Official Study Title: Effect of Empagliflozin on Hepatic Glucose Metabolism: Role of Autonomic Nervous System

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Unique Protocol ID: HSC20170214H

Protocol Template Form

Item 1 UTHSCSA Tracking Number	HSC20170214H

Item 2 Abstract / Project Summary	Provide a succinct and accurate description of the proposed research. State the purpose/aims. Describe concisely the research design and methods for achieving the stated goals. This section should be understandable to all members of the IRB, scientific and non-scientific. **DO NOT EXCEED THE SPACE PROVIDED**.

Purpose/Objectives: To investigate the effect of empagliflozin, an SGLT2 inhibitor on hepatic glucose production and the role of autonomic nervous system in mediating the increase in hepatic glucose production in response glucosuria

Research Design/Plan: the role of autonomic nervous system in the increase in hepatic glucose production caused by empagliflozin will be examined with norepinephrine (NE) turnover in two protocols. The first protocol is cross sectional, in which 36 T2DM patients will receive hepatic glucose production (HGP) and NE turnover will be measured before and after empagliflozin or placebo administration. In protocol 2, diabetic and non-diabetic subjects will receive baseline HGP, NE turnover,) and liver fat measurement before at 2 days after the start and 12 weeks after empagliflozin or placebo treatment.

Methods: the following techniques will be employed (1) Measurement of hepatic glucose production with ^{3}H-glucose infusion, with and without glucose clamp, (2) substrate oxidation with indirect calorimetry and plasma ketone/lactate/insulin/glucagon concentrations; (; (3) Measurement of whole body norepinephrine turnover with ^{3}H-norepinephrine infusion; (4) Measurement of heart rate variability; (5) Measurement of liver fat content with ^{1}H-MRS

Clinical Relevance: The results of the present studies will help identify the mechanism responsible for the increase in HGP caused by empagliflozin and the increase in ketone production. The first action of the drug ameliorates its clinical efficacy while the second increases the risk of adverse events (ketoacidosis). Identifying the mechanisms underlying these actions will help developing therapeutic strategies which increase the drug clinical efficacy and mitigates its adverse events.

Item 3 Background	
Describe past experimental and/or clinical findings leading to the formulation of your study. *For research involving unapproved drugs, describe animal and human studies.* *For research that involves approved drugs or devices, describe the FDA approved uses of this drug/device in relation to your protocol.*	Insert background: **Effect of SGLT2 Inhibitors in T2DM Patients:** inhibition of renal sodium-glucose contransport is a novel strategy for the treatment of T2DM (1). Currently 3 agents are approved by the FDA for the treatment of T2DM; dapagliflozin, canagliflozin and empagliflozin. Members of this class of drugs lower the plasma glucose concentration by inhibiting renal glucose uptake and causing glucosuria. Clinical studies have demonstrated that in drug naïve T2DM patients and in patients treated with stable dose of metformin and/or sulfonylurea, the addition of SGLT2 inhibitor causes a 25-35 mg/dl decrease in fasting plasma glucose (FPG) concentration (2-4). Because the primary determinant of the FPG concentration is hepatic glucose production (HGP) (5,6), and SGLT2i consistently reduce the FPG, we anticipated that dapagliflozin treatment would be associated with reduced hepatic glucose output. Surprisingly, when administrated to T2DM patients following an overnight fast, HGP increased markedly (7,8). In a double blind, placebo controlled study in 18 T2DM males (age=52±3, BMI=52±3, FPG=164±10, and HbA1c=8.6±0.5), 2 weeks of treatment with dapagliflozin (10 mg/day) caused 38 mg/dl reduction in fasting plasma glucose concentration (7). However, despite the decrease in FPG concentration, dapagliflozin caused 17% increase in the basal rate of HGP (bHGP) (from 2.17±0.11 to 2.53±0.16, $p<0.05$, Figure 1). A similar increase in bHGP following empagliflozin treatment in T2DM was reported by Ferrannini and colleagues (8). The increase in bHGP caused by dapagliflozin can be viewed as a compensatory mechanism to compensate for urinary glucose loss, maintain the plasma glucose concentration at the normal level, and prevent

hypoglycemia. However, in T2DM individuals, the rise in HGP took place while the plasma glucose concentration was well within the hyperglycemic range (FPG ~130 mg.dl). Thus, it is paradoxical.

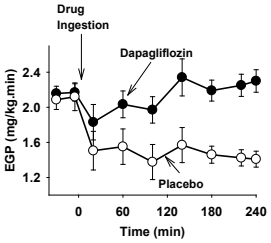
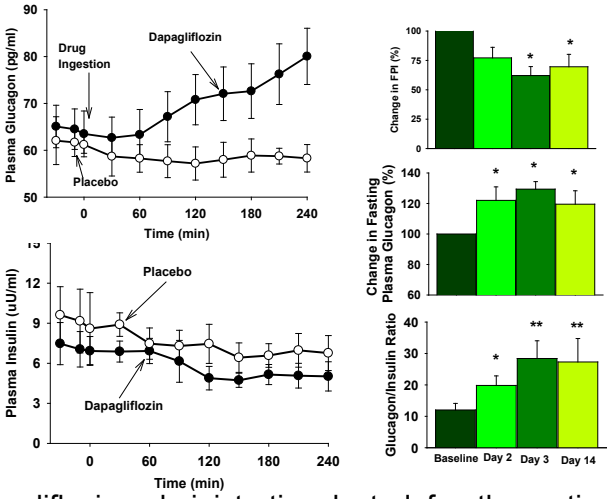


Figure 1: Acute Effect of dapagliflozin and placebo on HGP in T2DM patients

Figure 2: Effect of dapagliflozin and placebo on plasma insulin, glucagon and glucagon to insulin ratio in T2DM patients



The increase in bHGP following dapagliflozin administration lasted for the entire treatment period (2-weeks, and 4 weeks in the study by Ferrannini [8]). This paradoxical increase in bHGP has important clinical significance, since it returns to the systemic circulation approximately one half of the amount of glucose lost in the urine. Thus, it offsets urinary glucose loss caused by dapagliflozin and attenuates the decrease in plasma glucose concentration caused by the drug.

The rise in bHGP following dapagliflozin administration raises an important question about the signal which links SGLT2 inhibition/glucosuria to the liver. To investigate the cause of this paradoxical rise in bHGP, we measured plasma insulin and glucagon concentrations following dapagliflozin administration. Figure 2 demonstrates that the increase in bHGP cause by dapagliflozin is accompanied by a small decrease in plasma insulin concentration and a large increase in plasma glucagon concentration (32%) (Figure 2). Thus, the ratio of plasma glucagon to insulin, the principal determinant of the rate of HGP, markedly increased (from 12±2 to 28±7, $p<0.01$) following dapagliflozin administration (Figure 2) and this increase lasted for the entire treatment period (2 weeks). It should be noted that, because the increase in bHGP was associated with a reduction in fasting plasma insulin concentration, the increase in bHGP produced by dapagliflozin does not reflect worsening of hepatic insulin resistance. The hepatic insulin resistance index (the product of bHGP and fasting plasma insulin) was slightly reduced by dapagliflozin.

Dapagliflozin also decreased the postload plasma glucose concentration (9). Administration of 10 mg dapagliflozin caused 60% reduction in the incremental urea under the plasma glucose concentration curve during the OGTT (9). Since the disposal of the majority of an exogenous glucose load takes place in skeletal muscle (10), we measured whole bod insulin-mediated glucose uptake with the euglycemic insulin clamp. Dapagliflozin caused a 16% increase in insulin-mediated tissue glucose uptake (from 4.3±0.4 to 5.0±0.6 mg/kg.min, $p<0.05$) (7), suggesting improved peripheral insulin sensitivity. Further, dapagliflozin caused a doubling of beta cell function in T2DM patients (9). Since the primary site of SGLT2i action is on the kidney, and since SGLT2 transporters have not been demonstrated in skeletal muscle or beta cells, the improvements in insulin sensitivity and insulin secretion can be attributed to reversal of glucotoxicity (2,11).

We previously have shown that improving insulin sensitivity in T2DM individuals by lowering plasma free fatty acid concentration with acipimox (removal of lipotoxicity) resulted in an increase in glucose oxidation and decrease in fat oxidation and 50% increase in mitochondrial ATP synthesis rate (12). Unexpectedly, the improvement in insulin sensitivity following dapagliflozin treatment was associated with opposite effect on mitochondrial ATP synthesis and substrate oxidation (13). Dapagliflozin treatment

(2 weeks) caused a 20% reduction in glucose oxidation during the fasting state with concomitant a 14% increase in fat oxidation (Figure 3). Moreover, although dapagliflozin significantly increased insulin-stimulated glucose disposal (by 16%), glucose oxidation during insulin infusion was reduced by approximately 50% in association with a 15% increase in fat oxidation (13). Of note, no significant change in plasma FFA concentration or suppression of plasma FFA concentration during the insulin clamp was observed following dapagliflozin administration. Thus, the increase in fat oxidation can not be explained by oversupply of fat to peripheral tissue; rather, it must be due to an effect of the drug on metabolic pathway(s) regulating fat oxidation. Regardless of the mechanism for the increase in fat oxidation caused by SGLT2 inhibitors, we hypothesize that the increase in fat oxidation leads to depletion of intramyocellular and intrahepatic fat content and can explain the improvement in insulin sensitivity measured with the insulin clamp. In the present grant proposal, we will measure the effect of SGLT2 inhibitor, dapagliflozin on hepatic fat content measured with MRI spectroscopy.

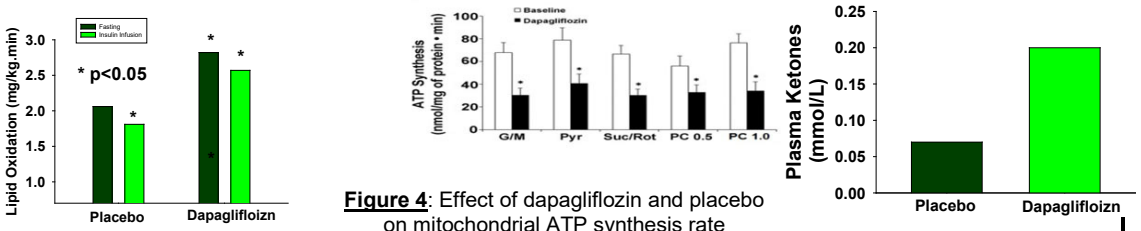


Figure 4: Effect of dapagliflozin and placebo on mitochondrial ATP synthesis rate measured ex vivo in isolated mitochondria from skeletal muscle. G/M=glutamate; pyr=pyruvate; suc=succinate; PC=palmitoyl carnitine

The increase in fat oxidation following dapagliflozin treatment was associated with a surprising 55% reduction in mitochondrial ATP synthesis rate (Figure 4) and a modest, but significant increase in plasma ketone concentration (Figure 5) (13). These changes in substrate oxidation, together with the increase in bHGP caused by dapagliflozin, suggest that dapagliflozin shifts substrates away from the TCA cycle (acetyl Co-A towards ketone production and oxaloacetate towards gluconeogenesis; Figure 6) and are consistent with the reduction in ATP synthesis rate caused by dapagliflozin (Figure 4). The increase in fat oxidation, decrease in ATP synthesis rate and increase in plasma glucagon and decrease in plasma insulin concentration explain the increased conversion of acetyl-CoA to ketones and increase in plasma ketone concentration, which under stress conditions can lead to the development of clinically significant ketoacidosis, as has been reported in patients treated with SGLT2 inhibitors (14,15).

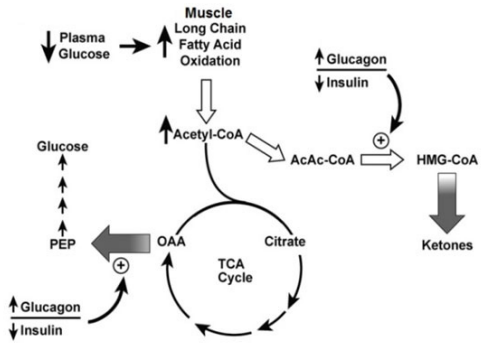


Figure 6: Scheme illustrating the metabolic and hormonal effects of dapgliflozin on glucose and fat metabolism in T2DM patients

Pathogenesis of Impaired Fasting Glucose

Impaired fasting glucose (FPG=100-125 mg/dl) (IFG) was introduced by the American Diabetes Association in 1997 as an intermediate stage in glucose tolerance between normal glucose tolerance and overt T2DM (16), and it was meant to identify subjects at increased future T2DM risk without the need for an OGTT. Compared to NGT, IFG

subjects manifest an increase in the fasting plasma glucose concentration and excessive rise in plasma glucose concentration following glucose load, e.g. at 60 and 75 minutes after OGTT and mixed meal (17). We (17-21) and others (22-26) have previously demonstrated that IFG subjects manifest moderate to severe insulin resistance in the liver with normal/near normal insulin sensitivity in skeletal muscle and impaired first phase but normal second phase insulin secretion (17-26). These metabolic defects in IFG subjects explain the shape of plasma glucose concentration following a glucose load. The normal/near normal insulin sensitivity in skeletal muscle and normal second phase insulin secretion in IFG subjects explains why the 2-hour plasma glucose concentration returns to the starting fasting, albeit elevated, level in IFG subjects.

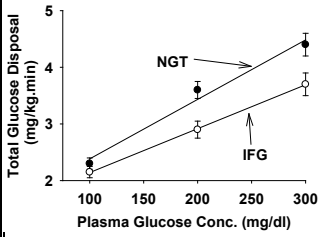
Despite a wealth of previous studies (17-32) which have examined the pathophysiology of IFG and which have identified multiple metabolic defects, including hepatic insulin resistance and impaired first phase insulin secretion, none of these defects can explain the elevated FPG concentration in IFG subjects. This question was the aim of my currently funded NIH R01 grant.

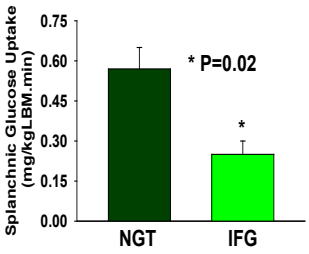
The basal rate of hepatic glucose production (bHGP) is the principal determinant of the FPG concentration (5,6). We (18) and others (23-25) previously have demonstrated that IFG subjects manifest moderate to severe hepatic insulin resistance. However, the elevated fasting plasma insulin concentration in IFG subjects offsets the hepatic insulin resistance and maintains the basal rate of HGP in the normal range (18, 23-25). Indeed the vast majority (18,22-25, 27, 28), though not all (29,32), of previous studies have failed to document a significant rise in bHGP in IFG compared to NGT subjects, and the investigators who reported an elevated bHGP in IFG in some studies (29,32) also reported a normal bHGP in other studies (22,23,28). A normal rate of bHGP in IFG subjects in the presence of an elevated FPG concentration suggests a decreased basal glucose clearance rate. Consistent with this hypothesis, we (33) previously have demonstrated that IFG subjects manifest a decreased basal rate of glucose clearance during the fasting state. The aim of the currently funded NIH grant was to directly quantitate the basal rate of glucose uptake in IFG subjects and compare the results to those in NGT individuals.

Whole Body Basal Glucose Uptake in NGT and IFG Subjects: Because the basal rate of glucose uptake is insulin independent (34,35) (driven by the mass action of glucose), we utilized the stepped hypeglycemic clamp in combination with the pancreatic clamp technique to quantitate whole body glucose-mediated glucose uptake in IFG and NGT subjects. 12 NGT (age 38±5, sex (f/m) 6/6, BMI=28.2±1.1, FPG=90±2) and 12 IFG (age 32±2, sex (m/f) 7/5, BMI=31.2.0±1.4, FPG=109±2) subjects participated in the study. Subjects received somatostatin infusion with replacement of basal plasma insulin and glucagon levels while the plasma glucose concentration was raised and maintained at +100 mg/dl (i.e. from ~100 mg/dl to 200 mg/dl) for 100 minutes (0-100 minutes). At 100 minutes, the plasma glucose concentration was raised and maintained by an additional 100 mg/dl (i.e, from 200 to 300 mg/dl). 120 minutes before the start of glucose infusion a prime (40 µCi) continuous (0.4 µCi/min) infusion of ^{3}H-glucose was started and continued until the end of the study to quantitate the rate of total body glucose appearance (Ra).

Consistent with previous studies (33), the basal rate of HGP was slightly but not significantly reduced in IFG compared to NGT subjects; 2.15±0.14 vs 2.30±0.16 mg/kg.min. However, the basal glucose clearance was significantly reduced in IFG subjects (2.61±0.11 vs 3.6±0.2 ml/kg.min). Compared to NGT, glucose-mediated glucose uptake was significantly reduced during both hyperglycemic clamp steps in IFG subjects (Figure 7). The rate of glucose disposal in IFG and NGT subjects was 2.89±0.10 vs 3.39±0.29 ($p=0.02$) at 200 mg/dl and 3.73±0.27 vs 4.15±0.21 mg/kg.min ($p<0.05$) at 300 mg/dl, respectively. Thus, the slope of line relating total body glucose-mediated glucose disposal and the plasma glucose concentration was 17% lower in

IFG compared to NGT. These results demonstrate impaired glucose-mediated glucose uptake (glucose resistance) in IFG subjects.





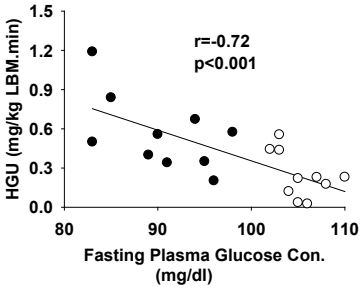
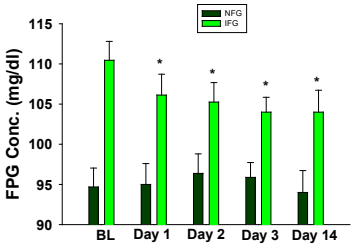


Figure 8: Hepatic and non-hepatic splanchnic glucose uptake measured with the Oral-IV double tracer technique in IFG and NGT subjects during the fasting state

Metabolic Effect of SGLT2 Inhibitor in NGT and IFG Subjects: To examine the effect of SGLT2 inhibition on FPG concentration in NGT, and IFG subjects compared to T2DM, we treated 8 NGT subjects (age= 58±2, m/f=6/2, BMI= 27.1±1.1, FPG=95±2, eGFR=120±10), 8 IFG subjects (age= 59±3, m/f=5/3, BMI= 30.3±0.8, FPG=110±2, eGFR=99±6) and 15 T2DM subjects (age= 55±2, m/f=12/3, BMI= 31.1±2.1, HbA1c=7.8±0.2, eGFR=107±7) with empagliflozin 25 mg/day for 2 weeks. Empagliflozin caused 45±4, 50±5 and 97±11 grams glucosuria per day in NGT, IFG and T2DM subjects, respectively. As anticipated, empagliflozin caused 32 mg/dl reduction in the FPG concentration in T2DM patients. Conversely, the fasting plasma glucose concentration in NGT subjects was not affected by empagliflozin treatment (95 mg/dl vs 94 mg/dl) despite 45 grams per day of urinary glucose loss. Although urinary glucose loss was comparable in IFG subjects and NGT subjects, empagliflozin caused a modest but significant reduction in the FPG concentration (from 110±2 to 103±3 mg/dl, p<0.01) in IFG subjects (Figure 10). Further, the magnitude of decrease in the FPG concentration in IFG subjects strongly and inversely correlated with the FPG concentration (r=-0.61, p<0.01) (Figure 11). These results are consistent with a recent study which demonstrated 6 mg/dl decrease in the FPG concentration (from 105 to 99 mg/dl) accompanied by a 34% increase in bHGP after 4 weeks empagliflozin treatment in non-diabetic obese IGT subjects (40). Collectively, these results suggest that: (1) HGP, though not measured in our study, similar to the previous study (40), and must have been markedly elevated in both NGT and IFG to compensate for the large urinary glucose loss and maintain the FPG concentration at or close to the normal fasting level (~90-100 mg/dl).



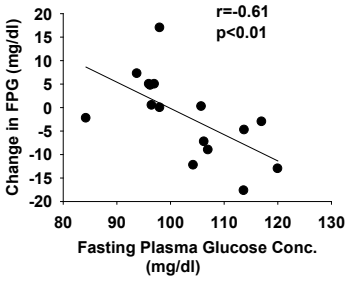


Figure 11: Relationship between the decrease in FPG conc. caused by empagliflozin treatment (25 mg/day) and the FPG conc.

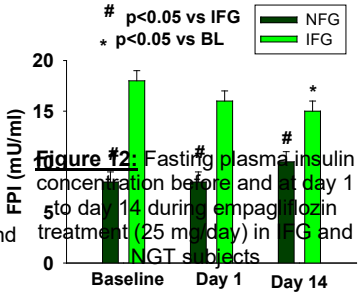


Figure 12: Fasting plasma insulin concentration before and at day 1 to day 14 during empagliflozin treatment (25 mg/day) in IFG and NGT subjects

As anticipated, empagliflozin caused a small decrease in the fasting plasma insulin concentration and a large increase in the fasting plasma glucagon concentration in T2DM patients. Thus, consistent with our previous study (7), the fasting plasma glucagon to insulin ratio was doubled (from 5 to 11) by empagliflozin in T2DM patients.

Surprisingly, however, empagliflozin did not affect the fasting plasma insulin and glucagon concentrations in NGT subjects and caused only a modest reduction in fasting plasma insulin concentration (Figure 12) with a small, transient, non-significant increase in fasting plasma glucagon in IFG subjects (Figure 13). Consistent with our previous study (13), empagliflozin caused a significant increase in plasma ketone concentration in T2DM patients (from 0.52±0.04 to 0.68±0.3, p=0.03), while plasma ketone concentration was not affected by empagliflozin treatment in either IFG (0.43±0.03 vs 0.43±0.03) or NGT (0.53±0.04 vs 0.45±0.07) subjects following empagliflozin treatment (Figure 14). The lack of increase in plasma ketone concentration in NGT subjects is surprising in light of the large shift in fuel consumption during the fasting state caused by empagliflozin in non-diabetic subjects (decrease in RQ from 0.81 to 0.74). Collectively, these results demonstrate that: (1) Although SGLT2 has been shown to be present in the alpha cell and SGLT2i have been shown to directly stimulate glucagon secretion from the alpha cell (41), it is likely that, in man *in vivo*, the decrease in plasma glucose concentration in T2DM subjects following SGLT2 inhibition is the principal signal causing the decrease in plasma insulin concentration and increase in plasma glucagon concentration, (2) mechanism(s) other than the increase in plasma glucagon and decrease in plasma insulin concentration (likely neuronal) stimulates HGP and cause a shift in substrate oxidation following SGLT2 inhibition in NGT subjects and IFG subjects, and (3) the increase in plasma glucagon concentration and/or decrease in plasma insulin concentration is an important signal for the increase in ketone production following SGLT2 inhibition.

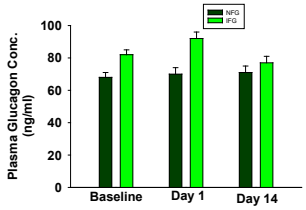


Figure 13: Fasting plasma glucagon concentration before (BL) and at day 1 to day 14 during empagliflozin treatment (25 mg/day) in IFG and NGT

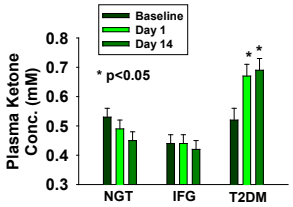


Figure 14: Effect of empagliflozin treatment (25 mg/day) on plasma ketone concentration in NGT, IFG and T2DM subjects

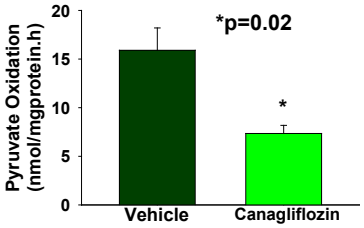
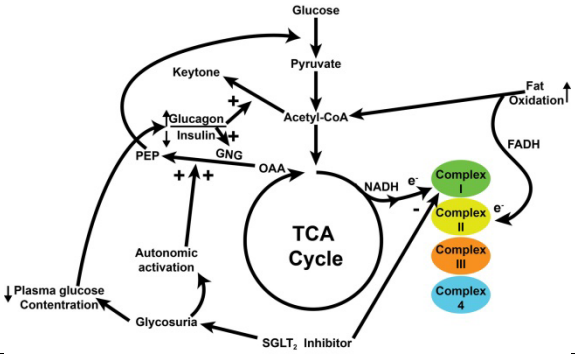


Figure 15: Effect of dapagliflozin 3 μM on pyruvate oxidation in hepatocytes in culture

A recent *in vitro* study (42) suggested that canagliflozin and dapagliflozin inhibit mitochondrial complex 1 activity in cultured HEK-293 cells, suggesting an OFF-target action of the drug and this could explain the decrease in glucose oxidation in *in vivo* studies in man. To examine a possible direct effect of SGLT2 inhibitors on glucose oxidation in hepatocytes, we examined the effect of dapagliflozin, empagliflozin and canagliflozin (3 μM) on pyruvate oxidation in cultured hepatocytes. Figure 15 demonstrates that 3 μM canagliflozin caused a 56% reduction in pyruvate oxidation in cultured hepatocytes, and similar results were obtained with dapagliflozin and empagliflozin. Of note, none of the 3 SGLT2 inhibitors affected ketone or glucose production in cultured hepatocytes. Inhibition of citrate synthase by SGLT2 inhibitor could explain the reduction in pyruvate oxidation and shift of OAA toward gluconeogenesis resulting in increased glucose production as was observed in *in vivo* studies in man. However, hepatic citrate synthase activity was not affected by canagliflozin. Inhibition of complex 1 in hepatocytes by SGLT2 inhibitor (42) could explain the inhibition of pyruvate oxidation in hepatocytes *in vitro*. A similar *in vivo* action of SGLT2 inhibitors potentially could explain the shift in substrate oxidation (decreased glucose and increased fat oxidation). The increase in fat oxidation provides an alternatve energy source to the cell by generating FADH which generates ATP by

contributing electrons directly to mitochondrial complex II, bypassing the inhibition of complex I by SGLT2 inhibitor. Such an action could cause an increase in plasma lactate concentration. A recent study has reported small, though non-significant increase in plasma lactate concentration in obese IGT individuals following empagliflozin treatment (40). An increase in plasma lactate concentration, in addition to the increase in ketone production, could contribute to the euglycemic acidosis reported in some patients receiving treatment with SGLT2 inhibitors (14,15). In the present study, we will measure the effect of dapagliflozin on plasma lactate, ketone, bicarbonate concentrations and plasma pH in NGT, IFG and T2DM patients.

Our recent findings about the effect of empagliflozin on glucose metabolism in NGT and IFG subjects (Figures 10-14) and the effect of SGLT2 inhibitors on pyruvate oxidation in hepatocytes *in vitro* (Figure 15) require an update of our previous scheme (13) (Figure 6) describing the mechanism(s) responsible for the metabolic actions of SGLT2 inhibitors in T2DM patients. We suggest the following working hypothesis (Figure 16) which we will be testing in the current grant request: (1) Because empagliflozin did not stimulate glucagon secretion or inhibit insulin secretion in NGT subjects, it is likely that glucosuria stimulates HGP, at least initially, via mechanisms other than the increase in plasma glucagon concentration and decrease in plasma insulin concentration. Of note, the increase in HGP begins rapidly following dapagliflozin administration and before there is a significant increase in plasma glucagon concentration (Figure 2). This suggests a neurogenic origin, which results from activation of the renal nerves which communicate directly with the liver via neural connections in the portal circulation or indirectly via connections to the CNS. Consistent with this hypothesis, a recent study has demonstrated that the increase in HGP in dogs following hypoglycemia is markedly influenced by the brain (43). Further, renal denervation in experimental animals caused a significant increase in hepatic norepinephrine content (44). In the present grant proposal, we will measure the effect of empagliflozin on sympathetic activity with ^{3}H-NE infusion; (2) In addition to inhibition of renal glucose absorption, SGLT2 inhibitors likely exert a direct inhibitory action on mitochondrial metabolism (possibly by inhibiting complex I[42]) causing inhibition of pyruvate oxidation and reduction in glucose oxidation. (3) The inhibition of glucose oxidation results in a reciprocal increase in fat oxidation and acetyl Co-A levels (4) The increase in fat oxidation aims to provide an alternative energy source to meet the metabolic demands of the cell. Because fat oxidation is associated with generation of FADH which can generate ATP via donating electrons to complex 2, thereby, bypassing the block at complex 1. (5) the decrease in the fasting plasma glucose concentration in T2DM patients following glucosuria stimulates glucagon secretion and inhibits insulin secretion resulting in an increase in plasma glucagon to insulin ratio; (6) the increase in glucagon to insulin ratio further stimulates and maintains the increase in HGP and stimulates the conversion of acetyl Co-A to ketones (possibly by stimulating the expression of hydroxyl methylglutaryl Co-A synthase [HMGS], the rate limiting step for the conversion of acetyl Co-A to beta hydroxybuterate [45,46]); (7) in subjects with NGT, SGLT2 inhibition does not affect the FPG concentration. Thus, plasma glucagon and insulin concentrations are not affected by SGLT2 inhibition, and the plasma ketone concentration does not increase (Figure 14). In the present grant request, we will test these hypotheses by: (1) examining the effect of empagliflozin on HGP, substrate oxidation, plasma ketone/lactate/insulin/glucagon concentrations, hepatic fat content, basal hepatic glucose uptake and sympathetic nervous system activity in NGT, IFG and T2DM patients with FPG concentration <160 mg/dl using state-of-the-art techniques. Because empagliflozin does not affect the FPG, insulin and glucagon concentrations in NGT subjects, this allows us to provide definitive evidence for the role of sympathetic activation in the increase in HGP caused by empagliflozin, and (2) examination of the effect of empagliflozin on substrate oxidation, hepatic fat content, FPG concentration, and hepatic glucose uptake during the fasting state in IFG

	subjects will provide insight about the pathophysiology of IFG and the therapeutic potential of this class of drugs in reversing IFG to NGT and reducing their risk of progression to T2DM.  Figure 16: Working hypothesis for the mechanism(s) responsible for the metabolic actions of SGLT2 inhibitors in T2DM
Item 4 Purpose and rationale *Insert purpose, objectives and research questions/hypotheses here.* *If you cut and paste from another document, make sure the excerpted material answers the question*	Insert purpose: Sodium-Glucose cotransport inhibitors (SGLT2i) are a novel class of antidiabetic agents which lower the plasma glucose concentration by inhibiting renal glucose reuptake and producing glucosuria. Hepatic glucose production (HGP) is the primary determinant of the fasting plasma glucose concentration. Although SGLT2i lower the fasting plasma glucose concentration in T2DM patients, we have demonstrated that SGLT2i produce a paradoxical increase in the rate of hepatic glucose production. Studies which have examined the mechanisms responsible for the increase in HGP caused by SGLT2i demonstrated a small decrease in plasma insulin concentration and a robust increase in plasma glucagon concentration. Thus, the plasma glucagon to insulin ratio was markedly increased and could explain, at least in part, the increase in HGP, while the decrease in plasma glucose concentration produced by SGLT2i improves insulin sensitivity by removal of glucotoxicity. The decrease in plasma glucose concentration results in decreased glucose influx to the cell which shifts fuel consumption from glucose to fat oxidation and cause an increase in ketone production. We and others recently have demonstrated that, despite the improvement in insulin sensitivity, SGLT2i inhibit mitochondrial ATP synthesis, suggesting a possible direct off-target effect of the drug on substrate oxidation independent of SGLT2 inhibition and glucosuria. Impaired fasting glucose (FPG=100-125 mg/dl) is an intermediate stage in the transition from normal glucose tolerance to overt diabetes. We have demonstrated that bHGP, which is the primary determinant of the FPG, is modestly decreased in IFG while the rate of basal glucose disposal is reduced in IFG subjects compared to NGT. Since approximately one half of basal glucose disposal takes place in the splanchnic area and skeletal muscle in an insulin-independent mechanism, we quantitated the rate of basal glucose disposal in IFG subjects and demonstrated that during the postabsorptive state total body glucose uptake is significantly reduced in IFG subjects. Interestingly, SGLT2i reduced the FPG in IFG subjects with no effect on the FPG in NGT subjects despite producing a similar amount of glucosuria in both groups. Moreover, SGLT2i inhibited glucose oxidation increased fat oxidation without affecting ketone production in IFG and NGT subjects. Further, SGLT2i did not affect the plasma insulin or glucagon concentrations in NGT subjects and caused only modest decrease in the fasting plasma insulin concentration and a small non-significant and transient increase in plasma glucagon concentration in IFG subjects. Lastly, we have demonstrated that in *in vitro* studies, all 3 SGLT2i inhibit pyruvate oxidation in hepatocytes in culture. Because SGLT2i did not affect the FPG concentration in NGT and caused only modest decrease in FPG in IFG despite large glucosuria, it has to cause a robust increase in HGP to maintain the plasma glucose concentration. Since SGLT2i increased decreased glucose oxidation and increased fat oxidation without increase in plasma ketone level or significant change in the plasma glucagon to insulin ratio in NGT and

	IFG individuals, we hypothesize that: (1) the change in plasma insulin and glucagon concentrations in T2DM individuals is likely the effect of the decrease in plasma glucose concentration; (2) signals other than the change in glucagon to insulin ratio (likely neuronal) are activated by glucosuria and stimulate HGP in NGT and IFG individuals; (3) SGLT2i cause an increase in basal hepatic glucose uptake in IFG subjects and lead to a decrease in FPG; and (4) to examine these hypotheses, we suggest the following specific Aims: **Specific Aim 1:** We will measure FPG, bHGP (^{3}H-glucose infusion), whole body fat oxidation (indirect calorimetry), plasma insulin, glucagon, FFA, ketone and lactate concentrations, and hepatic fat content (^{1}H-MRS) in IFG, NGT and T2DM patients (drug naïve with FPG <160 mg/dl) at baseline and at day 1 and 4 weeks after treatment with SGLT2i (empagliflozin) **Specific Aim 2:** Measure autonomic balance (with heart rate variability) and sympathetic nervous system activity (^{3}H-norepinephrine turnover) in IFG, NGT and T2DM subjects (drug naïve with FPG <160 mg/dl) at baseline and at day 1 and 4 weeks of treatment with SGLT2i (empagliflozin). **SIGNIFICANCE** The present study will add new insight about the mechanism(s) underlying the multiple metabolic actions of SGLT2 inhibitors in T2DM patients (1). Our preliminary results demonstrate that, this class of drugs, in addition to the inhibition of renal glucose transport, exerts a direct effect on glucose oxidation. We and others previously have shown that SGLT2 inhibitors exert multiple metabolic actions on glucose and fat metabolism with significant clinical implication in T2DM patients: (1) stimulate the basal rate of HGP which offset approximately 50% of urinary glucose loss produced by the drug and ameliorate the drug efficacy in lowering the plasma glucose concentration; (2) increase fat oxidation and ketone production, thus, increasing the susceptibility to diabetic ketoacidosis; and (3) increase plasma glucagon concentration which could contribute to the increase in HGP and ketone production. The results of the present study will define the mechanisms responsible for these actions of SGLT2 inhibitors and pave the rode for the development of therapeutic strategies to mitigate their adverse events, e.g. risk of ketoacidosis, and increase the efficacy of this class of drugs to lower the plasma glucose concentration in T2DM patients.

Item 5 Study Population(s) Being Recruited In your recruitment plan, how many different populations of prospective subjects do you plan to target? Provide number: 2	Identify the criteria for **inclusion**:	Identify the criteria for **exclusion**:

e.g., a population can be individuals with type 2 diabetes controlled with diet and/or a population of healthy controls. Or a population can be individuals attending an education program, etc. List each different population on a separate row and provide a short descriptive **label**: *(e.g., normal-healthy, diabetics, parents, children, etc.)* *To add rows use copy & paste*		
Type 2 diabetic subjects	1) FPG< 240 mg/dl 2) Age 18-70 years 3) eGFR>60 ml/min 4) weight stable (±4Lb) in the preceding 3 months 5) Good general health	1) Use of an insulin or SGLT2 inhibitor medications affects glucose tolerance 2) Major organ disease 3) eGFR <60 ml/min 4) type 1 diabetes
Subjects with FPG<126 mg/dl	1) FPG<126 and 2-h PG <200 mg/dl 2) Age 18-70 years 3) eGFR>60 ml/min 4) weight stable (±4Lb) in the preceding 3 months 5) Good general health	1) Use of medications that affect glucose tolerance 2) Major organ disease 3) eGFR <60 ml/min
Sub-Study: Type 2 diabetic	1) current diagnosis of Type 2 Diabetes 2) fasting plasma glucose concentration <300 mg/dl 3) Subjects must be drug naïve or on stable dose of metformin and/or sulfonylurea in the preceding 3 months 4) body weight has been stable (± 3 lbs) over the preceding three months 5) does not participate in an excessively heavy exercise 6) good general health as determined by physical exam, medical history, blood chemistries, CBC, TSH, urinalysis, and EKG	1) eGFR > 60 ml/min 2) hematocrit > 34%, 3) allergy to iodine 4) Major organ disease
Sub-Study: non-diabetic	1) No diagnosis of diabetes (FPG <126 mg/dl) 2) body weight has been stable (± 3 lbs) over the preceding three months 3) does not participate in an excessively heavy exercise 4) good general health as determined by physical exam, medical history, blood chemistries, CBC, TSH, urinalysis, and EKG	1) eGFR > 60 ml/min 2) hematocrit > 34%, 3) allergy to iodine 4) Major organ disease

Item 6
Research Plan / Description of the Research Methods *a.* *Provide a **comprehensive narrative** describing the **research methods**.*
*Provide the **plan for data analysis** (include as applicable the **sample size calculation**).*

Step-by-Step Methods: **Study Design:**

After screening, subjects will receive the following tests: (1) Measurement of hepatic glucose production, substrate oxidation and plasma ketone/lactate/insulin/glucagon concentrations; (2) (Measurement of whole body norepinephrine turnover with ^{3}H-norepinephrine infusion; (3) Measurement of heart rate variability; (4) Measurement of liver fat content with ^{1}H-MRS; (5) 75-grams oral glucose tolerance test (OGTT)

The studies will be performed in a random order.

After the completion of the above studies, subjects in each group will be randomized (in 2:1 ratio) to receive for 12 weeks, empagliflozin 25 mg per day (n=24) or placebo (n=12), in a double blind fashion. The drug treatment will be started (day 1) on the day of the second hepatic glucose measurement (see specific details below). On day 2, the measurement of heart rate variability and norepinephrine turnover will be repeated. Subjects will continue treatment with the drug (empagliflozin or placebo) that they received during the second hepatic glucose measurement for 12 weeks and at the end of 12 weeks, all baseline tests will be repeated.

The treatment period could be extended based upon the circumstances until the repeat studies are completed.

Tests	Baseline	Day 1-3	12 Weeks
OGTT	OGTT		OGTT
HGP	Short	Long	Short
NE	NE	NE	NE
MRI	MRI		MRI
DEXA	DEXA		
HRV	HRV	HRV	HRV

The metabolic effects of empagliflozin lasted for 2 weeks in our previous studies (7). However, Ferrannini and colleagues (40) reported that at 4 weeks, the glucose effects of empagliflozin (HGP) are attenuated while the fat effects (fat oxidation and ketone production) are enhanced. Therefore, to evaluate the long term metabolic impact of SGLT2 inhibition, we decided to extend the treatment period in the current study to 12 weeks.

Specific Methods:

Screening: Medical history and physical examination will be performed. Blood samples will be drawn for CBC, blood chemistries, lipid profile, thyroid function and HbA1c. EKG will be performed.

Measurement of Hepatic Glucose Production with ^{3}H-glucose infusion (HGP – *short*): The rate of endogenous (primarily reflecting liver) glucose production will be measured with ^{3}H-glucose infusion as previously described (7). After a 10 hour overnight fast, a prime (25 µCiXFPG/100) - continuous (0.25 µCi) infusion of ^{3}H-glucose will be started and continued for 3 (4 in T2DM patients) hours. After 2 hours (3 hours in T2DM patients) of tracer equilibration, blood samples will be drawn from the catheter on the dorsum of the hand at 120, 130, 140, 150, 160, 170 and 180 minutes (from 180-240 minutes in T2DM patients). Plasma glucose, insulin, glucagon, GLP-1, cortisol, growth

hormone, ketones, lactate, bicarbonate and catecholamine concentrations, and ^{3}H-glucose specific activity will be measured. Indirect calorimetry to measure energy expenditure and substrate oxidation rates will be performed between 60-90 minutes.

Measurement of Hepatic Glucose Production With Drug Administration (HGP – *Long*): On the day of starting the treatment, the rate of endogenous (hepatic) glucose production will be measured with ^{3}H-glucose as described above with one difference. The ^{3}H-glucose infusion will be continued for 7 hours (8 in T2DM patients). After 2 hours of tracer equilibration (3 hours in T2DM patients), blood samples will be drawn from the catheter on the dorsum of the hand at -30, -20,-15,-10,-5 and 0 (time zero is drug ingestion time) minutes. At time zero, subjects will receive the first dose of the drug (placebo or empagliflozin 25 mg) and blood samples will be obtained from the retrogradely-placed catheter on the dorsum of the hand every 20-30 minutes for 5 hours. Plasma glucose, insulin, C-peptide, glucagon, GLP-1, cortisol, growth hormone, ketone, lactate, bicarbonate, and catecholamine concentrations, and ^{3}H-glucose specific activity will be measured. Urine will be collected during the equilibration period and after drug administration. Urinary volume and glucose concentration will be measured and urinary glucose excretion rate will be calculated. Indirect calorimetry to measure energy expenditure and glucose and fat oxidation will be performed 3 times through the study, from -60 to -30, from 150 to 180 and from 270 to 300 minutes. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

Norepinephrine turnover rate: The rate of total body norepinephrine spillover (release), which reflects total body sympathetic nervous system activity, will be measured with 3H-norepinephrine (3H-NE) infusion. Subjects will report to the CRC at 7 AM after a 10 hour overnight fast, and a catheter will be placed into an anticubital vein for tracer (3H-NE) infusion. A second catheter will be placed retrogradely into a vein on the dorsum of the hand, which is placed in a heated box (60°C) for sampling of arterialized blood. The arterialized blood will be ascertained by measuring O2 saturation (>95%). After drawing 3 baseline blood samples, a prime (3.6 µCi) -continuous (0.36 µCi) infusion of 3H-NE will be started and continued for 2 hours. 4-6 arterialized blood samples will be drawn from the catheter on the dorsum of the hand at every 10-30 minutes. Plasma NE and 3,4-dihydroxyphenylglycol (DHPG) concentrations and 3H-NE and 3H-DHPG radioactivity will be measured. Previous studies (47,48) have demonstrated that this 3H-NE infusion rate produces a steady state of plasma 3H-NE radioactivity within 12 minutes without significant increase (<3%) in plasma 3H-DHPG (the primary intraneuronal metabolite of norepinephrine) radioactivity. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

Heart Rate Variability: Heart rate variability (HRV) will be measured in the morning after a 10-12 hour overnight fast. The measurement will be performed using the SphygmoCor device (AtCor Medical, Sydney, Australia) with the participant lying in the resting supine position for 10 min. This device takes into account only the normal heart beats, ignoring ectopic beats, and derives the time (SDNN and rMSSD) and frequency (LF and HF) domain parameters of the normal R-R intervals of the electrocardiogram (49). Parasympatheric nervous system (primarily is represented by decreased high frequency domain [0.15-0.4 HZ] and rMSSD), and sympathetic nervous (primarily is represented by decreased low frequency domain [0.04-0.15] and SDNN) system activity will be assessed before and at day 2 and week 12 of treatment with empagliflozin. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

The following additional measurements will be performed: 1) Central aortic pulse pressure, 2) Pulse wave velocity (PWV), 3) Ankle-Brachial Index (ABI), 4) Toe-Brachial Index (TBI), and 5) Below knee blood flow. All measurements will be done on the same day of the HRV. Central aortic pulse pressure and pulse wave velocity (PWV) are performed by the same instrument (Sphygmacore) with which heart rate variability is measured. They will be measured together with HRV with the patient lying supine in bed. For the central aortic pulse pressure, blood pressure is measured while patient is lying supine on bed, and a probe is placed on the radial pulse to detect radial pulse wave.

The measurement takes approximately 10 minutes. The PWV will be measured as carotid-femoral and carotid-radial PWV. The patient will be connected to ECG electrodes, and the probe will be placed on the carotid pulse, femoral pulse and radial pulse and the pulse wave at each site is recorded together with the ECG. This measurement will take approximately 20 minutes. Ankle-Brachial Blood Pressure Index (ABI) and Toe-Brachial Blood Pressure Index (TBI) are measured with an US machine with the patient lying supine on bed. During the measurement, a blood pressure cuff is placed around the arm and a second cuff around the leg, and a probe is connected to the toe. The measurement takes 10-15 minutes.

Blood flow in the leg is measured with the same machine with a special U/S probe which is place on the anterior tibial region below the knee. It measured blood flow in the anterior tibial artery. This measurement takes about 10-15 minutes. All vascular measurements will be performed on the same visit and will take one and a half to two hours to complete.

Hepatic Fat Content: Hepatic triglyceride content will be measured by magnetic resonance and proton spectroscopy (^{1}H-MRS) with 3-T magnetic resonance imaging scanner (Siemens, Germany) as previously described (50). Measurements will be performed in the morning after 10 h overnight fast. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

Follow Up Visits: at weeks 1, 2, 4, 6, 8 and 10 after the start of treatments, subjects will come to the CRC for follow-up visits. At week 4 and 8 visits, medical history and physical exam will be performed. Body weight, blood pressure and pulse will be measured. Blood sample will be drawn for the measurement of fasting plasma glucose, insulin, glucagon, FFA, HbA1c, Pro BNP and ketones. Blood will also be drawn to monitor renal function with ketones, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

If the treatment period is extended, patients will continue to be followed up every 2-4 weeks and provided with the study medications until the repeat tests are completed. If the circumstances prohibit clinic visits for follow-up, the study team will perform a phone follow-up visit to ensure patient adherence, information collection, and adverse events monitoring.

Sub-Study 1**:**
36 T2DM patients will participate in this study, The T2DM subjects enrolled in the main study will also be subjects enrolled into the sub-study. Each subject will receive a measurements of hepatic glucose production with stable isotope (6,6,-D2-glucose) plus indirect calorimetry with empagliflozin (n=24) or placebo (n=12) administration. Norepinephrine turnover measurement will be performed at baseline (before the empagliflozin or placebo administration) and 5 hours after the drug administration.
On a separate day subjects will return to the CRC and the measurement of hepatic glucose production and NE turnover studies will be repeated with a glucose clamp.

Specific Methods:
Measurement of Hepatic Glucose Production With 6,6,D2-glucose, ^{3}H-NE infusion and Drug Administration: In this study, HGP measurement will be done with 6,6,D2-glucose infusion and NE turnover measurement with ^{3}H-NE infusion. A prime (4 mg/kg) continuous (0.04 mg/kg.min) infusion of 6,6-D2-glucose (Cambridge Isotopes, Boston, MA) will be started and continued to the end of the study (8 hours). After a 3-hour basal tracer equilibration period, patients will ingest empagliflozin 25 mg (n=24) or placebo (n=12). The 6,6-D2-glucose will be continued for additional 5 hours after drug ingestion. Blood samples will be drawn from the catheter on the dorsum of the hand at -30, -20,-10, and 0 (time zero is drug ingestion time) minutes. At time zero, subjects will receive the drug (placebo or empagliflozin 25 mg) and blood samples will be obtained from the retrograde-placed catheter on the dorsum of the hand every 20 minutes for 6 hours. Plasma glucose, insulin, glucagon, GLP-1, cortisol, growth hormone, ketone, lactate, and bicarbonate, concentrations, and 6,6-D2-glucose enrichment will be measured.

At -60 minutes, ^{3}H-NE infusion will be started for 60 minutes (from -60 to 0 minutes) for the measurement of NE turnover as described above (4 blood samples are collected every 10-30 minutes between -60 and time zero). The ^{3}H-NE infusion will be repeated at 240 minutes and 4 blood samples are collected before and after 3H NE infusion. Urine will be collected during the equilibration period and after drug administration (0 to 5 hours). Urinary volume and glucose concentration will be measured and urinary glucose excretion rate will be calculated. Indirect calorimetry to measure energy expenditure and glucose and fat oxidation will be performed 2 times through the study, from -90 to -60, from and from 210 to 240 minutes. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

Measurement of Hepatic Glucose Production With 6,6,D2-glucose, ^{3}H-NE infusion and glucose clamp:

HGP will be measured with 6,6,D2-glucose and NE turnover will be measured as described above with one difference. After the drug ingestion, the plasma glucose concentration will be measured every 5 minutes and variable 20% dextrose solution will be adjusted to maintain the plasma glucose concentration at the fasting level. Blood will also be drawn to monitor renal function, electrolytes, glucosoria, and monitor and mitigate urinary tract infections.

Effect of Empagliflozin on EGP and NE turnover with Pancreatic Clamp:

In this study, HGP measurement will be done with 6,6,D2-glucose infusion and NE turnover measurement with ^{3}H-NE infusion as described in Study above, and plasma insulin and glucagon concentrations will be clamped at the basal level using the pancreatic clamp technique (17,62), while the plasma glucose concentration will be allowed to decrease spontaneously after empagliflozin and placebo administration. Somatostatin (750 µg/h) infusion will be started 15 minutes before the start of 6,6-D2-glucose infusion along with basal infusions of glucagon (0.3 ng/kg.min) and insulin (0.1 mU/kg.min) to replace basal plasma glucagon and insulin concentrations. The somatostatin, glucagon and insulin infusions will be continued for the entire duration of the study (until 2 PM). A prime (4 mg/kg) continuous (0.04 mg/kg.min) infusion of 6,6-D2-glucose (Cambridge Isotopes, Boston, MA) will be started and continued to the end of the study (9 hours). After a 3-hour basal tracer equilibration period (time zero), patients will ingest empagliflozin 25 mg (n=24) or placebo (n=12). The 6,6-D2-glucose will be continued for additional 5 hours after drug ingestion. Blood samples will be drawn from the catheter on the dorsum of the hand at -30, -20,-10, -5, and 0 (time zero is drug ingestion time) minutes. At time zero, subjects will receive the drug (placebo or empagliflozin 25 mg) and blood samples will be obtained from the retrograde-placed catheter on the dorsum of the hand every 20 minutes for 5 hours. Plasma glucose, insulin, glucagon, GLP-1, cortisol, growth hormone, ketone, lactate, and bicarbonate, concentrations, and 6,6-D2-glucose enrichment will be measured.

At -60 minutes, ^{3}H-NE infusion will be started for 60 minutes (from -60 to -0 minutes) for the measurement of NE turnover as described above (4 blood samples are collected every 10-30 minutes between -60 and time zero). The ^{3}H-NE infusion will be repeated at 240 minutes and 4 blood samples are collected before and after 3H NE infusion. Urine will be collected during the equilibration period and after drug administration (0 to 5 hours). Urinary volume and glucose concentration will be measured and urinary glucose excretion rate will be calculated. Indirect calorimetry to measure energy expenditure and glucose and fat oxidation will be performed 2 times through the study, from -90 to -60, and from 210 to 240 minutes.

Sub-Study 2 (only conducted at TDI):

In initial studies, we observed that the increase in EGP after empagliflozin administration is influenced by the amount of fluids (saline infused intravenously plus amount of water drank by patient) taken by the patient during the study. Because empagliflozin causes glucosuria, and increase in urinary sodium and fluid excretion, this observation raises the possibility that plasma volume is an important parameter in the action of SGLT2 inhibitors. Further, since decreased plasma volume is strong stimulus of sympathetic activity, the decrease in plasma volume could

provide the link between empagliflozin and sympathetic activation (which is the primary focus of the present study).

To explore possible role of change in plasma volume in the link between empagliflozin administration and increase in EGP, we request to add a pilot study (Sub-study 2) in which the effect of empagliflozin (or placebo) on EGP production is measured with and without replacement of fluids lost in the urine during the study.

Research Design and Methods:
Subjects: Two groups of subjects will participate in this sub-study: (1) 36 T2DM patients, and (2) 18 non-diabetic healthy subjects . T2DM subjects must have fasting plasma glucose concentration <300 mg/dl. There is no limit on the upper level of HbA1c. Subjects must be drug naïve or on stable dose of metformin and/or sulfonylurea in the preceding 3 months. Other than diabetes, subjects must be in good general health as determined by physical exam, medical history, blood chemistries, CBC, TSH, urinalysis, and EKG. Only subjects whose body weight has been stable (± 3 lbs) over the preceding three months and who do not participate in an excessively heavy exercise program will be included. Individuals with eGFR < 60 ml/min, hematocrit <34%, and subjects with allergy to iodine will be excluded.

Pregnant women and women planning to become pregnant will be excluded from the study. We will perform a pregnancy test at the time of screening. Women of child baring age will be requested to use at least one barrier method before being enrolled in the study.

Study Design:
After screening, eligible subjects will receive DEXA scan and 4 measurements of EGP as described below.

Each EGP measurement will be performed on a different day 1-3 weeks apart, after 10-12 hour overnight fast. Subjects will be instructed to ingest a weight maintaining diet containing 45% CHO, 25% fat, and 30% protein throughout the entire study period.

EGP Measurement
Subjects will fast after 8 PM on the preceding day and report to the Clinical Research Center at 6 AM on the following morning. Subjects will not take their metformin or sulfonylurea on the day of study. At 6 AM a catheter will be inserted into an antecubital vein for the infusion of all test substances. A second catheter will be placed retrogradely into a vein on the dorsum of the hand, which will be placed in to a heated box (60°C) for arterialized blood withdrawal. The retrograde catheter will be continuously infused with half normal saline solution at rate 2 ml/hour to maintain it patency. At 6 AM (-180 minute), a prime-(25 X FPG/100 µCi) continuous (0.25 µCi/min) infusions of 3H-3-glucose for determination of rates of total body glucose appearance and disappearance will be started and continued for 10 hours (420 minute).

Blood sample will be withdrawn at -180 minute to measure plasma glucose concentration and background 3H-3-glucose specific activity. Between -40 to -10 minutes, 5 additional blood samples will be withdrawn every 5-20 minutes to measure 3H-3-glucose specific activity, plasma glucose, insulin, glucagon, FFA, ketone, norepinephrine, ACTH, cortisol, renin, angiotensin-II, NT-proBNP, and electrolyte (Na, K, Cl, HCO3) concentrations.

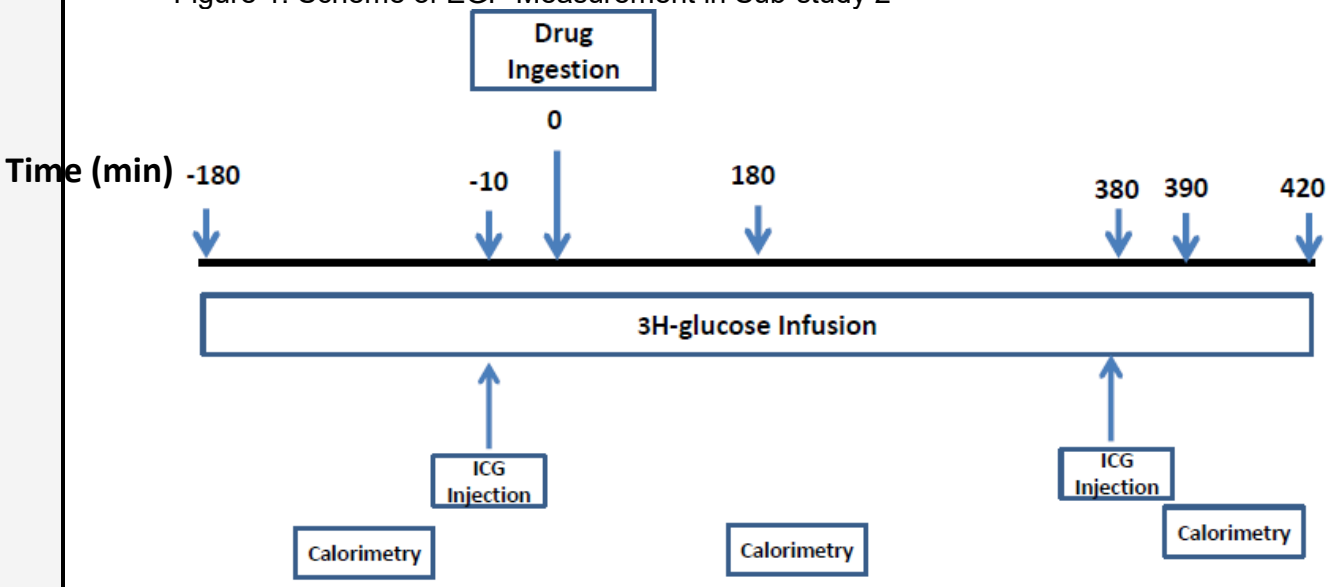
At time -10 minute, a bolus of (0.25 mg/kg) ICG will be injected and 8 blood samples (from -8 to zero minutes) will be withdrawn every 20 seconds to one minute to measure plasma volume.

At time zero each subject will ingest the treatment drug (empagliflozin 25 mg or placebo). Plasma volume measurements will be repeated with a bolus injection of ICG at 380 minute. 7 blood samples will be drawn after time zero for the measurement 3H-3-glucose specific activity, plasma glucose,

insulin, glucagon, FFA, ketone, norepinephrine, ACTH, cortisol, renin, angiotensin-II, NT-proBNP, and electrolyte (Na, K, Cl, HCO3) concentrations.

30-45 minutes of continuous indirect calorimetry will be performed at -60, 180, and 390 minutes to determine rates of glucose/lipid oxidation and energy expenditure. The total blood draw in this sub study will be 165 ml.
At 6 AM subjects will void (and discard) and urine will be collected from -180 to zero, and from time zero to 420 minute. Urine volume and concentrations of glucose, □-OH-B, sodium, and chloride will be measured.

Figure 1: Scheme of EGP Measurement in Sub-study 2



Measurement I. Subjects will ingest placebo at time zero, and blood and urine samples will be collected as described above. The retrograde line will be kept patent by infusing 0.5 N saline at 2 ml per hour using high precision infusion pumps (2ml/hour x 7 hours = 14 ml total over 7 hours).
Measurement II. Subjects will ingest empagliflozin (25 mg) at time zero, and blood and urine samples will be collected as described above. Each intravenous line will be kept patent as described in Measurement I. Using this protocol we previously have shown that SGLT2i (dapagliflozin, empagliflozin, and canagliflozin) stimulate endogenous glucose production (EGP) compared to placebo and increase plasma ketone concentration by 0.3-0.6 mmol/L, in association with a 4-7 uU/ml decrement in plasma insulin concentration that was paralleled by a similar decrease in plasma C-peptide concentration and increase in plasma glucagon concentration (3).
Measurement III. Subjects will ingest empagliflozin (25 mg) at time zero and blood and urine samples will be collected as described above. At time zero, an intravenous infusion of saline will be started to replace the amount of fluid and electrolites excreted in the urine during the EGP measurement without volume replacement. Approximately 70 meq of Na+ and 70 meq of Cl- are excreted in the urine by SGLT2i treatment within 6 hours. Thus, 2.2 ml/min (of half normal saline), or 1.1 ml/min of normal saline (0.9%) will be infused until the study end at 420 minute. In previous studies with empalgilfozin, as well as with dapagliflozin and canagliflozin (3,5), we demonstrated that 8-hour urine volume and 8-hour urine sodium chloride excretion increased by ~600 ml and ~70 meq, respectively. Thus, administration of 462 ml (or 924 ml if normal saline is infused) of fluid and 70 meq of Na+Cl- over a 7-hour period should be more than sufficient to prevent any decrease in plasma (represents 5% of body weight), and extracellular (represents 20% of body weight) fluid volume. The measurement of plasma volume with ICG will document the absence of any change (decrease) in fluid space. In addition we will measure NT proBNP throughout the study.

Measurement IV: This measurement will be performed similar to Measurement III with one difference. At time zero, subjects will ingest placebo instead of empagliflozin.
Data Analysis and Statistical Methods. Under steady-state postabsorptive conditions, the basal rate of endogenous glucose appearance (Ra = bEGP) equals 3-3H-glucsoe infusion rate divided by steady state plasma tritiated glucose specific activity. After drug administration, non-steady conditions for 3-3H-glucose specific activity prevail and the rate of whole body of glucose appearance (Ra) is calculated from the Steele equation. The difference in EGP during the last half hour of the study (390 to 420 minutes) between subjects receiving empagliflozin (Measurement II) vs placebo (Measurement I) represents the increase in EGP caused by empagliflozin. The difference in EGP between subjects receiving empagliflozin without volume repletion (Measurement II) versus with volume repletion (Measurement III) represents the role of intravascular volume depletion in the stimulation of EGP. The difference in the change in EPG between Measurement II and Measurement III will be compared to the difference between Measurement I and Measurement IV will be compared with ANOVA. Post hoc testing will be performed with Bonferroni correction for multiple comparisons.
The same statistical/analytical approach will be employed for the measurement of plasma FFA, and beta-OH-butyrate concentrations.
Sample Size Calculation. In previous studies, we demonstrated that the mean difference in EGP during the last hour of EGP measurement in empagliflozin-treated (Measurement II) and placebo-treated (Measurement I) subjects was 0.70±0.34 mg/kg.min (mean±SD) (3). To detect a 50% decrease in this difference at alpha <0.05 during the volume repletion study (Measurement III) with 90% power and alpha = 0.0125 (=0.05/4), we computed that 32 subjects are required. Therefore, to ensure 32 completers, we have set the sample size at 36.

Risks in Sub-Study 2:
Blood loss: blood loss in sub-study 2 will be 165 ml at each EGP measurement. Thus, the total blood loss will be 660 ml

Radiation: total amount of radiation in sub-study 2 will be 59.6 mrem (13.4 at each EGP measurement and 6 mrem at DEXA scan)

Indocyanine Green (ICG): is approved by the FDA for the measurement of cardiac output and hepatic function. It is cleared from the body by excretion to the biliary system. The only known adverse event is allergic reaction to iodine. Subjects with allergy to iodine will be excluded from the study. Although ICG is rapidly cleared from the body, it is recommended that subjects will not perform radioiodine uptake studies until one week after the use of ICG.

Empagliflozin: approved by the FDA for the treatment of T2DM. All patients in this sub-study have T2DM. The drug increases the risk of genital and urinary infections by ~5%. Rare adverse events (<1%) include volume depletion, decreased blood pressure, ketoacidosis, necrotizing fasciitis, and acute kidney injury. These adverse events were observed with long treatment (6 months) with the drug. However, subjects will receive only two tablets in the present study.

Assessment of adverse events
Definitions of adverse events:
Adverse event - An AE is defined as any untoward medical occurrence in a patient or clinical investigation subject administered a medicinal product and which does not necessarily have to have a causal relationship with this treatment.

An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

The following should also be recorded as an AE in the CRF and SAE form (if applicable):

- Worsening of the underlying disease or of other pre-existing conditions
- Changes in vital signs, ECG, physical examination and laboratory test results, if they are judged clinically relevant by the investigator

Serious adverse event:
A serious adverse event (SAE) is defined as any AE, which fulfils at least one of the following criteria:

- results in death,
- is life-threatening, which refers to an event in which the patient was at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if more severe,
- requires inpatient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity,
- is a congenital anomaly / birth defect,
- is deemed serious for any other reason if it is an important medical event when based on appropriate medical judgement which may jeopardise the patient and may require medical or surgical intervention to prevent one of the other outcomes listed in the above definitions. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm, blood dyscrasias or convulsions that do not result in hospitalisation or development of dependency or abuse.
- Cancers of new histology and exacerbations of existing cancer must be classified as a serious event regardless of the time since discontinuation of the drug and must be reported.

Adverse events of Special Interest (AESIs):

The term AESI relates to any specific AE that has been identified at the project level as being of particular concern for prospective safety monitoring and safety assessment within this trial, e.g. the potential for AEs based on knowledge from other compounds in the same class. AESIs need to be reported to the Pharmacovigilance Department of Boehringer Ingelheim within the same timeframe that applies to SAEs.

Patients with AESIs need to be followed up appropriately, regardless of the origin of the laboratory data (e.g. central, local etc.). The Investigator should consider which, if any, concomitant therapies should not be taken during evaluation. Discontinued treatments can be reintroduced per Investigator discretion.

The following are considered as AESIs:
Hepatic injury - A hepatic injury is defined by the following alterations of hepatic laboratory parameters after randomisation:

- an elevation of AST and/or ALT ≥ 3 fold ULN combined with an elevation of total bilirubin ≥ 2 fold ULN measured in the same blood sample
- an isolated elevation of ALT and/or AST ≥ 5 fold ULN

These laboratory findings constitute a hepatic injury alert and the patients showing these abnormalities need to be followed up according to medical judgement. .

In case of clinical symptoms of hepatic injury (icterus, unexplained encephalopathy, unex-plained coagulopathy, right upper quadrant abdominal pain, etc.) without laboratory results (ALT, AST, total

bilirubin) available, the Investigator should make sure these parameters are analysed, if necessary in an unscheduled blood test.

Decreased renal function

Decreased renal function is defined by a creatinine value showing a ≥ 2 fold increase from baseline and is above the ULN.

For the AESI “decreased renal function” the Investigator shall collect an unscheduled laboratory sample for creatinine as soon as possible and initiate follow-up laboratory tests of creatinine according to medical judgement.

Metabolic acidosis, ketoacidosis and diabetic ketoacidosis (DKA)
In case of metabolic acidosis, ketoacidosis and DKA further investigations should be done according to the medical judgment and the clinical course until a diagnosis is made and/or the patient is recovered.
DKA is defined by the diagnostic criteria in the table below, and as defined by the American Diabetes Association (ADA).

Investigators should note that not all criteria in the table below need to apply for the diagnosis of DKA, and clinical judgment should also be taken into consideration. Due to its mechanism of action, empagliflozin may potentially modify the clinical presentation of DKA which may occur at lower plasma glucose levels than stated in the table below.

Table 1 Diagnostic criteria for DKA

	DKA		
	Mild	Moderate	Severe
Plasma glucose (mg/dL)	>250	>250	>250
Arterial pH	7.25-7.30	7.00-7.24	<7.00
Serum bicarbonate (mEq/L)	15-18	10 to <15	<10
Urine ketones*	Positive	Positive	Positive
Serum ketones*	Positive	Positive	Positive
Effective serum osmolality (mOsm/kg)**	Variable	Variable	Variable
Anion gap***	>10	>12	>12
Alteration in sensoria or mental obtundation	Alert	Alert/drowsy	Stupor/coma

* Nitroprusside reaction method
** Calculation: 2[measured Na (mEq/L) + glucose (mg/dL)/18
*** Calculation: (Na+) – (Cl- + HCO3-) (mEq/L)

Intensity of adverse event
The intensity of the AE should be judged based on the following:

- Mild: Awareness of sign(s) or symptom(s) which is/are easily tolerated
- Moderate: Enough discomfort to cause interference with usual activity
- Severe: Incapacitating or causing inability to work or to perform usual activities

Causal relationship of adverse event
Medical judgment should be used to determine the relationship, considering all relevant factors, including pattern of reaction, temporal relationship, de-challenge or re-challenge, confounding factors such as concomitant medication, concomitant diseases and relevant history. Assessment of causal relationship should be recorded in the case report forms.

Yes: There is a reasonable causal relationship between the investigational product administered and the AE.

Arguments that may suggest that there is a reasonable possibility of a causal relationship could be:

- The event is consistent with the known pharmacology of the drug.
- The event is known to be caused by or attributed to the drug class.
- A plausible time to onset of the event relative to the time of drug exposure.
- Evidence that the event is reproducible when the drug is re-introduced.
- No medically sound alternative aetiologies that could explain the event (e.g. pre-existing or concomitant diseases, or co-medications).
- The event is typically drug-related and infrequent in the general population not exposed to drugs (e.g. Stevens-Johnson syndrome).
- An indication of dose-response (i.e. greater effect size if the dose is increased, smaller effect size if dose is reduced).

No: There is no reasonable causal relationship between the investigational product administered and the AE.

Arguments that may suggest that there is no reasonable possibility of a causal relationship could be:

- No plausible time to onset of the event relative to the time of drug exposure is evident (e.g. pre-treatment cases, diagnosis of cancer or chronic disease within days / weeks of drug administration; an allergic reaction weeks after discontinuation of the drug concerned).
- Continuation of the event despite the withdrawal of the medication, taking into account the pharmacological properties of the compound (e.g. after 5 half-lives).

Of note, this criterion may not be applicable to events whose time course is prolonged despite removing the original trigger.

- Additional arguments amongst those stated before, like alternative explanation (e.g. situations where other drugs or underlying diseases appear to provide a more likely explanation for the observed event than the drug concerned).
- Disappearance of the event even though the trial drug treatment continues or remains unchanged.

Responsibilities for SAE reporting to Boehringer Ingelheim
The Sponsor shall report (i.e., from signing the informed consent onwards through the trial defined 12-week follow-up period) all SAEs and non-serious AEs which are relevant for a reported SAE and Adverse Events of Special Interest (AESI) by fax or other secure method using BI IIS SAE form to the BI Unique Entry Point in accordance with timeline specified below

- within five (5) calendar days upon receipt of initial and follow-up SAEs containing at least one fatal or immediately life-threatening event;
- within ten (10) calendar days upon receipt of any other initial and follow-up SAEs.

BIPI Unique Entry Point:
Boehringer Ingelheim Pharmaceuticals, Inc.
900 Ridgebury Road, Ridgefield, CT
Fax: 1-203-837-4329

For each adverse event, the investigator will provide the onset date, end date, intensity,treatment required, outcome, seriousness, and action taken with the investigational drug. The investigator will determine the expectedness of the investigational drug to the AEs as defined in the Listed Adverse Events section of the Boehringer Ingelheim's (BI's) Investigator Brochure for the Productor BI Drug

Information e.g. Summary of Product Characteristics (SmPC) or Product Information (PI) for the authorised Study Drug provided by BI,

After the individual patient's end of trial: the investigator does not need to actively monitor the patient for new AEs but should only report any occurrence of cancer and trial treatment related SAEs which the investigator may become aware of by any means of communication.

Data Analysis Plan: **Data Analysis and Statistical Plan:** Glucosuria Induced Increase in Hepatic Glucose Production: Under steady-state postabsorptive conditions, the basal rate of endogenous (primarily reflecting hepatic) glucose appearance (Ra = bHGP) equals the ^{3}H-glucose infusion rate divided by steady state plasma ^{3}H-glucose specific activity. After drug (empagliflozin) administration, non-steady state conditions for 3H-glucose specific activity prevail and the of rate whole body glucose appearance (Ra) is calculated from Steele's equation (52). The difference between HGP in empagliflozin-treated and placebo-treated subjects, in each group (i.e. NGT, IFG and T2DM), represents the increase in HGP stimulated by glucosuria.

During the Oral-IV double tracer infusion study, only the ^{14}C-glucose which escapes the liver and non-hepatic splanchnic tissues appears in the systemic circulation, while all of the infused ^{3}H-glucose appears in the systemic circulation. Therefore, the difference between Ra calculated with ^{3}H-glucose and Ra calculated with ^{14}C-glucose represents the basal rate of hepatic plus non-hepatic splanchnic tissue glucose uptake. Thus, the basal rate of EGP will be calculated for ^{3}H-glucose as the ^{3}H-glucose infusion (dpm/min) divided by the steady-state plasma ^{3}H-glucose specific activity (dpm/mg). The rate of ^{14}C-glucose appearance will be calculated as the ^{14}C-glucose infusion rate (dpm/min) divided by the steady-state plasma ^{14}C-glucose specific activity (dpm/mg). The hepatic and non-hepatic splanchnic glucose uptake equals $Ra_{3H\text{-}glucose}$ minus $Ra_{14C\text{-}glucose}$ Norepinephrine clearance rate will be calculated as ^{3}H-NE infusion rate (dpm/min) divided by the steady state plasma ^{3}H-NE radioactivity (dpm/ml) (between 12 and 20 min). Norepinephrine spill over (release) rate (pg/min), which reflects the sympathetic nervous system activity (53), will be calculated as the product of plasma norepinephrine concentration (pg/ml) and norepinephrine clearance rate (ml/min). The difference between norepinephrine spill over in empagliflozin-treated and placebo-treated subjects, in each group (i.e. NGT, IFG and T2DM), represents the effect of glucosuria on the sympathetic nervous system activity. The ratio between plasma ^{3}H-DHPG to ^{3}H-NE (between 20 to 120 minutes) will be used as an index of neuronal uptake of norepinephrine (54).

Values will be presented as mean±SD. Difference between means will be compared by ANOVA. Post hoc testing will be performed with the Bonferroni test.

The increase in HGP caused by glucosuria in each treatment group will be related to the change in norepinephrine spill over rate with Pearson's correlation in each treatment group. We anticipate a strong correlation between the two variables.

Anticipated Results and Potential Problems: Based upon our preliminary results, we anticipate that empagliflozin will produce a large increase in HGP in NGT subjects which will maintain the FPG concentration at the fasting level. Further, because the FPG concentration does not change following empagliflozin administration, we anticipate that neither the fasting plasma insulin nor glucagon concentrations will be affected by empagliflozin. Likewise, we anticipate a large increase HGP in IFG subjects with no significant change in the fasting glucagon to insulin ratio. We also anticipate a shift in substrate oxidation (i.e. decrease in glucose oxidation and increase in fat oxidation) in NGT and IFG subjects with no significant increase in plasma ketone concentration. These results will provide strong evidence against a significant role of the increase in plasma glucagon to insulin ratio in the increase in HGP and shift in fuel consumption from glucose to fat oxidation following SGLT2 administration. Further, we anticipate that SGLT2 inhibitors will alter autonomic balance and increase sympathetic nervous system activity in NGT and IFG subjects and that the increase in sympathetic nervous system activity will be, at least in part, responsible for the increase in HGP. Thus, we anticipate a strong correlation between the increase in sympathetic nervous system activity and the magnitude of increase in HGP in IFG and NGT subjects.

It will be of great interest to examine the effect of empagliflozin on plasma lactate concentration in NGT subjects. If, as anticipated, empagliflozin causes a robust increase in fat oxidation, decrease in glucose oxidation and increase in lactate concentration in NGT subjects, without a change in ketones, FPG, FFA, insulin, and glucagon concentrations, this will provide a strong evidence in support of a direct effect of the drug on mitochondrial substrate oxidation, independent of SGLT2 inhibition. In such a scenario, future studies with MRI spectroscopy and ^{13}C-acetate infusion to quantitate hepatic TCA cycle activity (55,56) before and immediately after the administration of SGLT2 inhibitor in NGT subjects will provide direct evidence in support of an inhibitory effect of SGLT2 inhibitor on mitochondrial function independent of SGLT2 inhibition.

Consistent with previous studies (57), we anticipate that IFG subjects will manifest increased hepatic fat content and reduced HGU. Further, we anticipate that SGLT2 inhibitors will cause a significant decrease in hepatic fat content, increase in basal HGU and reduction in FPG concentration back/close to the normal level. Because MRI spectroscopy quantitates hepatic triglyceride content, while toxic fat metabolites (e.g. fatty acyl Co-A, DAG and ceramide), are responsible for the inhibition of HGU, it is possible to observe an improvement in HGU and decrease in the FPG concentration in IFG and T2DM patients on day 3 without a significant reduction in hepatic fat content measured with MRI. Nonetheless, in such a scenario, the reduction in hepatic fat content should be detected after 12 weeks of treatment.

Lastly, we anticipate that, in addition to the increase in HGP, shift in fuel consumption, and change in autonomic nervous system activity, SGLT2 inhibitors will produce a significant decrease in FPG concentration in T2DM patients and the decrease in the FPG concentration will be accompanied with a small decrease in the fasting plasma insulin concentration and large increase in plasma glucagon concentration in association with an increase in plasma ketone concentration in T2DM subjects following empagliflozin administration. These results will provide strong evidence in support of an important role of the changes in plasma insulin and glucagon concentrations in the increase in plasma ketone concentration following SGLT2i administration. It follows, therefore, that combination therapy with antidiabetic agents which inhibit glucagon secretion and increase insulin secretion, e.g. GLP-1 RA, plus SGLT2i will decrease the risk of ketoacidosis associated with SGLT2i therapy in T2DM patients (14,15).

We anticipate that empagliflozin will increase norepinephrine spill over to the plasma in T2DM patients as well, and that the increase in norepinephrine spillover will strongly correlate with the increase in HGP. Because the methodology utilized in the present study measures the rate of total body norepinephrine spill over, it is possible that the increase in epinephrine spill over from specific organs, e.g. kidney and splanchnic (primarily hepatic) tissue, will be diluted in the systemic circulation. Thus, it is possible that only a modest effect of empagliflozin on sympathetic activity will be detected. In such a scenario, measurement of organ specific (58), e.g. renal norepinephrine spill over (with renal vein catheterization) and splanchnic (with intradoudenal ^{3}H-NE infusion and arterial or hepatic vein sampling) can be employed to quantitate the change in sympathetic activity in the kidney and liver.

Sample Size Calculation We powered the study to detect a significant increase in the basal rate of HGP following empagliflozin administration versus placebo. In previous studies, the increase in the rate of HGP versus placebo following empagliflozin administration was 0.34±0.26 mg/kg.min. Therefore, we computed that 30 subjects in 2:1 randomization (20 empagliflozin and n=10 placebo) provide >90% power to detect a significant difference in HGP between empagliflozin-treated and placebo-treated subjects (in 3 groups comparison) at alpha <0.05. Therefore, to ensure 30 completers, we have set the sample size at 36 in each group.

Item 7 Risks Section:

Complete the following table to describe the risks of all **research procedures** listed in Step 2, Institutional Form (items 28-34). *Do not list risks of Routine care procedures here.*

X N/A, Risks are described in the informed consent document – do not complete this table.

Research procedures *example:* • History and physical • Questionnaire • Laboratory tests *Add or delete rows as needed*	**Risks** List the reasonably expected risks under the following categories as appropriate:
	○
	○
	○
	○
	○
	○