Simultaneous SGLT2 inhibition and caloric restriction improves insulin resistance and kidney function in OLETF rats

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1. Introduction

Sodium-glucose cotransporter 2 (SGLT2) is highly expressed in the renal proximal tubule and inhibition of SGLT2 decreases plasma glucose by increasing urinary excretion. For this reason, SGLT2 inhibitors (SGLT2i) are rapidly emerging as a novel therapy for type 2 diabetes due to their effective hypoglycemic and potential cardio- and nephroprotective effects, while caloric restriction (CR) is a common behavioral modification to improve adiposity and insulin resistance. Therefore, both interventions simultaneously may potentially further improve metabolic syndrome by enhancing carbohydrate metabolism. To test this hypothesis, cohorts of 10-week old, male Long Evans Tokushima Otsuka (LETO) and Otsuka Long Evans Tokushima Fatty (OLETF) rats were treated with SGLT2i (10 mg luseoglifozin/kg/day x 4 wks) (OLETF only) and/or 30% CR (2 wks at 12 weeks of age). CR maintained body mass in both strains while SGLT2i alone did not have any effect on body mass. Simultaneous treatments decreased SBP in OLETF vs SGLT2i alone, decreased insulin resistance index (IRI), and increased creatinine clearance vs OLETF ad lib. Conversely, CR decreased albuminuria independent of SGLT2i. In conclusion, SGLT2i treatment by itself did not elicit significant improvements in insulin resistance, kidney function or blood pressure. However, when combined with CR, these changes where more profound than with CR alone without inducing chronic hypoglycemia.

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muscle AMPK phosphorylation in a dose-dependent manner, which may facilitate increased fatty acid oxidation in muscle (Xu et al., 2017). These findings are consistent with a reduction in carbohydrate oxidation and an increase in lipolysis and ketogenesis found in T2DM patients on SGLT2i therapy that was proportional to the duration of the therapy (Ferrannini et al., 2016). Collectively, these studies suggest that altered glucose metabolism during treatment with SGLT2i during conditions of metabolic derangement may alter lipid and protein metabolism as well.

Furthermore, applying metabolomic analyses demonstrated that 12 weeks of SGLT2i treatment in patients with T2DM or non-alcoholic liver disease improved metabolic pathways related to energy metabolism, and mitochondrial and endothelial function suggesting that impaired SGLT2 activity associated with metabolic defects contributes to a decline in kidney function (Muldner et al., 2020).

Severe (70%), long-term (7 wks) caloric restriction (Nakano et al., 2011) and chronic SGLT2i treatment (3 mo) (Kojima et al., 2013a) improved glucose tolerance and reduced proteinuria and glomerular damage in rat models of metabolic derangement (OLETF and T2DN, respectively); however, the potential benefits following more acute interventions of either or combined have not been examined during similar conditions. Therefore, we hypothesized that acute (10 d), mild caloric restriction (30%) combined with SGLT2i treatment improve insulin resistance and kidney function and health associated with improved glucose metabolism without inducing hypoglycemia. Furthermore, studies of the potential benefits of SGLT2i and/or CR in a model of metabolic syndrome (MetS), which is more complicated than T2D alone, are scarce, and those examining the combined effects are unrealized.

2. Results

2.1. CR blunts the incremental increase in BM and is not altered further with SGLT2i

Slope analysis was performed to better appreciate the impacts of all the treatments on the changes in BM (Fig. 1). Between 7 and 10 wks of age (before starting treatments), the incremental increase in BM was greater (p = 0.031) in OLETF (8.8 ± 0.8 g/d, r² = 0.99) compared to LETO (5.8 ± 1.0 g/d, r² = 0.99). After 10 wks of age, mean incremental increase was not different (p = 0.962) between ad lib-fed LETO (3.8 ± 0.3 g/d, r² = 0.98) and ad lib-fed OLETF (4.6 ± 0.9 g/d, r² = 0.98). The incremental change in BM in LETO CR (−1.1 ± 0.9 g/d, r² = 0.60) was lower (p = 0.003) compared to ad lib-fed LETO. CR in OLETF without (−0.1 ± 1.1 g/d, r² = 0.04) and with SGLT2i (−0.9 ± 1.5 g/d, r² = 0.16) decreased (p < 0.001) the incremental change in BM compared to ad lib-fed OLETF, and the slopes between the two CR groups were not different (p = 0.995). In ad lib-fed OLETF, the incremental increases in BM without SGLT2i (4.6 ± 0.9 g/d, r² = 0.98) was the same (p = 1) compared to OLETF SGLT2i (4.6 ± 0.7 g/d, r² = 0.98). The relatively low coefficients of determination (r²) and shallower slopes in all the CR groups suggest that BM was maintained relatively constant (incremental increase blunted) rather than reduced over time.

2.2. Simultaneous SGLT2i and CR reduced MetS-induced increase in SBP

Before the SGLT2i treatment at 9 wks of age, SBP was higher (p < 0.001) in ad lib-fed OLETF (140 ± 3 mmHg) compared to ad lib-fed LETO (118 ± 5 mmHg) confirming the MetS-induced increase in SBP (Fig. 2). At the end of the study, CR did not change SBP in either LETO (p = 0.995) or OLETF (p = 0.985). Moreover, SGLT2i without CR did not alter SBP in OLETF (p = 0.931). However, the simultaneous treatment of SGLT2i and CR decreased (p = 0.038) SBP compared to OLETF SGLT2i without CR by the end of the study.

2.3. Simultaneous SGLT2i and CR improved MetS-associated insulin resistance index

Glucose tolerance tests with corresponding insulin measurements and subsequent IRI calculations were performed to quantify the functional metabolic effects of both treatments alone and in combination. Mean glucose peak during oGTT (at 30 min) was reduced by 27% in OLETF SGLT2i CR rats compared to OLETF CR (p = 0.022) (Fig. 3A). Mean AUCglucose was 97% higher (p < 0.001) in ad lib-fed OLETF compared to ad lib-fed LETO and was not reduced by either SGLT2i or CR alone; however, the OLETF SGLT2i CR group was not different from LETO CR suggesting that the simultaneous treatment decreased AUCglucose (Fig. 3B). There were no significant differences in basal insulin between strains. However, mean basal insulin was 44% lower in OLETF SGLT2i CR compared to OLETF CR (p = 0.018) and OLETF SGLT2i (p = 0.008) (Fig. 3C). Moreover, neither of the treatments elicited a change in AUCinsulin in OLETF. AUCinsulin was 33% lower in LETO CR compared to OLETF CR (p = 0.041) (Fig. 3D). Mean IRI was 98% greater (p < 0.001) in ad lib-fed OLETF vs ad lib-fed LETO and was not reduced by SGLT2i or CR alone. However, the combined treatments reduced (p = 0.019) mean IRI in OLETF by 33% compared to CR alone (Fig. 3E). At 14 wks of age, basal fasting blood glucose concentrations were 35% greater (p < 0.001) in ad lib-fed OLETF compared to ad lib-fed LETO. Neither treatments reduced basal fasting blood glucose concentrations in either strain. SGLT2i treatments in OLETF did not reduce static fasting blood glucose concentrations. However, CR in LETO increased (p = 0.024) basal

![Fig. 1. Mean ± SE incremental change in body mass per day (n = 7). Data was not recorded during urine collection or after oGTTs and is denoted by gaps in data between one day to the next. m = mean slope of the line ± SE (g/d).](image1)

![Fig. 2. Mean ± SE systolic blood pressure (SBP) by weeks of age (n = 6) *p < 0.05 vs LETO, †p < 0.05 OLETF SGLT2i vs OLETF SGLT2i CR (by t-test).](image2)
glucose:insulin ratio by 66% (Table 1).

2.4. CR reduced SGLT2i-induced glycosuria

Urinary glucose excretion was minimally detectable (<2 mg/d) in all non-SGLT2i-groups without significant differences between strains. CR reduced (p < 0.001) the SGLT2i-induced glycosuria by 64% in OLETF (Fig. 4A). The robust glycosuria observed in the SGLT2i groups provides evidence of the drug’s effectiveness.

2.5. CR increased urinary Na\(^+\):K\(^+\) ratio and decreased fractional excretion of K\(^+\) (FE\(_{\text{K}}\)) in OLETF

Serum Na\(^+\) concentration was greater in LETO CR compared to OLETF CR (p = 0.021), without significant changes with either CR or SGLT2i. Neither treatment alone or in combination changed urinary electrolyte excretion; however, CR increased (p < 0.001) urinary Na\(^+\): K\(^+\) ratio by 90% in OLETF. FE\(_{\text{K}}\) was greater in ad lib-fed LETO compared to OLETF CR (p = 0.029) and OLETF SGLT2i CR (p < 0.001). Moreover, FE\(_{\text{K}}\) decreased in OLETF CR (p = 0.003) and OLETF SGLT2i CR (p = 0.008) compared to OLETF ad lib-fed SGLT2i control. (Table 2).
2.6. SGLT2i treatment resulted in negative correlations between aldosterone and Na\(^+\) excretion and urine volume without changes in circulating aldosterone

Plasma aldosterone was not significantly different between ad lib LETO and OLETF (\(p = 0.552\)) nor after CR (\(p = 0.494\)), SGLT2i (\(p = 0.913\)), or combined treatment (\(p = 0.506\)) in OLETF (Supplemental Fig. 1). Nevertheless, a moderate negative correlation was observed between aldosterone and urine volume (\(r = -0.759\)) in OLETF SGLT2i ad lib. In addition, a moderate positive correlation (\(r = 0.528\)) and a strong negative correlation (\(r = -0.852\)) between aldosterone and Na\(^+\) excretion was observed in OLETF CR and OLETF SGLT2i CR, respectively (Supplemental Table 1).

2.7. Uromodulin and NGAL excretion remained unaltered in OLETF compared to LETO, and after both CR and SGLT2i treatments

24 h uromodulin excretion showed a high intra-variability among all the groups, without significant difference between strains or treatments (Supplemental Fig. 2). In addition, mean 24 h NGAL excretion increased with SGLT2i treatment alone in OLETF and decreased in two CR groups. However, none of these changes reached significance (\(p = 0.083\) OLETF ad lib vs OLETF SGLT2i, \(p = 0.295\) LETO ad lib vs LETO CR and \(p = 0.058\) OLETF SGLT2i ad lib vs OLETF SGLT2i CR) (Supplemental Fig. 3).

Fig. 4. Mean ± SE 24 h (A) urinary glucose excretion (mg/d), (B) urinary albumin excretion (mg/d), (C) plasma creatinine (mg/dl), (D) urinary creatinine excretion (mg/d), (E) plasma/urine creatinine ratio (relative units), and (F) creatinine clearance (C\(_{cr}\)) (mL/min). \(^a\)\(p < 0.05\) vs. LETO, \(^b\)\(p < 0.05\) vs. Ad lib, \(^c\)\(p < 0.05\) vs. SGLT2i, \(^d\)\(p < 0.05\) vs. LETO CR. \(^*\)\(p < 0.05\) vs OLETF SGLT2i CR (by \(t\)-test).

2.8. CR improved albuminuria (UalbV) and combined treatment increased creatinine clearance (C\(_{cr}\)) in OLETF

Albuminuria was 15.6-fold greater (\(p < 0.001\)) in ad lib-fed OLETF compared to ad lib-fed LETO characteristic of the renal injury associated with the strain (Fig. 4B). Although SGLT2i alone did not reduce the albuminuria in OLETF, CR reduced mean UalbV by 74% (\(p = 0.003\)) and by 61% (\(p = 0.009\)) with SGLT2i (Fig. 4B). No profound changes in plasma creatinine were detected among the groups although levels tended to decrease (\(p = 0.062\)) with the combined treatment compared to OLETF SGLT2i. (Fig. 4C). Urinary creatinine excretion (UcrTV) was 31% greater (\(p = 0.001\)) in ad lib-fed OLETF compared to ad lib-fed LETO, and levels were similarly elevated with CR and/or SGLT2i (Fig. 4D). Combined treatment in OLETF increased (\(p = 0.015\)) Cr by 71% compared to LETO CR (Fig. 4E), which is reflective of the trending decrease in plasma creatinine in this group.
fed OLETF (0.4-fold increased glucose:G6P ratio in OLETF SGLT2i CR (1.1-fold compared to LETO, independent of CR (Fig. 5B). This translated into an SGLT2i CR vs 83% in OLETF SGLT2i (P < 0.001) and 87% in OLETF CR compared to LETO CR; however, SGLT2i treatment reduced IR expression by 104% (P < 0.029) (Fig. 6A). AMPK expression was consistent among groups (Fig. 6B); however, pAMPK increased by 56% (P < 0.002) in OLETF CR and 44% (P < 0.019) in OLETF SGLT2i CR compared to ad lib-fed OLETF (Fig. 6C). Despite these changes, the p-AMPK:AMPK ratio did not change significantly amongst groups (Fig. 6D). Relative Akt expression increased by 54% (P < 0.022) in OLETF CR compared to ad lib-fed OLETF (Fig. 6E). On the other hand, p-Akt:Akt ratio doubled in OLETF SGLT2i compared to ad lib-fed OLETF (2.3 ± 0.4 vs 1.0 ± 0.0, P = 0.022) (Fig. 6F). These changes were not observed with CR. No differences in GLUT4 translocation nor phosphorylation were detected among the groups (Fig. 6H–J).

### 2.9. Combined treatment increased hepatic Glucose:G6P ratio in OLETF, while hexokinase activity is reduced by CR in LETO and by SGLT2i in OLETF

We measured hepatic glucose and glucose-6-phosphate (G6P) content, the cytosolic content of the gluconeogenic enzymes, G6Pc and PCK1, and GLUT2 translocation. We also measured hexokinase (HK) activity, which is the first committed step in glycolysis. Mean hepatic G6P content was two-fold higher in LETO and by 104% (P < 0.001) and 87% in OLETF SGLT2i CR compared to LETO CR; however, SGLT2i treatment reduced IR expression by 104% (P < 0.029) (Fig. 6A). AMPK expression was consistent among groups (Fig. 6B); however, pAMPK increased by 56% (P < 0.002) in OLETF CR and 44% (P < 0.019) in OLETF SGLT2i CR compared to ad lib-fed OLETF (Fig. 6C). Despite these changes, the p-AMPK:AMPK ratio did not change significantly amongst groups (Fig. 6D). Relative Akt expression increased by 54% (P < 0.022) in OLETF CR compared to ad lib-fed OLETF (Fig. 6E). On the other hand, p-Akt:Akt ratio doubled in OLETF SGLT2i compared to ad lib-fed OLETF (2.3 ± 0.4 vs 1.0 ± 0.0, P = 0.022) (Fig. 6F). These changes were not observed with CR. No differences in GLUT4 translocation nor phosphorylation were detected among the groups (Fig. 6H–J).

### 2.10. SGLT2i decreased insulin receptor expression and increased Akt phosphorylation in OLETF independent of changes in GLUT4 translocation or phosphorylation in gastrocnemius muscle

Given that skeletal muscle is the primary site of glucose utilization and could partially explain the improvement in glucose tolerance, we measured insulin receptor (IR) abundance in the plasma membrane, as increased translocation to the plasma membrane is inversely correlated to receptor phosphorylation, and thus, linked to insulin resistance. In addition, we measured the phosphorylation of AMPK (pAMPK) and Akt (pAkt) as these are downstream events of IR activation. We also measured GLUT4 translocation to assess the potential for increased glucose absorption, and GLUT4 phosphorylation to assess transporter activation via insulin stimulation (Sadler et al., 2013). Relative IR expression was 2.3-fold greater (P = 0.011) in ad lib-fed OLETF compared to ad lib-fed LETO; however, SGLT2i treatment reduced IR expression by 104% (P < 0.029) (Fig. 6A). AMPK expression was consistent among groups (Fig. 6B); however, pAMPK increased by 56% (P < 0.002) in OLETF CR and 44% (P < 0.019) in OLETF SGLT2i CR compared to ad lib-fed OLETF (Fig. 6C). Despite these changes, the p-AMPK:AMPK ratio did not change significantly amongst groups (Fig. 6D). Relative Akt expression increased by 54% (P < 0.022) in OLETF CR compared to ad lib-fed OLETF (Fig. 6E). On the other hand, p-Akt:Akt ratio doubled in OLETF SGLT2i compared to ad lib-fed OLETF (2.3 ± 0.4 vs 1.0 ± 0.0, P = 0.022) (Fig. 6F). These changes were not observed with CR. No differences in GLUT4 translocation nor phosphorylation were detected among the groups (Fig. 6H–J).

### 2.11. CR increased the difference in primary carbon metabolism between strains, while combined treatments increased carboxylic acids metabolism in OLETF compared to SGLT2i

A plasma metabolomic analysis was performed to further elucidate which metabolic pathways were changed by SGLT2i and/or 30% CR, as we have previously found that short term CR induces strain-dependent changes (e.g. increased glycolysis, lipolysis, and proteolysis in OLETF but not LETO) (Cornejo et al., 2021). Additionally, metabolomics analyses enhance our understanding of the mechanisms regulating cellular, substrate metabolism. In this study, CR induced some static shifts in the plasma metabolome in OLETF: glycemic acid decreased 1.9-fold (q = 0.027) with CR (Fig. 5D) and 1.6-fold (q = 0.159) with SGLT2i + CR (Fig. 8F) compared to OLETF ad lib-fed controls. At basal levels, plasma oleic acid (4.8-fold, q = 0.102), ribitol (2.2-fold, q = 0.005) and ribose (1.9-fold, q = 0.031) were greater in ad lib-fed OLETF compared to ad lib-fed LETO (Fig. 7B). This difference was accentuated when comparing both strains after CR, where OLETF had higher concentrations of the carbohydrates, glucose (1.3-fold, q = 0.158), ribitol (3.1-fold, q < 0.001), ribose (2.4-fold, q < 0.001), xylulose (1.2-fold, q = 0.138), and sorbitol (1.7-fold, q = 0.126), the fatty acids, oleic (2.2-fold, q = 0.091) and palmitoleic (1.9-fold, q = 0.049), and the organic compounds, phenylglycinamide (2.4-fold, q = 0.126) and tyrosine (6.5-fold, q = 0.010). On the other hand, OLETF CR had lower concentrations of compounds related to amino acid metabolism including putrescine (22.2-fold, q = 0.153), glycine (2.1-fold, q < 0.001), and urea (1.3-fold, q = 0.072), and the fatty acids, azelaic acid (2-fold, q = 0.091), heptadecanoic acid (1.5-fold, q < 0.001), octadecanol (1.6-fold, q = 0.126), and stearic acid (1.4-fold, q = 0.060), and cholesterol (1.2-fold, q = 0.111) compared to LETO CR (Fig. 5C). Moreover, combined treatments in OLETF increased the carboxylic acids, cinnamic (4.8-fold, q = 0.106), nicotinic (3.5-fold, q = 0.155), and salicylic (4.4-fold, q = 0.129), and levoglucosan (1.5-fold, q = 0.147) and succinate semialdehyde (3-fold, q = 0.122), precursors of glucose and sucinic acid, respectively, compared to OLETF SGLT2i (Fig. 7I). Conversely, the concentrations of oleic (6-fold, q = 0.155), glucuronic (1.7-fold, q = 0.155), and glutaric acids (1.6-fold, q = 0.155), and phenylalanine (1.7-fold, q = 0.182), putrescine (1.6-fold, q = 0.155), and valine (1.3-fold, q = 0.155) were
3. Discussion

3.1. SGTL2i therapy does not further improve CR-mediated body mass loss

Slope analysis of body mass changes over time revealed that SGTL2i did not significantly alter CR-induced decrease in BM in the OLETF animals. Previous studies have suggested that SGTL2i may decrease BM initially due to increased diuresis (Washburn and Poucher, 2013); however, we did not find significant differences in urinary volume among the groups in our study. Other studies suggested that SGTL2i either increased fatty acid metabolism and/or reduced lipogenesis in diet-induced obese rats, decreasing liver and adipose mass (Liang et al., 2012). This decrease in organ masses could ultimately account for reduced BM, but likely beyond the duration of the time frame of our study duration. However, the maintenance of body mass despite the combined treatments suggests that, at least in our model, there are other mechanisms that prevent the reduction of basal glucose beyond a certain threshold even in the presence of glycosuria. We suggest that two factors in the liver have a major influence in maintaining a normal basal glucose in OLETF: (1) increased gluconeogenesis as a response to the combination of treatments, observed in the form of increased liver glucose: G6P ratio, and (2) a reduction of glycolysis in response to SGTL2i, shown as a reduction in hexokinase activity in the liver, and in response to CR, shown as a reduction in the concentration of glyceraldehyde, a product of glycolysis, in plasma.

3.2. Combined SGTL2i and CR treatment has potential anti-hypertensive and nephroprotective effects

Several clinical trials have shown a modest but consistent reduction in SBP in SGTL2i treated patients compared to other antidiabetic drugs after one to two weeks of treatment (Oliva and Bakris, 2014). However, the mechanisms are not well-defined, but may not be associated with reducing extracellular volume or a reduction of the RAAS activity in the present study. This is supported by the urinary results where FE\(\text{Na}\) and \(U_{\text{Na}}\) were unaffected by SGTL2i, and by plasma aldosterone concentration, which did not show a significant decrease with any of the treatments (Supplemental Fig. 1). Despite this, a moderate negative correlation was observed between aldosterone concentration and urinary volume after SGTL2i treatment, and a strong negative correlation between aldosterone and \(\text{Na}^{+}\) excretion after combination treatment, suggesting a normalization of the aldosterone function in SLGT2i treated OLETF rats (Supplemental Table 1). Moreover, the lack of an increase in \(U_{\text{Na}}\) in conjunction with the SGTL2i-induced glycosuria may reflect compensatory increases in distal tubule \(\text{Na}^{+}\) reabsorption via multiple channels (i.e., ENaC, NCC, NKCC etc.) that is further accomplished by CR via reduced dietary \(\text{Na}^{+}\) intake. An increase in NHE3 and NCC expression has been observed in the renal cortex of luseogliflozin-treated spontaneously hypertensive rats (Ansary et al., 2019). Additionally, FE\(\text{K}\) tended to increase with SGTL2i alone suggesting that K\(^{+}\) reabsorption was decreased, which may reflect a potential downstream effect on Na\(^{-}\)-K\(^{+}\) exchange in the distal tubule that would be characterized by an increase in Na\(^{+}\) reabsorption at the expense of K\(^{+}\) (Maciel et al., 2014). In our study, neither SGTL2i nor CR alone reduced SBP.
However, when comparing the combined treatment in OLETF to the untreated ad lib-fed control group at the end of the study, we observed a significant reduction in SBP comparable to that previously reported in the literature (Guthrie, 2013) suggesting that longer duration treatments could have had a more robust effect on SBP.

We also assessed basal renal function between strains, as the OLETF develops nephropathy over time (Nakano et al., 2011), with marked proteinuria after 25 weeks of age (Nagai et al., 2005; Kawano et al., 1994) and impaired function, which may diminish the benefits of SGLT2 inhibition. Moreover, previous clinical studies revealed that SGLT2i decreased urinary albumin-to-creatinine ratio, a marker of diabetic nephropathy (DN) (Fioretto et al., 2016). The maintenance of plasma creatinine concentrations across the groups suggests that, at this stage of the MetS, the renal injury was not sufficient to substantially reduce kidney function. In addition, the excretion of uromodulin and neutrophil gelatinase-associated lipocalin (NGAL), both biomarkers of kidney function, were not significantly reduced in OLETF groups suggesting that interstitial fibrosis or tubular atrophy in young OLETF rats is absent or at least undetectable (Supplemental Figs. 2 and 3) (Rysz et al., 2017; Dekkers et al., 2018). Nonetheless, increased albuminuria was detectable in the OLETF compared to the LETO, which was ameliorated with CR, independent of treatment with SGLT2i. These observations reinforce the notion that CR has potential nephroprotective effects, especially when accompanied by a net decrease in protein intake (Brenner, 1983).
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which would be associated with the CR in the present study. Reduced protein intake has been associated with amelioration of glomerulosclerosis in some non-obese rat models, while overall CR has a beneficial effect on aging in general (Stern et al., 2001). Moreover, a reduction in protein intake (either isocaloric or in combination with 20% CR) starting at 8 w.o. provided nephron-protection in the form of reduced urinary albumin excretion at 29 w.o. in OLETF rats (Sakamoto et al., 2006). However, independent of the CR-associated benefits on glomerular injury (Cherney et al., 2014; Škrtič et al., 2014), the nephro-protective effects were not further enhanced with SGLT2i in our model suggesting that the suite of defects associated with MetS prohibit further improvements in the condition with the combined treatments. This is significant because it re-enforces the idea that early detection and intervention are critical to maximize the effectiveness of treatments when combating the multitude of defects that are associated with MetS.

3.3. Urinary glucose excretion was proportional to caloric intake and independent of fasting glucose

We measured 24 h glucose excretion to assess the efficacy of the SGLT2i treatment. Although fasting glucose concentration was not different between the untreated OLETF and SGLT2i treated rats, excreted glucose was several orders of magnitude greater in the SGLT2i treated groups compared to the others, clearly indicating the effectiveness of the drug. A negligible amount of excreted glucose was detected in the other groups, consistent with our previous results from similarly aged rats (Choi et al., 2020), demonstrating that the severity of the hyperglycemia at this stage of the disease is not sufficient to induce robust glycosuria. Interestingly, the reductions in excreted glucose with CR did not translate to detectable changes in fasting glucose. These data may seem to conflict with previous findings where fasting serum glucose in dapagliflozin-treated patients was positively correlated with cumulative urine glucose (Komoroski et al., 2009). Moreover, results from SGLT2-deficient mice suggest that an increase in caloric intake and/or a potential increase in glucose absorption via SGLT1 would be sufficient to

Fig. 7. Metabolomic maps of 9 comparisons of interest, comparing fold-changes in mean plasma concentrations of detected metabolites. Only metabolites with p < 0.05 were mapped per comparison, and fold-changes are shown for metabolites with q < 0.2. Fold-changes are proportional to figure size.
normalize blood glucose and maintain body mass similar to wild type mice, without changes in electrolytes, blood pressure, or GFR (Vallon et al., 2011). Furthermore, OLETF rats have increased intestinal expression of SGLT1 compared to LETO rats (Fujita et al., 1998), which may partially explain the differential responses and lack of glycosuria-induced decrease in fasting blood glucose. We propose that compensatory mechanisms including an increase in SGLT1-mediated systemic absorption and release of hepatic glucose (gluconeogenesis and/or glycogenolysis) were sufficient to maintain circulating levels. This is of clinical relevance because the combination of SGLT2i and CR can potentially induce bouts of hypoglycemia, which was one of the safety issues we intended to address in this study. Although there is evidence of increased gluconagon:insulin ratio and ketosis in SGLT2i treated TD2 patients, this is not consistently found in clinical trials and could be due to several negative feedback mechanisms (Zhu et al., 2021). Ironically, despite the insulin resistant condition in the OLETF, intact and fully functional compensatory mechanisms may prohibit the combined therapies from completely ameliorating the strain-associated hyperglycemia at this stage.

### 3.4. Combined treatments improved the insulin resistance independent of sustained improvements in IR signaling or Akt activation in skeletal muscle

Insulin resistance index, a surrogate measure for systemic insulin resistance in the pre-diabetic state, was reduced by the combined treatments. We measured IR expression as well as AMPK and Akt phosphorylation in skeletal muscle to help elucidate mechanisms associated with insulin signaling (Coughlan et al., 2014). Moreover, impaired IR, and by consequence, Akt activation, decrease glucose infusion rate (a measure of insulin sensitivity) and endogenous glucose production (Kubota et al., 2011). Although we measured insulin receptor expression (specifically in the membrane) rather than phosphorylation, previous studies with 30% CR in Fischer 344 x Brown Norway showed that glucose uptake was increased in slow and fast twitch muscle via increased Akt phosphorylation independent of insulin receptor (IR) phosphorylation (Sequea et al., 2012).

However, the decrease of IRI in our study could not be completely explained by the decrease in insulin receptor expression or Akt activation in the muscle, as these changes paradoxically occurred only after treatment with SGLT2i alone, but not with the combined treatment. Although Akt phosphorylation was not increased in the CR groups, 40% CR stimulated Akt2 phosphorylation, thus was isofrom specific (Mecurdy et al., 2003), which was associated with increased GLUT4 translocation (Calera et al., 1998; Hajduch et al., 2001). Nevertheless, our results suggest that SGLT2i, rather than CR, may be more beneficial in activating total Akt.

A previous study with 30% CR in OLETF showed stable levels of fasting glucose and circulating glucose over time, even after 42 weeks of age, reportedly due to a higher expression and translocation of adipocyte GLUT4 in the CR group (Park et al., 2005). In our study, however, GLUT4 translocation in the muscle was not different across groups. These results coincided in part with previous results which showed that long-term 30% CR in OLETF reduced the expression of GLUT4 mostly at the intracellular membranes rather than the plasma membrane (Okauchi et al., 1995). One distinction of our study, however, is that we measured GLUT4 in the fasted state when adaptation to the perturbation may have already occurred and not post-prandially, which would have stimulated a potential response. The potential changes in postprandial GLUT4 translocation are dependent of acute insulin stimulation and at baseline (i.e. fasted state) the rate of GLUT4 endocytosis may exceed exocytosis (Yang and Holman, 1993). Nonetheless, our data confirms that the rate of GLUT4 translocation is similar in both strains regardless of treatment in the fasted state. Furthermore, the improvements in insulin resistance index observed with CR and SGLT2i as calculated from the oGTT data suggest that insulin signaling and GLUT4 translocation (and potentially GLUT2 in those tissues) at the time of the GTT were enhanced and that these changes were not captured by the end of study analyses. Thus, the interventions performed here did not induce sustained improvements in IR signaling and glucose uptake (as measured by end of study analyses), which may reflect adaptations to the treatments resulting in glucose homeostasis regardless of the metabolic condition at the time of sampling.

### 3.5. 30% CR did not induce proteolysis and elicited more changes in the metabolome compared to SGLT2i alone

In our previous study, a 50% CR increased the concentration of several plasma amino acids along with an acute reduction in BM that could not be completely accounted for by the reductions in adipose and organ mass losses suggesting that lean BM loss and proteolysis were sufficient to contribute to total BM loss (Cornejo et al., 2020; Luan et al., 2002). In contrast, 30% CR with or without SGLT2i did not significantly
increase plasma amino acid concentrations in OLETF but rather a modest decrease in glyceric acid, a product of glycogenolysis, was detected suggesting that glycogen stores may have been sufficiently reduced at this stage of the CR. Moreover, CR induced an initial reduction in body mass that was maintained during the entire intervention, regardless of treatment with SGLT2i. These changes suggest that the degree of CR was not sufficient to induce significant proteolysis in OLETF rats. Furthermore, the decrease in total adipose with CR suggests that lipolysis was increased and was sufficient to ameliorate the potential for promoting proteolysis (and thus lean tissue catabolism), which is beneficial to maintain lean tissue and ameliorate the nitrogen load on the kidneys especially in the presence of existing glomerular injury.

3.6. Limitations

The present study focuses on comparing different endpoints between either strain or treatment to assess the degree of improvements in insulin resistance and nephroprotective and anti-hypertensive potential. However, because of some study limitations, the interpretation of some results must be made with balanced caution. Presentation of end-of-study results without including changes over time do not provide a complete examination of the changes. For example, the modest changes in estimated GFR and albumin-creatinine ratio at the end of the study are important, but do not provide insight on the progression of these changes over time. Moreover, serum creatinine measurements could confound the assessment of AKI in the context of SGLT2i therapy because SGLT2i and RAAS disruptors can increase creatinine initially (Chu et al., 2022). More robust conclusions could be drawn by measuring eGFR over time to assess the potential induction of AKI prior to acute luseogliflozin treatment. It should be noted that, although SGLT2 inhibitors as a drug class reduce macroalbuminuria and improve GFR, they also present different degrees of AKI risk at the beginning of the regimen due to their pharmacokinetic profile, SGLT2 selectivity, and potential off-target effects. Some of these off-target effects include greater SGLT2 vs SGLT1 selectivity of empagliflozin than canagliflozin, and canagliflozin’s ability to activate AMPK, which can increase the respiratory burden in the tubular cells (Chu et al., 2019). Regarding luseogliflozin specifically, short term treatment acutely reduced eGFR in several patients in clinical trials, possibly due to functional changes in renal hemodynamics. Nonetheless, this acute decline was also correlated to patient BP and BMI, and eGFR decline decreased in the long term (Kohagura et al., 2020). In short, we caution against drawing conclusions from luseogliflozin as a representative of this class of drug (SGLT2), but rather suggest making conclusions on nephroprotective effects on a drug-to-drug basis.

Moreover, analyzing the expression of renal genes contributing to endothelial function and oxidative stress (e.g. Nox1 and Dusp15) (Zeng et al., 2022) would be of additive value to this study, especially when considering the short duration of the manipulation. The short-study duration could also be a limitation, but this was purposeful as, at the time of the study, the effects of combined therapy by SGLT2i and CR were not sufficiently established to determine if the potential for sustained hypoglycemia was realistic. Thus, the short-duration manipulations were purposeful to assess the effects independent of sustained hypoglycemia. Lastly, robust histological evaluation of the kidney at the end of the treatments would provide additional insights, which are missing in the literature.

3.7. Conclusions

The present study demonstrated that the combined treatment of 30% CR with SGLT2i ameliorated insulin resistance and provided modest nephroprotective and anti-hypertensive benefits without the deleterious effects of sustained hypoglycemia and lean tissue catabolism. The lack of sustained and consistent changes in cellular insulin signaling and the metabolome with treatments between strains may reflect compensatory changes in cellular metabolism in response to caloric restriction and minute perturbations in glucose metabolism over time that were not captured by our end of study measurements. Nonetheless, the results of the present study suggest that modest caloric restriction can be safely combined with SGLT2 inhibition to ameliorate most of the consequences of the metabolic syndrome such as hypertension, impaired glucose tolerance, and insulin resistance, adiposity, and potentially the dyslipidemia (over a longer duration intervention). Ironically, the initial perturbations in glucose metabolism associated with both treatments may initiate compensatory mechanisms that mask more profound benefits suggesting that a longer duration intervention of both may be necessary to allow for greater benefits once re-adaptation of these mechanisms has been achieved to overcome the compensation.

4. Methods

Animals. Male, lean strain-control LETO (n = 15) and obese, insulin resistant Otsuka Long Evans Tokushima Fatty (OLETF) (n = 29) rats (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) were fed ad libitum with standard laboratory rat chow (MF, Oriental Yeast Corp., Tokyo, Japan) until 10 weeks of age. Both strains of rats were matched for age through the study. OLETF rats were then randomly assigned to either (1) untreated (n = 14; equivalent volume of saline solution (0.9%) x 4 wks) or (2) SGLT2i treated (n = 15; 10 mg luseogliflozin/kg/d x 4 wks by gavage) groups. Luseogliflozin was dissolved in deionized water (0.25% w/v) prior to dosing. At 12 wks of age, LETO rats were randomly assigned to either (1) untreated, (ad libitum-fed, strain controls (LETO Ctrl; n = 8) or (2) untreated, calorie restricted (LETO CR; n = 7; 30% CR x 2 wks) groups. Likewise, both OLETF groups were randomly assigned to (1) untreated, (ad libitum-fed, within-strain controls (OLETF Ctrl; n = 8), (2) SGLT2i treated, (ad libitum-fed (OLETF SGLT2i; n = 7), (3) untreated, 30% CR (OLETF CR; n = 7), or (4) SGLT2i treated + 30% CR (OLETF SGLT2i CR; n = 7) (Fig. 8). A 10 mg/kg/day dosing regime was established based on a previous, longer term study in T2DN rats (Kojima et al., 2013b). Although lower doses of the SGLT2i empagliflozin (0.6 mg/kg/day) have demonstrated cardio-renal protective effects (Zeng et al., 2022), higher doses have resulted in more profound effect on glucose excretion without considerable deleterious effects in a diabetic condition. SGLT2 inhibitors were developed to ameliorate the consequences of T2D, thus there is no expectation that SGLT2i would be prescribed to healthy, non-diabetic individuals. Therefore, providing SGLT2i to the healthy LETO rats would not provide further insights to the undiscovered, biomedical benefits of such a treatment during metabolic derangement, which is a principal outcome of the present study. Although we recognize the potential to enhance our knowledge of the basic biology of the SGLT2 transporter by providing the inhibitor to healthy animals, the focus of the present study was on the biomedical implications during a condition of impaired glucose metabolism, which is associated with metabolic syndrome, for which the drug was produced. Thus, the exclusion of both SGLT2i treated LETO groups (ad libitum and CR) was intentional for the sake of practicality and safety as the potential risk of hypoglycemia-induced perturbations in this strain surpasses the potential benefits to enhancing our knowledge in our estimation. All animals were maintained in groups of two animals per cage for the entire study to minimize stress (Manouze et al., 2019) except during CR, which was necessary to ensure the appropriate degree of CR. Ad libitum food intake per rat was calculated as the mean intake for double occupancy cages. Mean food intake for all the groups at the start (12 w.o.) and the end of CR (14 w.o.) can be found in Supplemental Table 2. Two days before oral glucose tolerance test (oGTT), all animals were transferred to metabolic cages for 24 h to collect urine for further biochemical analyses, while maintaining their previous level of food intake. Animals were maintained in a specific pathogen-free facility under controlled temperature (23°C) and humidity (55%) with a 12:12 light-dark cycle. All animals were given free access to water for the
4.1. Blood pressure

Systolic blood pressure (SBP) was routinely measured in triplicate in conscious rats by tail-cuff plethysmography (Bp- 98A; Softron Co., Tokyo, Japan) as previously described (Rodriguez et al., 2012; Thorwald et al., 2019). Rats were acclimated to the restraint tube prior to measurements. Measurements were made every other day after treatment for the entire study. Repeated measures with a percent coefficient of variability (CV) > 15% were excluded. Measurements were averaged by week and analyzed by one-way ANOVA.

4.2. oGTT

oGTTs were performed in all animals at 14 wks of age as previously detailed in our hands (Vazquez-Medina et al., 2013). Briefly, a 2 g/kg glucose bolus was given by gavage to overnight-fasted (14 h) rats. Blood was collected via the caudal vein before gavage and at 5, 10, 30, 60, and 120 min.

4.3. Dissections

Three days after oGTTs, animals were fasted overnight, and tissues collected the subsequent morning. After body mass (BM) measurements were obtained, animals were anesthetized with 100 mg pentobarbital/kg and collected the subsequent morning. After body mass (BM) measurements were made and analyzed by one-way ANOVA. Measurements were performed in all animals at 14 wks of age as previously detailed in our hands (Vazquez-Medina et al., 2013). Briefly, a 2 g/kg glucose bolus was given by gavage to overnight-fasted (14 h) rats. Blood was collected via the caudal vein before gavage and at 5, 10, 30, 60, and 120 min.

4.4. Biochemical analyses

Plasma creatinine, and urine glucose, creatinine, albumin, Na⁺ and K⁺, were measured using a Hitachi 7020 chemistry analyzer (Diamond Diagnostics, Massachusetts, USA). Serum Na⁺ and K⁺ measurements were outsourced to a local commercial lab (Central Valley Diagnostic Lab, Merced, CA, USA). Plasma insulin (Wako, Osaka, Japan), and plasma aldosterone, urine uromodulin and urine neutrophil gelatinase-associated lipocalin (NGAL) (Abcam, Boston, MA, USA) were measured using commercially available ELISA kits. Liver glucose (Wako, Osaka, Japan), glycogen (Cell Biolabs, San Diego, USA), and glucose-6-phosphate (G6P) content, and hexokinase (HK) activity (Sigma-Aldrich, St. Louis, USA) were measured by commercial available colorimetric kits, with values normalized to total protein (TP) content. All samples were analyzed in duplicate and run in a single assay with intra-assay percent CV of <10% for all assays. Creatinine clearance (Ccr) was calculated to estimate glomerular filtration rate (GFR) as: Ccr (mL/min) = urine creatinine (mg/dl) x urine flow (mL/min)/plasma creatinine (mg/dl).

(Bazzano et al., 2015). Fractional excretion of Na⁺ and K⁺ (FE(\text{Na}⁺/\text{K}⁺)) was calculated as:

\[ \text{FE} = \left( \frac{\text{urinary excretion of } [x]}{\text{serum } [x]} \right) \times \left( \frac{\text{TP} \times 100}{\text{urinary excretion of } [x]} \right) \]

4.5. Protein expression by Western blot

Prior to processing samples for Western blot analyses, they were washed with PBS to minimize the potential impacts of residual blood on the analyses. Homogenates of gastrocnemius muscle and liver were separated into cytosolic and plasma membrane fractions. The cytosolic fraction was obtained by homogenizing 15–25 mg of tissue in 200 μl KPi buffer containing 1% protease and 3% phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, USA) and centrifuging (15,000 rpm × 15 min) to separate. The pellet was then homogenized and sonicated (20 s) in 150 μl KPi buffer with 1% Tween-20, 1% protease and 3% phosphatase inhibitor cocktail, and centrifuged in the same conditions to extract the membrane fraction. The purity of the fractions was assessed by measuring fraction-specific proteins (α-tubulin for cytosol and Na⁺-K⁺ATPase for membrane). Supernatant total protein content was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Predetermined amounts of total protein (TP) were resolved in a 10% Tris-HEC SDS gel. Proteins were electroblotted using the Invitrogen™ Mini Blot module onto a 0.45 μm Immobilon-FL polyvinylidene difluoride (PVDF) membrane for 1 h at 20V. Membranes were blocked 1 h with Odyssey blocking buffer (TBS) and incubated 1 h at 4 °C with primary antibodies diluted in TBS with 0.2% Tween-20 targeted against GLUT2 (RRID: AB_2890623) (1:1000 dilution, 20 μg TP), GAPC (RRID: AB_10883319) (1:500 dilution, 40 μg TP), PKC1 (RRID: AB_2160031) (1:4000 dilution, 6 μg TP), AMPK (RRID: AB_915794) and p-AMPK (RRID: AB_330330) (1:1000 dilution, 40 μg TP), Akt (RRID: AB_915783) and p-Akt (RRID: AB_2315049) (1:1000 dilution, 30 μg TP), IR (RRID: AB_2280448) (1:1000 dilution, 12.5 μg TP), GLUT4 (RRID: AB_2191441) and p-GLUT4 (RRID: AB_2890624) (1:1000 dilution, 8 μg TP), α-tubulin (RRID: AB_2210057) (1:40,000 dilution, 5–20 μg TP), and Na⁺-K⁺ATPase (RRID: AB_1310695) (1:40,000 dilution, 5–20 μg TP). Membranes were washed with TBS with 1% Tween-20 and incubated for 1 h with a secondary antibody (LI-COR Biosciences, Lincoln, USA) diluted 1:20,000 in TBS with 0.2% Tween-20 and 0.1% SDS, rewashed and visualized using a Li-Cor Odyssey Imaging System.

4.6. Metabolomic analysis

Aliquots of plasma (n = 6–7/group) were analyzed for primary carbon metabolites by gas chromatography (GC) time-of-flight (TOF) mass spectrometry (MS) data acquisition and processed at the NIH West Coast Metabolomics Center at UC Davis as previously described (Fiehn, 2016), generating a dataset of 136 consistently identified metabolites. Data were reported as the mTIC normalized (Borrego et al., 2016) quantitative ion peak heights of all structurally annotated compounds. Data was rank transformed (Conover and Iman, 1981) as previously described (Dhillon et al., 2018). Student’s t-test was performed in 9 previously selected pairwise comparisons (Table 3) and Benjamini-Hochberg False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995) q-values were calculated for each metabolite per comparison. Fold changes were calculated per comparison as mean group B/mean group A, after mTIC normalization. Network maps were plotted per comparison using MetaMapp (Barupal et al., 2012) and visualized in Cytoscape 3.7.1.

Table 3

| Comparison | Group A | Group B |
|------------|---------|---------|
| A          | LETO Ad | LETO CR |
| B          | LETO Ad | OLETF Ad |
| C          | LETO CR | OLETF CR |
| D          | OLETF Ad | OLETF CR |
| E          | OLETF Ad | OLETF SGLT2i |
| F          | OLETF Ad | OLETF SGLT2i + CR |
| G          | OLETF CR | OLETF SGLT2i |
| H          | OLETF CR | OLETF SGLT2i + CR |
| I          | OLETF SGLT2i | OLETF SGLT2i + CR |
4.7. Statistics

Means (±SE) were compared by one-way ANOVA, with the Holm-Sidak method for post-hoc multiple comparison after excluding outliers by extreme studentized deviate test with α = 0.05 in order to preserve statistical power without violating the assumption of normality. Glucose tolerance was assessed by comparing mean AUC values obtained from the glucose profiles during the GTT. The accompanying AUCs of the insulin response during the GTT were also calculated. The AUC values were also compared by one-way ANOVA. The insulin resistance index (IRI) was calculated as the product of AUCglucose and AUCinsulin divided by 100 as previously described (Cornejo et al., 2020; Rodríguez et al., 2012) and used to assess the effects on MetS-associated insulin resistance observed in OLETF. It should be noted that IRI is an accurate surrogate measure for glucose tolerance for our animal model, as older (23 w.o.) OLETF rats still retain insulin secretion capacity, although with decreased insulin sensitivity (Kanazawa et al., 1997).

Relationships between dependent and independent variables were evaluated by simple regression, except for insulin, where a 4th order regression was used. Correlations were evaluated using Pearson correlation coefficients. Means, regression, and correlations were considered significantly different at p < 0.05. Statistical analyses were performed with JMP 15 software (Cary, USA).

Ethics approval

The Animal Experimentation Ethics Committee at Kagawa University, Kagawa, Japan approved the experimental protocols (ID: 21614). All experimental procedures were conducted in accordance with the guidelines for care and use of animals established by Kagawa University.

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CRediT authorship contribution statement

Manuel A. Cornejo: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. Eira Jardines: Formal analysis, Investigation. Akira Nishiyama: Conceptualization, Methodology, Supervision, Resources. Daisuke Nakano: Conceptualization, Methodology, Supervision, Resources. Rudy M. Ortiz: Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors do not declare any conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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step of glucose intolerance in Otsuka Long-Evans Tokushima Fatty rats. Diabetes 41, 1459-1466. https://doi.org/10.2337/diabetes.41.11.1459.

Guthrie, R.M. 2013. Sodium glucose co-transporter 2 inhibitors and the potential for cardiovascular risk reduction in patients with type 2 diabetes mellitus. Postgrad. Med. 125 (3), 21-32. https://doi.org/10.3810/pgm.2013.05.2654.

Hadjis, E., Luther, F., Huland, H.S., 2001. Protein kinase B (PKB/Akt) - a key regulator of glucose homeostasis? FEBS Lett. 513, 1-8.

Hoehrer, B., Tsuchiya, O. 2017. Diabetic nephropathy: reparative effects of glucagon-like peptide 1 agonists and SGLT2 inhibitors. Nat. Rev. Nephrol. 13 (12), 3-4. https://doi.org/10.1038/nrneph.2017.140.

Kalra, S., Jacob, J.J., Gupta, Y., 2016. Newer antidiabetic drugs and calorie restriction mimicry. Indian J Endocrinol Metab. https://doi.org/10.4103/2231-8486.185022.

Kanazawa, M., Tanaka, A., Nomoto, S., et al., 1997. Alterations of insulin and glucagon secretion from the perfused pancreas before, at the onset and after the development of diabetes in male Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Diabetes Res. Clin. Pract. 38 (3), 161–167. https://doi.org/10.1016/S0168-8227(97)00126-2.

Kawano, K., Hirashima, T., Mori, S.N.T., 1994. OLETF (Otsuka Long-Evans Tokushima Fatty) rat: a new NIDDM rat strain. Diabetes Res. Clin. Pract. 24, S317–S320.

Kohagura, K., Yamasaki, H., Takano, H., Ohya, Y., Seino, Y., 2020. Luseogliflozin, a sodium-glucose cotransporter 2 inhibitor, preserves renal function irrespective of acute changes in the estimated glomerular filtration rate in Japanese patients with type 2 diabetes. Hypertens. Res. 43 (9), 876-883. https://doi.org/10.1080/14737160.2020.1840262.

Kojima, N., Williams, J.M., Takahashi, T., Miyata, N., Roman, R.J., 2013a. Effects of a new SGLT2 inhibitor, luseogliflozin, on diabetic nephropathy in T2DN rats. J. Pharmacol. Exp. Therapeut. 345 (3), 464–472. https://doi.org/10.1124/jpet.113.209869.

Komoroski, B., Vachharajani, N., Feng, Y., Li, L., Kornhauser, D., Pfister, M., 2009. Flavin-adenine dinucleotide-dependent enzymatic browning and attenuates inflammation and insulin resistance by polarizing M2 macrophages in type 2 diabetes. J. Biol. Chem. 285 (15), 11237–11244. https://doi.org/10.1074/jbc.M808515200.

Kubota, T., et al., 2011. Impaired insulin signaling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. Cell Metabol. 13 (3), 294–307. https://doi.org/10.1016/j.cmet.2011.01.018.

Li, J., et al., 2012. Effect of canagliflozin on renal threshold for glucose, glycemia, and body weight in normal and diabetic animal models. PLoS One 7 (2), 2–8. https://doi.org/10.1371/journal.pone.0030555.

Luan, Y., Hirashima, T., Man, Z.W., Wang, M.W., Kawano, K., Sumida, T., 2002. Pathogenesis of obesity by food restriction in OLETF rats: Increased intestinal monosaccharide acyltransferase activities may be a crucial factor. Diabetes Res. 57 (2), 75–82. https://doi.org/10.1016/S0168-8227(02)00026-6.

Maccil, A.T., Park, M., Maceo, E., 2014. Fractional excretion of sodium in the course of acute kidney injury in critically ill patients: potential monitoring tool? Rev Bras. Ter Intensiva 26 (2), 143–147. https://doi.org/10.1590/S0100-71582014000200003.

Manouze, H., Ghenem, A., Poisset, V., Bennis, M., Be-Athman, S., Benoile, J.J., Becker, C., Bernard, C., 2019. Effects of single cage housing on stress, cognitive, and body weight in normal and diabetic animal models. PLoS One 7 (2), 259–268. https://doi.org/10.1371/journal.pone.0025995.

Martoń, A., Akehi, Y., Minuma, G., et al., 2006. The suppressive effects of dietary calorie restriction: effects of body composition, insulin signaling and aging calorie restriction in obesity: prevention of kidney disease in rodents I. J. Nutr. 131 (3), 913S–917S. https://doi.org/10.1093/jn/131.3.913S.

Tanaka, S., Sugiyama, Y., Saito, H., Sugahara, M., Higashiyama, Y., Yamaguchi, J., Inagi, R., Suzuki, M., Nagakura, M., Tanaka, T., 2018. Sodium-glucose cotransporter 2 inhibition normalizes glucose metabolism and suppresses oxidative stress in the kidneys of diabetic mice. Kidney Int. 94 (5), 912–925. https://doi.org/10.1016/j.kint.2018.04.025.

Thorwald, M.A., Godoy-Lugo, J.A., Rodriguez, G.J., Rodriguez, M.A., Jamal, M., Kinoshita, H., Nakano, D., Nishiyama, A., Forman, H.J., Ortiz, R.M., 2019. Nrf2-related gene expression is impaired during a glucose challenge in type II diabetic rat hearts. Free Radic. Biol. Med. 130 (July 2018), 306–317. https://doi.org/10.1016/j.freeradbiomed.2018.04.005.

Vallon, V., Platt, A.K., Cunard, R., Schroth, J., Whaley, J., Thomson, S.C., Koepell, H., Tieg, N., 2011. SGLT2 mediates glucose reabsorption in the early proximal tubule. J. Am. Soc. Nephrol. 22 (1), 104–112. https://doi.org/10.1681/ASN.2010030246.

Vazquez-Medina, J.P., Popovich, I., Thorwald, M.A., Viscarra, J.A., Rodriguez, R., Sonnance-Organs, J.G., Laim, I., Pet-Periardi, J., Nakano, D., Nishiyama, A., Ortiz, R., 2013. Angiotensin receptor-mediated oxidative stress is associated with impaired cardiac redox signaling and mitochondrial function in insulin-resistant rats. AJP Hear Circ Physiol 305, 599–513. https://doi.org/10.1152/ajpheart.00101.2013.

Washburn, W.N., Poucher, S.M., 2013. Differentiating sodium-glucose co-transporter 2 inhibitors in development for the treatment of type 2 diabetes mellitus. Expert Opin. Invest. Drugs 22 (4), 463–486. https://doi.org/10.1517/13543784.2013.774392.

Xu, L., Nagata, N., Nagashimada, M., Zhuge, F., Ni, Y., Chen, G., Mayoux, E., Kaneko, S., Wu, H., 2013. Angiotensin receptor-mediated oxidative stress is associated with impaired cardiac redox signaling and mitochondrial function in insulin-resistant rats. AJP Hear Circ Physiol 305, 599–513. https://doi.org/10.1152/ajpheart.00101.2013.

Yamada, K., et al., 2015. Effects of a sodium-glucose co-transporter 2 selective inhibitor, ipragliflozin, on the diurnal profile of plasma glucose in patients with type 2 diabetes: a study using continuous glucose monitoring. J Diabetes Invest 6 (6), 699–707. https://doi.org/10.1111/jdi.12577.

Yang, J., Holman, G.D., 1993. Comparison of GLUT4 and GLUT1 subcellular trafficking in basal and insulin-stimulated 3T3-L1 cells. J. Biol. Chem. 268 (7), 4600–4603. https://doi.org/10.1074/jbc.268.7.4600.

Zeng, S., Delic, D., Chu, C., et al., 2012. Antiinfective effects of dose SGLT2 Inhibition with empagliflozin in comparison to Ang II receptor blockade with telmisartan in 5/6 nephrectomised rats on high salt diet. Biomed. Pharmacother. 66 (3), 317–322. https://doi.org/10.1016/j.biopha.2011.07.006.

Zou, X., Lin, C., Li, H., Su, C., X., J., 2021. SGLT2 increased the plasma fasting glucose level in patients with diabetes: a meta-analysis. Eur J. Pharmacol. 903 (May). https://doi.org/10.1016/j.ejphar.2021.174145.