration, in contrast to empagliflozin treatment, does not have a beneficial effect on methylglyoxal levels in the kidney cortex (51). Thus, the amelioration of dicarbonyl stress in the kidneys may be one of the important renoprotective effects of empagliflozin. To our knowledge, no study to date has been investigating the effect of empagliflozin on dicarbonyl stress. The role of methylglyoxal in the pathogenesis of renal microvascular complications a finding by the clinical ADDITION-DK study (52) that methylglyoxal levels are associated with detrimental changes in kidney function in individuals with T2D.

In our study, empagliflozin improved markers of renal function, leading to reduced levels of albuminuria and NGAL. In another study of obese diabetic rats, SGLT2 inhibition along with empagliflozin attenuated the renal injury markers NGAL and cystatin C (53). Proven to be a novel marker for diabetic nephropathy, NGAL is also associated with cardiovascular events and serves as a more reliable early renal impairment parameter than albuminuria or serum creatinine (54). Given that KIM-1 is a marker of acute hypoxic injury to proximal tubular cells (55), this may explain why we observed no differences in urinary KIM-1 after empagliflozin treatment.

According to our result, the alleviation of oxidative and dicarbonyl stress and decreased urinary tubular injury markers are probable renoprotective mechanisms of empagliflozin.

Ketone bodies can also be metabolised and utilised in the kidneys (56). In our study, in the renal cortex, unlike in the myocardium, empagliflozin increased gene expression of BDH1, an enzyme that promotes BHB oxidation. Increased ketone body utilisation in the kidneys can contribute to improved renal function and efficiency. In one study of diabetic mice, empagliflozin increased BHB and BDH1 gene expression in the kidneys (56); however, ketone body metabolism in the myocardium was not investigated.

To the best of our knowledge, no study has examined ketone body levels and utilisation in both the myocardium and kidneys. On the other hand, our study has also some limitation, we did not focus on blood or intraglomerular pressure, RAAS system or erythropoietin production that could be involved in potential mechanism of pleiotropic effects of empagliflozin on renal function.

According to our results in the heart and kidneys, empagliflozin may alter fuel metabolism and regulate ketone body metabolism, mechanisms linked to cardiovascular and renal protection. Higher ketones utilization in kidney can contribute to the improvement in renal fuel efficiency while increased ketone bodies in myocardium can be due to increased circulating levels as well as decreased utilization. The impacts of empagliflozin on substrate and glycogen utilisation, lipid mobilisation, and oxidation and ketogenesis stimulation in tissues serve to induce a “pseudo-fasting state”, which can be considered the main systemic metabolic effect of this medication.

In conclusion, our results reveal that empagliflozin administration in prediabetic rats has an important systemic metabolic effect on alterations in substrate utilisation, diverting from glucose oxidation to fatty acid oxidation and increasing ketone body use. Improved oxidative and dicarbonyl stress and decreased uric acid seem to play an important role in the cardio- and reno-protective effects of empagliflozin.

*Abbreviations:* AGEs, advanced glycation end products; BDH1, β-hydroxybutyrate dehydrogenase; BHB, β-hydroxybutyrate; CML, carboxymethyl lysine; NEFA, non-esterified fatty acids; GSH, reduced form of glutathione; GSSG, oxidised form of glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; MCP-1, monocyte chemoattractant protein-1; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; SGLT2i, sodium-glucose cotransporter 2 inhibitors; NRF2, nuclear factor-erythroid 2-related factor-2.

*Authors’ contribution:* H. Malinska and M. Huttl were responsible for the conception and design of the study, drafted and wrote the manuscript. M. Huttl, I. Markova, D. Miklankova, O. Oliyarnyk, J. Trnovska, J. Kucera, R. Sedlacek and M. Haluzik contributed to the analysis and interpretation of the data.

All authors revised the manuscript critically for intellectual content and approved the final version to be published.

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