ΠΑΝΕΠΙΣΤΗΜΙΟ ΙΩΑΝΝΙΝΩΝ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΠΑΘΟΛΟΓΙΚΟΣ ΤΟΜΕΑΣ

Β' ΠΑΘΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

ΕΠΙΔΡΑΣΗ ΤΩΝ ΑΝΑΣΤΟΛΕΩΝ ΤΩΝ SGLT-2 ΣΥΜΜΕΤΑΦΟΡΕΩΝ ΣΤΙΣ ΜΕΤΑΒΟΛΙΚΕΣ ΠΑΡΑΜΕΤΡΟΥΣ ΤΟΥ ΟΡΟΥ ΚΑΙ ΤΩΝ ΟΥΡΩΝ ΣΕ ΑΣΘΕΝΕΙΣ ΜΕ ΣΑΚΧΑΡΩΔΗ ΔΙΑΒΗΤΗ ΤΥΠΟΥ 2

Σεμπάστιαν Φίλιππας-Ντεκουάν

Ιατρός

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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SCHOOL OF HEALTH SCIENCES

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EFFECT OF SGLT-2 INHIBITORS ON THE SERUM AND URINE METABOLOME IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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M.D.

PhD THESIS

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«Η έγκριση της διδακτορικής διατριβής από το Τμήμα Ιατρικής του Πανεπιστημίου Ιωαννίνων δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα Ν. 5343/32, άρθρο 202, παράγραφος 2 (νομική κατοχύρωση του Ιατρικού Τμήματος)».

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Σεμπάστιαν Φίλιππας-Ντεκουάν

ΠΕΡΙΕΧΟΜΕΝΑ

Introduction

Diabetes Mellitus

Diabetes mellitus (DM) refers to a group of common metabolic disorders which share the phenotype of hyperglycemia. Several distinct types of DM are caused by a complex interplay of genetic and environmental factors. Depending on the etiology of DM, the pathophysiological factors which contribute to the development of hyperglycemia include reduced insulin secretion, decreased glucose utilization and increased glucose production (1). The metabolic dysregulation in DM causes secondary pathophysiologic changes in multiple organ systems. In the Western hemisphere, DM is a leading cause of end-stage renal disease, nontraumatic lower extremity amputation and adult blindness. Furthermore, DM is a major cardiovascular risk factor associated with acute coronary syndromes, stroke and peripheral arterial disease. With an increasing incidence worldwide, with more than 500 million persons predicted to be diagnosed with DM by the year 2030 (1), DM will be a leading cause of morbidity and mortality in the near future.

According to the diagnostic criteria endorsed by the American Diabetes Association (ADA), DM is diagnosed when one or more of the following criteria are met (2)

1) Serum fasting glucose levels >125 mg/dl.
2) Random glucose levels >199 mg/dl with symptoms suggestive of chronic hyperglycemia (e.g. poyuria, polydipsia, weight loss with increased appetite).
3) Glucose levels >199 mg/dl, 2 hours after the ingestion of 75g of glucose.
4) Glycated hemoglobin (HbA1c) >6.4 %.

Diabetes can be classified into 4 broad categories (2):

1. Type 1 DM (due to autoimmune β-cell destruction, usually leading to absolute insulin deficiency), including latent autoimmune diabetes of the adult (LADA).
2. Type 2 DM (due to progressive loss of insulin secretion, usually on the basis of chronic insulin resistance). Type 2 DM (due to progressive loss of insulin secretion, usually on the basis of chronic insulin resistance). Type 2 DM is usually preceded by a period of abnormal glucose homeostasis, characterized by impaired glucose tolerance (IGT) or/and impaired fasting glucose (IFG).
3. Specific types of diabetes due to other causes, e.g. monogenic diabetes syndromes (such as maturity onset diabetes of the young, neonatal diabetes),

diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis) and drug related diabetes (on the context of glucocorticoid use, in the treatment of HIV/AIDS, after organ transplantation).

4. Gestational DM (diabetes diagnosed in the second or in the third trimester of pregnancy that was not overt diabetes before the pregnancy).

Traditionally, type 1 diabetes is usually manifested during childhood and a significant number (approximately 1/3 of patients) present with diabetic ketoacidosis. In contrast, type 1 diabetes may be more difficult to diagnose in adults, who may also experience temporary remission from the need of insulin. On the other hand, type 2 diabetes is usually manifested during adulthood, although cases are increasing in children due to the obesity epidemic, is most often related to chronic insulin resistance. Here we will focus on type 2 diabetes (2).

Classification and diagnosis of insulin resistance

Glucose tolerance is classified into 3 broad categories. Normal glucose homeostasis, DM, or impaired glucose homeostasis. DM can be diagnosed by any one of the aforementioned ADA endorsed criteria. IFG is diagnosed when fasting serum glucose levels are 100-125 mg/dl. IGT is diagnosed when serum glucose levels after an oral glucose tolerance test are 140-199 mg/dl. Both IFG and IGT diagnosis carry an increased risk of cardiovascular disease, and persons should be counseled about ways to decrease their cardiovascular and diabetes related risks (2).

Regulation of glucose homeostasis

Glucose homeostasis reflects a balance between glucose ingestion, hepatic glucose production and peripheral glucose uptake and utilization. The most important regulator of glucose homeostasis is insulin (a hormone secreted by the pancreatic beta cells), while other hormones (e.g. glucagon), metabolic signals and neural input result in integrated control of glucose supply and utilization. In the fasting state, low insulin levels increase hepatic glucose production by promoting hepatic and renal gluconeogenesis and hepatic glucogenolysis while at the same time decrease glucose uptake in peripheral tissues (especially skeletal muscle and fat) (3). At the same time, low insulin levels increase the utilization of other substrates for energy production, such as free fatty acids and amino acids. Glucagon is the counter hormone of insulin, is secreted by the pancreatic alpha cells when blood glucose levels and insulin levels are

low. After its secretion glucagon stimulates hepatic and renal gluconeogenesis and glycogenolysis. Postprandially, the glucose load leads to insulin secretion from the pancreatic islets, while at the same time glucagon secretion is hampered, leading to a reversal of these processes. Insulin, an anabolic hormone, leads to increased synthesis of lipids and proteins (3). Peripheral utilization of glucose in the postprandial state is mainly driven by skeletal muscle. Other tissues, most notably the brain, use glucose in an insulin-independent fashion.

Insulin action

Insulin is produced in the beta cells of the pancreatic islets. The main regulator of insulin secretion is glucose, although other substances and molecules such as amino acids, ketones, gastrointestinal peptides and others may also lead to insulin secretion. Glucose is transported inside the beta cell through the facilitative transporter (GLUT1 and/or GLUT2) (4). Once glucose enters the cell, it is phosphorylated to glucose-6-phosphate through the rate limiting enzyme glucokinase. Further metabolism of glucose-6-phosphate through glycolysis generates energy in the form of adenosine triphosphate (ATP). The ATP generated, inhibits an ATP sensitive potassium channel on the cell membrane leading to depolarization of the cell membrane and opening of a voltage dependent calcium channel leading to calcium influx and insulin secretion. The ATP sensitive potassium channel is the binding site of sulfonylureas and meglitinides, the common antidiabetic drugs which act through glucose independent insulin secretion. On the other hand, incretins, which are hormones secreted by intestinal cells, such as glucagon like peptide-1 (GLP-1), increase glucose dependent insulin secretion through increased calcium dependent insulin secretion .

Insulin is secreted in the portal system. Half of the secreted insulin is cleared by the liver, while the other half enters the systemic circulation where it binds to receptors in various sites (4). The insulin receptor is a glycoprotein which consists of two subunits, an extracellular α subunit and a β- transmembrane subunit which is a tyrosine kinase. The α-subunit inhibits the β-subunit. The binding of insulin to its receptor inhibits the α-subunit leading to receptor autophosphorylation and the recruitment of intracellular signaling molecules (such as the insulin receptor substrate 1 and 2-IRS1 and IRS2) (4). The activation of IRS1 and IRS2 lead to a complex cascade of phosphorylation and dephosphorylation and ultimately activate the ras/map kinase and PI3K/Akt pathway. Activation of the ras/map kinase pathway leads to cellular growth and explains the

mitogenic effects of insulin. On the other hand, the metabolic actions of insulin are mediated by the PI3K/Akt pathway. Activation of this pathway, leads to multiple actions in insulin sensitive cells such as:

- Activation of protein synthesis through the mTORC1/S6k pathway (5)
- Activation of de novo lipogenesis (DNL) and cholesterol synthesis through the sterol regulatory binding protein-1c (SREBP-1c) (6)
- Translocation of GLUT4 receptors to the cell membrane which mediate the insulin dependent glucose cell entry (7)
- Inhibition of FoxO1 with downstream inhibition of hepatic gluconeogenesis and glucogenolysis (8)

Pathogenesis of type 2 Diabetes Mellitus

Insulin resistance and impaired insulin secretion are central to the pathogenesis of DM2. Most studies have shown that the primary defect is insulin resistance, which usually precedes by many years the clinical development of DM2. DM2 is not evident until insulin secretion is also hampered. DM2 has also a strong genetic component, with a concordance of DM2 in identical twins of 70-90%. Furthermore, persons with a parent with DM2 have increased risk of developing DM2, while if both parents have DM2 then the risk approaches 40%. The disease is polygenic and multifactorial while environmental factors (such as obesity, decreased physical activity and poor dietary habits) also contribute to the clinical phenotype. Although a large number of genes and genetic polymorphisms have been associated with increased risk of DM2, <10% of the genetic risk is associated with loci identified so far (2).

Pathophysiology

DM2 is characterized by cellular insulin resistance, impaired insulin secretion, increased hepatic glucose production, abnormal fat metabolism and systemic low grade inflammation. Obesity, particularly central (or visceral) obesity as measured by the hip/waste ratio, is very common in DM2 as >80% of persons with DM2 are obese. As mentioned earlier, insulin resistance is usually preceded by many years before the development of DM2. To compensate for the cellular insulin resistance the pancreatic beta cells respond by increasing insulin secretion leading to a relative hyperinsulinemic state to maintain normal glucose tolerance. As insulin resistance and the compensatory hyperinsulinemia progress, the ability of the pancreatic beta cell to sustain the

hyperinsulinemic state weaves in certain individuals. IGT is then developed, by the inability of the beta cell to maintain normal glucose tolerance following a glucose load. If the insulin resistance and IGT is sustained, insulin secretion is further decreased and increased hepatic glucose production is also evident, with the development of overt hyperglycemia and DM2. Although both impaired insulin secretion and increased hepatic glucose production are keys to the development of DM2, the relative contribution of each mechanism may vary in each individual (2).

Insulin resistance

Insulin resistance, defined as the impaired insulin action in insulin sensitive tissues such as the muscle, fat and the liver, is central to the pathogenesis of DM2. Insulin resistance is determined by genetic susceptibility and environmental factors as previously discussed. However, insulin resistance is relative, as excessive insulin secretion can maintain normal or near normal glucose utilization. Insulin resistance results in decreased peripheral glucose utilization and increased hepatic glucose production; both mechanisms contribute to the development of hyperglycemia. Increased fasting plasma glucose (IFG) is mainly associated with an increased production of glucose by liver cells due to decreased inhibition of hepatic gluconeogenesis. On the other hand, impaired post prandial glucose tolerance is mainly attributed to decreased glucose entry in peripheral tissues such as muscle and fat. Both mechanisms are present in DM2. On the other hand, non insulin dependent glucose utilization is not altered in DM2 (9).

Insulin resistance may be observed at different sites, from the cell membrane and the insulin receptor to the cell nucleus and the responsible genes. Indeed, it has been shown that insulin resistance may be secondary to decreased function of the insulin receptor, inhibition of the insulin receptor substrate (IRS-1 and IRS-2), inhibition of the PI3K/Akt pathway and increased FoxO1 function. All of the above may be observed when inhibition of the IRS-1 and IRS-2 occurs (10). The insulin receptor substrates consist of serine and threonine residues which can be phosphorylated by various molecules, such as, MAP kinase, JNK (c-Jun N-terminal Kinases), PKC (protein kinase c) and mTOR. The phosphorylation of the insulin receptor substrates either targets them for degradation or inhibits downward pathway activation through PI3K (8).

The pathogenesis of insulin resistance is incompletely understood and has not been elucidated. Obesity and ectopic fat accumulation, i.e. accumulation of fat in tissues who

normally are fat-free (such as the liver, the muscles and the pancreas), seem to play a major role in the development of insulin resistance.

The increased adipocyte mass leads to increased levels of circulating free fatty acids and other fat cell products. For example, adipocytes and the immune cells that infiltrate the inflamed visceral adipose tissue secrete a number of biologic products (nonesterified free fatty acids, retinol-binding protein 4, leptin, TNF-α, resistin, IL-6, and adiponectin) (11). The increased production of free fatty acids and some adipokines may cause insulin resistance in skeletal muscle and liver. Free fatty acids also impair glucose utilization in skeletal muscle, promote glucose production by the liver, and impair beta cell function. In contrast, the production by adipocytes of adiponectin, an insulin-sensitizing peptide, is reduced in obesity, and this may contribute to hepatic insulin resistance. Diacylglyceroles (DAGs) and ceramides have been recognized as important lipid mediators of insulin resistance (12). The obesity induced intracellular DAG accumulation leads to the activation of PKCε and ultimately to the phosphorylation of IRS and cellular insulin resistance (13). The observed ceramide intracellular deposition also leads to the activation of another isomorph of PKC, PKCζ, leading to the phosphorylation of other substrates and the development of insulin resistance. Furthermore, ceramide accumulation in fat tissue leads to inflammation of fat tissues through the NLRP3 infammasome (14). The subsequent release of various cytokines and chemokines from the activated macrophages leads to worsening of insulin resistance in all tissues (15).

Decreased insulin secretion

Insulin secretion and sensitivity are interrelated. In DM2, insulin secretion initially increases in response to insulin resistance to restore normal glucose tolerance. Initially, the insulin secretory defect is mild and selectively involves glucose-stimulated insulin secretion, including a greatly reduced first secretory phase. The response to other nonglucose secretagogues, such as arginine, is preserved, but overall beta cell function is reduced by as much as 50% at the onset of type 2 DM. Eventually, the insulin secretory defect progresses (15). The reason(s) for the decline in insulin secretory capacity in type 2 DM is unclear. A second genetic effect, superimposed upon insulin resistance, may lead to decreased beta cell mass and/or impaired insulin synthesis and secretion. Beta cell mass is decreased by ~50% in individuals with long-standing type 2 DM. Islet amyloid polypeptide or amylin, co-secreted by the beta cell, forms amyloid

fibrillar deposits found in the islets of individuals with long-standing type 2 DM. Furthermore, chronic hyperglycemia paradoxically impairs islet function ("glucotoxicity") and leads to a worsening of hyperglycemia. Improvement in glycemic control is often associated with improved islet function and lower requirement for exogenous insulin administration in insulin treated patients. In addition, elevated levels of free fatty acids ("lipotoxicity"), and systemic and local elevations in pro-inflammatory cytokines from increased numbers of islet-associated macrophages, may also worsen islet function. Reduced GLP-1 action also contribute to the reduced insulin secretion (16).

Increased hepatic glucose and lipid production

Insulin resistance in the liver leads to a failure of the hyperinsulinemic state to suppress hepatic gluconeogenesis while also leading to glycogen depletion resulting in fasting hyperglycemia. Fasting hyperglycemia is usually evident after peripheral insulin resistance and impaired glucose tolerance has occurred. In adipocytes (fat cells), insulin resistance leads to a failure to suppress beta oxidation and lipolysis resulting in increased free fatty acid flux to the liver. Combined with increased hepatic de novo lipogenesis, the free fatty acid flux from adipocytes results to increased lipid accumulation within the liver parenchyma and also to increased production of very low density lipoprotein (VLDL) and secretion from the liver (17). This forms the pathophysiologic basis for the dyslipidemia found in DM2 which consists of increased VLDL-cholesterol, reduced high density lipoprotein cholesterol (HDL-C) and increased small dense low density lipoprotein cholesterol (sdLDL-C). Furthermore, the increased lipid accumulation within the liver parenchyma is responsible for non alcoholic fatty liver disease, a disorder recently recognized as an important liver related and cardiovascular related adverse outcome determinants.

Renal handling of glucose

The role of the kidney in the homeostasis of glucose has been recently elucidated and will be discussed in depth later. In normoglycemic individuals, glucose is freely filtered through the glomerulus. However, 99% of the filtered glucose load is then reabsorbed by two glucose-sodium co transporters located at the proximal renal tubule. In persons with DM2, due to chronic hyperglycemia, these transporters are overexpressed leading to increased glucose reabsorption at the proximal renal tubule and worsening

hyperglycemia (18, 19). Furthermore, increased glucose reabsorption may play a part in the pathogenesis of diabetic kidney disease.

Management of DM2

The goals of therapy for type 1 or type 2 diabetes mellitus (DM) are to (1) eliminate symptoms related to hyperglycemia, (2) reduce or eliminate the long-term microvascular and macrovascular complications of DM, and (3) allow the patient to achieve as normal a lifestyle as possible (**table 1**). To reach these goals, the physician should identify a target level of glycemic control for each patient, provide the patient with the educational and pharmacologic resources necessary to reach this level, and monitor/treat DM-related complications. Symptoms of diabetes usually resolve when the plasma glucose is <200 mg/dL, and thus most DM treatment focuses on achieving the second and third goals (20).

Table 1. Comprehensive medical care in patients with DM
Individualized glycemic control and therapeutic plan
Self monitoring of blood glucose (individualized frequency)
HbA1c testing (2-4 times per year)
Lifestyle management in the setting of diabetes; nutrition plan, physical activity, self management education, psychosocial management
Detection, prevention or management of diabetes related complications; diabetes related eye examination (annual or biannualy), diabetes related neuropathy examination (annual examination), diabetes related kidney disease testing (annual), diabetes foot examination (1-2 times/year)
Manage or prevent diabetes relevant conditions; blood pressure, lipids, antiplatelet medication, pneumococcal-influenza-hepatitis B immunization

Pharmacological treatment

DM2 management should begin with lifestyle changes. An exercise regimen to increase insulin sensitivity and promote weight loss should also be instituted. Pharmacologic approaches to the management of type 2 DM include oral glucose-lowering agents, insulin, and other injectable glucose lowering agents; most physicians and patients prefer oral glucose-lowering agents as the initial choice. Any therapy that improves

glycemic control reduces "glucotoxicity" to beta cells and improves endogenous insulin secretion. However, type 2 DM is a progressive disorder and ultimately requires multiple therapeutic agents and often insulin in most patients.

Advances in the treatment of DM2 have generated oral glucose-lowering agents that target different pathophysiologic processes in DM2. Based on their mechanisms of action, glucose-lowering agents are subdivided into agents that increase insulin secretion, reduce glucose production, increase insulin sensitivity, enhance GLP-1 action, or promote urinary excretion of glucose. Insulin is sometimes the initial glucose-lowering agent in DM2 especially when HbA1c is >10% at diagnosis (21).

Biguanides

Metformin, the representative of this class of glucose lowering agents, reduces hepatic glucose production and increases peripheral glucose utilization. Metformin activates AMP dependent protein kinase and enters cells through organic anion transporters. Metformin reduces fasting glucose levels, insulin levels, improves the lipid profile and promotes modest weight loss. Gastrointestinal side effects are common with the use of metformin (diarrhea, bloating, anorexia, nausea) and in 10% of cases are severe enough to warrant discontinuation of metformin. These side effects may be reduced by slow titration of metformin dosage over a period of 2-3 weeks. Vitamin B12 are 30% lower, during metformin treatment. The major toxicity of metformin, lactic acidosis, is very rare and usually presents when eGFR<30 ml/min/m^2. Thus metformin is not indicated in chronic kidney disease patients with eGFR<30 ml/min/m^2 and treatment should be discontinued in all patients admitted to the hospital, in patients receiving nothing orally and in patients receiving radiographic contrast material (21).

Insulin secretagogues-agents that enhance the ATP sensitive potassium channel

Insulin secretagogues stimulate insulin secretion by interacting with the ATP-sensitive potassium channel on the beta cell. These drugs are most effective in individuals with DM2 of relatively recent onset (<5 years) who have residual endogenous insulin production. First - generation sulfonylureas (chlorpropamide, tolazamide, tolbutamide) have a longer half-life, a greater incidence of hypoglycemia and more frequent drug interactions and are no longer used. Second-generation sulfonylureas have a more rapid onset of action and better coverage of the postprandial glucose rise, but the shorter half-life of some agents may require more than once-a-day dosing. Sulfonylureas reduce both fasting and postprandial glucose and should be initiated at low doses and increased

at 1 - to 2-week intervals based on SMBG (22). In general, sulfonylureas increase insulin action and thus should be taken shortly before a meal; with chronic therapy, though, the insulin release is more sustained. Glimepiride and glipizide can be given in a single daily dose and are preferred over glyburide, especially in the elderly. Repaglinide, nateglinide and mitiglinide are not sulfonylureas but also interact with the ATP-sensitive potassium channel (23). Because of their short half-life, these agents are given with each meal or immediately before to reduce meal-related glucose excursions. Insulin secretagogues have the potential to cause hypoglycemia, especially in elderly individuals. Hypoglycemia is usually related to delayed meals, increased physical activity, alcohol intake, or renal insufficiency. Individuals who ingest an overdose of some agents develop prolonged and serious hypoglycemia and should be monitored closely in the hospital for at least 48 hours. Most sulfonylureas are metabolized in the liver to compounds (some of which are active) that are cleared by the kidney. Thus, their use in individuals with significant hepatic or renal dysfunction is not advisable. Weight gain, a common side effect of sulfonylurea therapy, results from the increased insulin levels and improvement in glycemic control. Some sulfonylureas have significant drug interactions with alcohol and some medications including warfarin, aspirin, ketoconazole, α glucosidase inhibitors and fluconazole. A related isoform of ATP-sensitive potassium channels is present in the myocardium and the brain. All of these agents except glyburide have a low affinity for this isoform. Despite concerns that this agent might affect the myocardial response to ischemia and observational studies suggesting that sulfonylureas increase cardiovascular risk, studies have not shown an increased cardiac mortality with glyburide or other agents in this class (21, 24).

Insulin secretagogues-agents that enhance GLP-1 receptor signaling

Incretins amplify glucose stimulated insulin secretion. Two categories of drugs are in circulation, either direct GLP-1 receptor agonists, or agents that inhibit dipeptidyl peptidase IV (DPP4) which leads to increased endogenous GLP-1 levels. Agents in this class do not cause hypoglycemia because of the glucose dependent insulin secretion nature of their effect. GLP-1 receptor agonists are, to this time, subcutaneously administered agents with strong HbA1c lowering capacity. Adverse effects are usually mild, and include nausea and vomiting which can be decreased by slow dosing titration. Furthermore, some of these agents (including liraglutide, semaglutide and dulaglutide) have proven cardiovascular benefits in large randomized controlled trials. On the other

hand, DPP4 inhibitors are orally administered drugs that inhibit degradation of endogenous GLP-1 and thus enhance its action. DPP4 inhibitors promote insulin secretion in the absence of hypoglycemia and weight gain and appear to have a preferential effect on postprandial blood glucose. These agents are used alone, or in combination with other oral antidiabetic drugs. Large randomized controlled studies of DPP4 inhibitors (including linagliptin, sitagliptin, vildaglipitin and saxagliptin) have shown that these agents have a neutral effect regarding cardiovascular disease. Past concerns about possible pancreatitis and increased risk of pancreatic cancer appear to be unfounded (21).

a-Glucosidase inhibitors

a-Glucosidase inhibitors reduce postprandial glucose by delaying glucose absorption; they do not affect glucose utilization or insulin secretion. These drugs, taken before each meal, reduce glucose absorption by inhibiting the enzyme that cleaves oligosaccharides into simple sugars in the intestinal lumen. Side effects include diarrhea, flatulence, abdominal distention which are related to the increased delivery of oligosaccharides to the large bowel and can be reduced by gradual upward dose titration. They should not be used in the presence of inflammatory bowel disease, gastroparesis or kidney disease. This class is not as potent as other antidiabetics in lowering HbA1c, but can be useful in lowering the postprandial glucose load (21).

Thiazolidinediones

Thiazolidinediones reduce insulin resistance by binding to the PPAR-γ (peroxisome proliferator receptor γ) nuclear receptor. This receptor is found in many tissues, although highest levels are found in adipocytes. PPAR-γ agonists regulate a large number of genes, promote adipocyte differentiation, reduce hepatic fat content and promote fatty acid storage. These agents promote redistribution of fat from central to peripheral tissues. Furthermore, agents of this class decrease insulin levels possibly by increasing insulin sensitivity in peripheral tissues (25). Troglitazone, the prototype of this class, was withdrawn from circulation due to reports of hepatotoxicity and an association with idiosyncratic reaction leading to hepatic failure. Rosiglitazone, the second drug in this class, was withdrawn from circulation in 2007 due to concerns of increased cardiovascular risk. Although the FDA cleared its name, its use has been largely abandoned. Pioglitazone, is the representative of this class in circulation. This agent has been shown to be safe from a cardiovascular disease point of view, while

recent studies have shown that it might reduce the incidence of stroke (25). Furthermore pioglitazone use has been associated with reduced liver fat and increased high density lipoproterin-cholesterol (HDL-C) levels and reduced triglyceride levels. On the other hand, pioglitazone has been associated with modest weight gain (2-3 kgs) due to increased water retention and peripheral oedema. Furthermore, this agent is contraindicated in patients with NYHA III or IV cardiac failure (26). Pioglitazone has also been associated with an increased risk of fractures, especially in women. Past concerns of an increased risk of bladder cancer appear to be unfounded, yet pioglitazone is part of an ongoing FDA safety review.

Sodium-Glucose cotransporter 2 (SGLT-2) inhibitors

SGLT-2 inhibitors reduce plasma glucose by inhibiting its reabsorption in the proximal convulated tubule of the kidney. Although seemingly simple, they seem to have a detrimental effect in the metabolism of every tissue. Randomized, placebo-controlled studies have shown a reduced risk of cardiovascular disease in patients treated with these agents (EMPA-REG study, the CANVAS program). Furthermore, recent studies have shown a benefit in patients with heart failure and chronic kidney disease, independently of the presence of diabetes. More about SGLT-2 inhibitors will be discussed later (27).

Bile acid binding resins

Bile acid, by acting through nuclear receptors, have a role in metabolism. Colesevelam, a bile acid binding resin, has been approved for the treatment of type 2 diabetes. Colesevelam increase plasma triglycerides and also cause gastrointestinal side effects such as constipation, abdominal pain and nausea. Although this agent has been approved for the treatment of type 2 diabetes, its use is currently limited (21).

Insulin therapy

Insulin should be considered as the initial treatment for type 2 diabetes in patients with high HbA1c levels (>10%) at presentation, in lean individuals with severe weight loss (indicating high glucose levels and insulinopenia), or in individuals hospitalized or acutely ill. Ultimately, every person with type 2 diabetes will require insulin because of the progressive nature of the disorder and the relative insulin deficiency that develops in patients with long standing diabetes. Both patients and physicians are reluctant in initiating insulin therapy, but glucose control and well being are improved after insulin

initiation. Because endogenous insulin secretion continues, patients are usually initially started at once daily insulin injection to reduce fasting hyperglycemia. As diabetes progresses and postprandial insulin secretion is reduced, these persons may require addition of meal time insulin (once daily or before each meal, as in persons with type 1 diabetes). Oral agents are usually maintained during insulin treatment, as they decrease insulin requirements during the day, while also preserving their desirable off target effects (such as decreased cardiovascular risk etc) (21).

Choice of initial agent

Metformin could be the initial agent at the time of DM2 diagnosis, unless there are any contraindications according to the latest guideline issued by the American Diabetes Association (ADA) (21). For most patients, this will be the initial treatment, with additional lifestyle modifications. Additional treatment may be considered depending on the patient's characteristics (e.g. established cardiovascular disease, renal disease). Metformin is effective, safe, inexpensive and may reduce cardiovascular risk and death.

In patients with contraindications to metformin, or metformin intolerance, initial therapy should be based on patient's characteristics (hypoglycemia risk, cardiovascular disease status, weigh, cost, heart failure, renal disease). When the HbA1c is >1.5 % off target, then initial combination treatment is warranted. In patients with hyperglycemia symptoms (polyuria, polydipsia), catabolic state (weight loss, hypertriglyceridemia, ketonemia) or in patients with HbA1c >10%, initial insulin treatment is warranted. As glucose toxicity resolves, simplifying the regimen, or switching to oral agents is possible.

Combination therapy

Because type 2 diabetes is a progressive disease in many patients, most will require intensification of treatment after a few years. Current recommendation is to use stepwise addition of medications to maintain HbA1c targets. The choice of medication depends of various factors including the patient's preference, age, hypoglycemia risk, weight, comorbidities and others. Treatment intensification should not be delayed in the presence of increased HbA1c. This applies to the early and prompt insulin initiation (21).

Assessment of glycemic control

Optimal monitoring of glycemic control involves plasma glucose measurements by the patient and an assessment of long-term control by the providers on the diabetes management team (measurement of hemoglobin A1c [HbA1c] and review of the patient's SMBG). These measurements are complementary: the patient's measurements provide a picture of short-term glycemic control, whereas the HbA1c reflects average glycemic control over the previous 2–3 months (28).

Measurement of SMBG is the standard for short term glycemic control, while measurement of the patient's glycated hemoglobulin level is the gold standard for long term glycemic control (over the previous 3 months). When plasma glucose is consistently elevated, there is an increase in nonenzymatic glycation of hemoglobin; this alteration reflects the glycemic history over the previous 2–3 months, because erythrocytes have an average life span of 120 days (glycemic level in the preceding month contributes about 50% to the HbA1c value). HbA1c should be measured in all individuals with DM during their initial evaluation and as part of their comprehensive diabetes care. As the primary predictor of long-term complications of DM, the HbA1c should mirror, to a certain extent, the short-term measurements of SMBG. These two measurements are complementary in that recent intercurrent illnesses may impact the SMBG measurements but not the HbA1c. Likewise, postprandial and nocturnal hyperglycemia may not be detected by the SMBG of fasting and preprandial capillary plasma glucose but will be reflected in the HbA1c. The HbA1c is an "average" and thus does not detect glycemic variability in the way SMBG and CGM can. In standardized assays, the HbA1c approximates the following mean plasma glucose values: an HbA1c of 6% = 7.0 mmol/L (126 mg/dL), 7% = 8.6 mmol/L (154 mg/dL), 8% = 10.2 mmol/L (183 mg/dL), 9% = 11.8 mmol/L (212 mg/dL), 10% = 13.4 mmol/L (240 mg/dL), 11% = 14.9 mmol/L (269 mg/dL), and 12% = 16.5 mmol/L (298 mg/dL). Clinical conditions leading to abnormal RBC parameters such as hemoglobinopathies, anemias, reticulocytosis, transfusions, and uremia may alter the HbA1c result. In patients achieving their glycemic goal, the ADA recommends measurement of the HbA1c at least twice per year. More frequent testing (every 3 months) is warranted when glycemic control is inadequate or when therapy has changed. Laboratory standards for the HbA1c test have been established and should be correlated to the reference assay of the Diabetes Control and Complications Trial (DCCT). The degree of glycation of other proteins,

such as albumin, or measurement of 1,5-anhydroglucitol can be used as an alternative indicator of glycemic control when the HbA1c is inaccurate. The fructosamine assay (measuring glycated albumin) reflects the glycemic status over the prior 2 weeks (28).

Individualization is the key to the management of diabetes. Management of blood glucose should aim for long periods of normal, or near normal glucose levels, although this has been shown to be quite difficult in DCCT and the UKPDS trials. Regardless of the level of hyperglycemia, improvement of glucose level will invariably result in decrease of diabetes related complications, especially microvascular complications. The target for glycemic control must be individualized and the goals of therapy should be discussed with the patient taking into account his/her medical, social and lifestyle issues. In general, the ADA suggests an HbA1c target of <7% with a more stringent target of <6.5% for some patients or a looser target of <7.5% for other patients. Fasting and postprandial glucose levels should also be taken into consideration with a target of 80-130 mg/dl for fasting glucose levels and <160-180 mg/dl for postprandial glucose levels. Hypoglycemia (glucose levels <70 mg/dl in persons with DM) is a major limiting factor to the intensification of glucose lowering therapy and should be strictly avoided in high risk populations such as the very young, the elderly and in patients with a history of cardiovascular disease (28).

SGLT-2 inhibitors

There are six Sodium-Glucose cotransporters identified in human cells. SGLT-1 through 6, although the first two have been extensively studied in the last years (29). In healthy adults the kidney reabsorbs all of the filtered glucose (approximately 180 gr/day) in the proximal convoluted tubule. An electrochemical gradient of sodium (Na^+) is required for glucose reabsorption. The Na^+/K^+ ATPase removes Na^+ at the basolateral membrane which generates the electrochemical driving force for apical glucose entry via Na^+-driven sodium–glucose cotransport (30). On the basolateral side, glucose exits the cell via the GLUT2 receptor and re-enters the bloodstream (31). Studies have shown that SGLT-2 is expressed at the early proximal tubule (the S1 segment) and that is responsible for approximately 97% of the whole kidney glucose reabsorption. On the other hand, SGLT-1 are mainly expressed in the intestine and in the S2 segment of the distal part of the proximal convoluted tubule of the kidney and is responsible for all

glucose absorption in the intestinal lumen following a glucose load, while it is also responsible for approximately 3% of the reabsorption of glucose from the kidney. Thus, under normal circumstances, SGLT-1 and SGLT-2 are responsible for all glucose reabsorption from the kidney (32, 33, 34).

In 1927 a person with familial renal glycosuria resulting from mutations of the SGLT-2 was described which resulted in urinary glucose losses ranging from 1-150 g/1.73 m^2 per day (35). Familial renal glycosuria is considered a benign condition but can be associated with polyuria, polydipsia, nocturia and recurrent urinary tract infections. However, no serious infections, or other serious complications have been observed in persons with SGLT-2 mutations, thus providing supporting evidence for the use of SGLT-2 inhibitors (36). In transgenic SGLT2 -/- null mice (33) glucose is reabsorbed in nephron segments downstream of the early proximal tubule, including distal aspects of the proximal convoluted tubule. This reabsorption may be mediated by SGLT1, the high-affinity/low-capacity co-transporter (as opposed to SGLT-2 which are low affinity/high capacity co-transporters), and/or by NaGLT1, the low-affinity co-transporter.

When plasma glucose levels exceed 180 mg/dl, the renal maximum transport maximum is reached and then the surplus of glucose spills into the urine. In persons with diabetes the renal maximum glucose handling can be increased in the context of tubular growth or increased SGLT-1 and SGLT-2 expression (37, 38, 39).

SGLT-2 inhibitors are a relatively novel glucose lowering drug class which includes 5 currently approved drugs including dapagliflozin, empagliflozin, canagliflozin, sotagliflozin and ertugliflozin. They act by inhibiting the SGLT-2 on the S1 segment of the proximal renal tubule which in turn leads to increased urinary glucose loss and decreased plasma glucose, which is insulin independent (40). SGLT-2inhibitor administration leads to a modest decrease of HbA1c of 0.80–1.03 in monotherapy and by 0.71–0.93 in combination with other antihyperglycemics (41). Their main characteristic which could prove clinically relevant is their differential ability to inhibit not only SGLT-2 but also SGLT-1 (42). The most selective SGLT-2 inhibitors are empagliflozin and ertugliflozin, while on the other hand, sotagliflozin is the least selective SGLT-2 inhibitor (43). Their main pharmacokinetic characteristics are outlined in **table 2.**

Table 2. Clinical and pharmacokinetic characteristics of currently approved SGLT-2 inhibitors					
	Empagliflozin	**Canagliflozin**	**Dapagliflozin**	**Ertugliflozin**	**Sotagliflozin**
Absorption (Tmax)	1.5 hrs	1-2 hrs	2 hrs	1 hr	1.25-3 hrs
Bioavailability	78%	65%	78%	100%	25%
Fraction bound to protein	86%	99%	91%	93.6%	>93%
Volume of distribution	73.8 L	83.5 L	118 L	86 L	9000 L
Total body clearance	10.6 L/hr	192 ml/min	207 ml/min	11 L/hr	300 L/hr
Cmax	259 nmol/l for the 10 mg dose 687 nmol/l for the 25 mg dose	2465 nmol/l for the 100 mg dose 7828 nmol/l for the 300 mg dose	460 nmol/l	8 ng/ml for the 5 mg dose 268 ng/ml for the 15 mg dose	127 ng/ml for the 200 mg dose 241 ng/ml for the 400 mg dose
T1/2	12.4 hrs	10.6 hrs for the 100 mg dose 13.1 hrs for the 300 mg dose	12.9 hrs	17 hrs	21-35 hrs
SGLT-2 inhibition IC50	3.1 nM	2.7 nM	1.2 nM	0.9 nM	1.8 nM
SGLT-1 inhibition IC50	8300 nM	710 nM	1400 nM	1960 nM	36 nM
Metabolism	Glucoronidation	Glucoronidation	Glucoronidation	Glucoronidation	Glucoronidation
Dosing	10 mg/day 25 mg/day	100 mg/day 300 mg/day	10 mg/day	5 mg/day 15 mg/day	200-400 mg/day
Elimination route	41% faeces 54% urine	52% faeces 33% urine	21% faeces 75% urine	41% faeces 50% urine	37% faeces 57% urine

These drugs also affect a number of metabolic and anthropometric parameters with various mechanisms of action (including body weight, serum lipids, arterial pressure, serum uric acid, hematocrit and others).

Effects on body weight

SGLT-2 inhibitors can invoke a decrease in body weight, mainly due to a reduction of total fat both at the visceral and at the subcutaneous area. This loss has been shown to maintained throughout the treatment period (44, 45). Empagliflozin administration led to a mean weight loss of 1.84 kg [95% confidence interval (CI): 1.38–2.3 kg] in a meta-analysis of 10 studies with 6,203 participants (46). Furthermore, a meta-analysis of 10 trials showed that canagliflozin could evoke an important weight loss (by 2.81 kg vs. placebo and by 3.49 kg vs. comparators) (47). Finally a meta-analysis of 12 trials also showed that dapagliflozin administration was associated with a significant weight loss of 2.10 kg (95% CI: 1.88–2.32) with a trend for a greater weight loss with the dose of 10 mg/day (48). A common characteristic of the weight loss with the drugs of this class is its durability during long-term administration. Thus, body weight reaches a nadir after 3–6 months of therapy and stabilizes thereafter (49, 50, 51). Furthermore, this weight loss is substantially greater as compared with the most of the other glucose–lowering drugs (52). Canagliflozin may be associated with an even greater weight loss compared with other SGLT-2 inhibitors due to its relatively higher affinity for SGLT-1 inhibition and thus decreased intestinal glucose absorption (53).

Weight loss and body fat reduction with SGLT2 inhibitors have been attributed to: (a) the glucosuria-associated calorie loss (by 200–300 kcal/day) and (b) the increased diuresis and osmotic diuresis, which result in volume depletion by 5–10% (45, 53). However, the weight loss observed in clinical trials with these compounds is substantially lower than that predicted from the loss of calories due to glucosuria. Energy balance studies have elucidated that a compensatory increase in energy intake can limit the body weight loss attributable to the effect of these drugs (54). It has been shown that empagliflozin-induced weight loss (−3.2 ± 4.2 kg) was substantially lower than that predicted by the observed calorie loss (206 kcal/day), whereas an increased calorie intake (by 269 kcal/day) coupled with a 2% increase in daily energy expenditure was evident (54). Thus, the administration of SGLT2 inhibitors is associated with increased food intake. In this context, a combination of SGLT2 inhibitors with GLP-1 agonists, which can decrease food intake, is a reasonable therapeutic option in patients with type 2 diabetes and obesity. In addition, it has been suggested that an increase in water consumption can augment weight loss during a hypocaloric diet. This observation may be helpful since it might help patients to overcome, at least in part, the above-mentioned homeostatic mechanism (55). On the other hand, the SGLT2 inhibitors-

associated weight loss can prevent the undesirable weight gain of the simultaneous administration of pioglitazone (56). Finally, it has been speculated that this weight loss (by approximately 2 kg), also noticed in the EMPA-REG OUTCOME trial, may have played a role in the beneficial cardiovascular effects of empagliflozin (57).

Effect on serum lipids

In the EMPA-REG OUTCOME trial, a small increase in LDL-C and HDL-C levels compared with placebo was evident with empagliflozin administration (57). A meta-analysis of empagliflozin trials provided similar results, showing an increase of LDL-C by 4.5–6.5% in the empagliflozin-treated arm (46). In the CANVAS Program, similar results were obtained in canagliflozin treated patients; a small increase of LDL-C (by 5–6 mg/dL) and HDL-C (by 2 mg/dL) levels compared with the placebo group was evident (58). In another study, a small dose-dependent increase of LDL-C levels by 4.5% in the 100 mg group and by 8% in the 300 mg group after 26 weeks of treatment wasreported in canagliflozin-treated patients compared with placebo, along with an increase in HDL-C and a decrease in triglycerides (59). However, canagliflozin was associated with an increase in LDL-C concentrations only in patients with LDLC > 120 mg/dL as another study concluded (60). Another study examined the effect of dapagliflozin on serum lipids in patients with or without elevated triglycerides and with or without decreased HDL-C levels (61). Dapagliflozin increased both HDL-C and LDL-C levels in both patient groups. On the other hand, triglyceride levels decreased only in patients with baseline increased triglycerides and decreased HDL-C levels. A retrospective study compared the effect on serum lipids of dipeptidyl peptidase-4 (DPP-4) inhibitors linagliptin/gemigliptin and dapagliflozin (62). Dapagliflozin increased significantly HDL-C (baseline levels 45.3 ± 10.6 mg/dL, posttreatment levels 50.3 ± 11.3 mg/dL, $p < 0.05$) and apolipoprotein A1 levels (baseline levels 126.1 ± 17.9 g/L, posttreatment levels 139.8 ± 28.8 g/L, $p < 0.05$). Furthermore, dapagliflozin increased significantly HDL-C levels compared with the DPP4 inhibitors. On the other hand, DPP4 inhibitors significantly decreased LDL-C levels compared with dapagliflozin. Finally, ertugliflozin 5 and 15 mg increased LDL-C compared with placebo (by 5.8% and 8.4% compared with 3.2%, respectively), increased total cholesterol (by 2.8% and 5.7% versus 1.1%, respectively) and HDL-C (by 6.2% and 7.6% compared with 1.9%, respectively). On the other hand, triglycerides decreased by 3.9% and 1.7% with ertugliflozin 5 and 15 mg, respectively, while placebo increased triglycerides by 4.5%.

A systematic review and meta-analysis comparing various SGLT-2 inhibitors with placebo on their effect on serum lipids showed, an increase of HDL-C (n = 4698, mean difference 1.93 mg/dL) and LDL-C (n = 5431, mean difference 3.5 mg/dL) levels, as well as a decrease of triglyceride levels (n = 4704, mean difference 7.8 mg/dL) in patients treated with SGLT-2 inhibitors (63). Canagliflozin was associated with the largest effects on serum lipids. Finally, in the same analysis, SGLT-2 inhibitors increased both HDL-C and LDL-C compared with other oral antidiabetic drugs (such as sulfonylureas and DPP4 inhibitors), although triglycerides were not significantly decreased. Another meta-analysis compared SGLT-2 inhibitors as add-on to metformin monotherapy with other antidiabetics. In this meta-analysis, both LDL-C levels and HDL-C levels were elevated in the SGLT-2 inhibitor group by 2.5–9% and 7–9%, respectively (64). A recent systematic review and meta-analysis showed that LDL-C levels were increased significantly by SGLT-2 inhibitors (n = 18,684, mean difference 3.5 mg/dL) (65). HDL-C levels were also increased significantly (n = 18,684, mean difference 3.9 mg/dL). The effects were more pronounced in patients treated with canagliflozin, while dapagliflozin administration failed to show any change in LDL-C or HDL-C levels, possibly due to the rather small number of patients (n = 808). On the other hand, in a meta-analysis of 13 trials, dapagliflozin induced an increase of 2–9% in LDL-C levels (66). Another meta-analysis evaluated the efficacy and safety of adding an SGLT-2 inhibitor on top of metformin and sulfonylurea treatment compared with placebo. The study found that when SGLT-2 inhibitors were added to metformin and sulfonylurea treatment, HDL-C levels increased by 5% and triglyceride levels decreased by 5% compared with placebo (67).

Effect on lipoprotein subfractions

A study in Japanese patients with T2DM assessed the effects of dapagliflozin compared with sitagliptin on serum lipids, LDL subfractions, and HDL subfractions. Small-dense LDL cholesterol (sdLDL) and HDL3 cholesterol concentrations were measured using a homogeneous method established by the authors. Dapagliflozin administration resulted in a significant reduction of sdLDL cholesterol by 20% (pretreatment levels 54.4 ± 24.6 mg/dL, posttreatment levels 43.6 ± 24.4 mg/dL, $p = 0.005$; $p = 0.003$ compared with the sitagliptin group) (68). On the contrary, large buoyant LDL-C levels increased significantly in the dapagliflozin-treated arm (pretreatment levels 63.8 ± 27.6 mg/dL, posttreatment levels 75.1 ± 34.1 mg/dL, $p = 0.026$; $p = 0.029$ compared with

sitagliptin group). Furthermore, dapagliflozin administration increased HDL-C levels (pretreatment levels 48.4 ± 11.1 mg/dL, posttreatment levels 53.5 ± 13.0 mg/dL, $p < 0.001$), mainly due to an increase of large HDL particles (HDL2) by 18% without affecting HDL3 particles. Apolipoprotein A1 levels were also significantly increased by dapagliflozin (pretreatment levels 133.5 ± 21.6 mg/dL, posttreatment levels 143.5 ± 22.6 mg/dL, $p = 0.002$). Apolipoprotein CIII levels increased significantly by dapagliflozin administration (pretreatment levels 10.5 ± 3.2 mg/ dL, posttreatment levels 11.4 ± 4.3 mg/dL, $p = 0.021$), while sitagliptin had no effect on apolipoprotein CIII levels. Triglyceride levels nonsignificantly decreased in both treatment groups. Another study aimed to evaluate the effect of dapagliflozin on cholesterol efflux capacity of HDL particles (69). No effect of dapagliflozin on cholesterol efflux capacity was evident after adjusting for age and body mass index compared with placebo.

Effect on serum electrolytes

Administration of SGLT-2 inhibitors has been associated with a small increase of serum potassium, mainly with high doses of canagliflozin (70). However clinically significant hyperkalemia was evident in patients on 300 mg canagliflozin with other risk factors for increased potassium such as decreased renal function and patients on medications affecting potassium excretion (71). The pathophysiological basis for the observed small increase of serum potassium levels is not well understood since the osmotic diuresis induced by SGLT-2 inhibition should lead to decreased serum potassium. Increased serum potassium could be attributed to the hemoconcetration following SGLT-2 inhibitor administration and also to potassium redistribution. Indeed, the increased urinary glucose loss leads to decreased serum glucose thus resulting to lower insulin levels and to increased potassium efflux from the intracellular to the extracellular space (72). An analysis of the CREDENCE trial with canagliflozin, revealed that compared with placebo, canagliflozin administration led to a decreased risk of investigator reported hyperkalemia or initiation of potassium binders (occurring in 32.7 vs. 41.9 participants per 1000 patient-years; hazard ratio (HR) 0.78, 95% confidence interval (CI) 0.64-0.95, P = 0.014), without increasing the risk of hypokalemia (73).

A small increase in serum phosphate concentration has been reported with SGLT2 inhibitors administration due to increased renal phosphate reabsorption. In fact, the drug-induced reduction of proximal sodium transport leads to an increased availability

of sodium to be reabsorbed with phosphate via the Na+–PO4 3– co-transporters at the proximal tubules (74).

Similar findings have been reported with serum magnesium levels, since an increase has been observed in patients prescribed SGLT-2 inhibitors (40). No serum sodium disturbances have been observed with the initiation of SGLT-2 inhibitors.

Effect on renal function

By inhibiting sodium-glucose reabsorption at the proximal tubule, SGLT-2 inhibitors were initially thought to adversely affect renal function by increased volume loss. Furthermore, the hypoglycemic effect of this class is directly correlated to renal function. However, in the 3 large randomized controlled cardiovascular outcome (CVOT) trials, the EMPA-REG (57, 75), the DECLARE-TIMI 58 (76) and the CANVAS (58), empagliflozin, dapagliflozin and canagliflozin administration led to a reduction of renal events in patients with diabetic kidney disease (**table 3**).

Table 3. Large RCTs of SGLT-2 inhibitors

	EMPA-REG OUTCOMES	CANVAS PROGRAM	DECLARE-TIMI 58
Year	2015	2017	2019
Drug	Empagliflozin	Canagliflozin	Dapagliflozin
Comparator	Placebo	Placebo	Placebo
n=	7020	10142	17160
Renal composite outcome	• incident or worsening nephropathy (progression to macroalbuminuria, doubling of the serum creatinine level, initiation of renal-replacement therapy, or death	• progression of albuminuria • sustained 40% reduction in the eGFR, the need for renal-	• sustained decrease of 40% or more eGFR to less than 60 ml/min/1.73 m^2 • new end-stage renal disease

	from renal disease) • incident albuminuria.	replacement therapy, or death from renal causes	• death from renal or cardiovascular causes.
Results (compared with placebo)	Hazard ratio in the, 0.61; 95% CI, 0.53 to 0.70; P<0.001	• Hazard ratio, 0.73; 95% CI, 0.67 to 0.79 • Hazard ratio, 0.60; 95% CI, 0.47 to 0.77	Hazard ratio, 0.76 (95% CI, 0.67–0.87)

In light of these results, studies were undertaken to test if the administration of SGLT-2 inhibitors in patients with chronic kidney disease, with or without type 2 diabetes, could result in clinically significant renal protection. Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE), a placebo-controlled trial of canagliflozin in 4.401 adults with type 2 DM, urinary albumin to creatinine ratio (UACR) of at least 300 mg/g Cr, and mean eGFR 56 ml/min/1.73 m^2 with a mean albuminuria level of over 900 mg/day had a primary composite end point of End Stage Renal Disease (ESRD), doubling of serum creatinine or renal or cardiovascular death (77). The study was stopped early due to positive efficacy and a 32% RR risk reduction for development of ESRD over control. Additionally, the development of the primary end point, which included chronic dialysis for over 30 days, kidney transplantation or eGFR <15 ml/min/1.73 m^2 sustained for at least 30 days, or renal death or cardiovascular death was reduced by 30%. This benefit was on top of an ACE (angiotensin converting enzyme) inhibitor or ARB (angiotensin receptor blocker) therapy in over 99% of the patients. The Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE) trial randomized 4,401 patients with type 2 diabetes and chronic diabetes-related kidney disease (UACR

>300 mg/g and eGFR 30 to <90 mL/ min/1.73 m2) to canagliflozin 100 mg daily or placebo (78). The primary outcome was a composite of end-stage kidney disease, doubling of serum creatinine, or death from renal or cardiovascular causes. The trial was stopped early due to conclusive evidence of efficacy identified during a prespecified interim analysis with no unexpected safety signals. The risk of the primary composite outcome was 30% lower with canagliflozin treatment when compared with placebo (HR 0.70 [95% CI 0.59–0.82]). Moreover, it reduced the prespecified end point of end-stage kidney disease alone by 32% (HR 0.68 [95% CI 0.54–0.86]). Canagliflozin was additionally found to have a lower risk of the composite of cardiovascular death, MI, or stroke (HR 0.80 [95% CI 0.67–0.95]), as well as lower risk of hospitalizations for heart failure (HR 0.61 [95% CI 0.47–0.80]) and of the composite of cardiovascular death or hospitalization for heart failure (HR 0.69 [95% CI 0.57–0.83]). In terms of safety, no significant increase in lower-limb amputations, fractures, acute kidney injury, or hyperkalemia was noted for canagliflozin relative to placebo in CREDENCE. An increased risk for diabetic ketoacidosis was noted, however, with 2.2 and 0.2 events per 1,000 patient-years noted in the canagliflozin and placebo groups, respectively (HR 10.80 [95% CI 1.39–83.65]) (187). The Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD) trial (79), 4,304 patients with chronic kidney disease (UACR 200–5,000 mg/g and eGFR 25–75 mL/min/1.73 m^2), with or without diabetes, were randomized to dapagliflozin 10 mg daily or placebo. The primary outcome was a composite of sustained decline in eGFR of at least 50%, endstage kidney disease, or death from renal or cardiovascular causes. Over a median follow-up period of 2.4 years, a primary outcome event occurred in 9.2% of participants in the dapagliflozin group and 14.5% of those in the placebo group. The risk of the primary composite outcome was significantly lower with dapagliflozin therapy compared with placebo (HR 0.61 [95% CI 0.51– 0.72]), as were the risks for a renal composite outcome of sustained decline in eGFR of at least 50%, endstage kidney disease, or death from renal causes (HR 0.56 [95% CI 0.45–0.68]), and a composite of cardiovascular death or hospitalization for heart failure (HR 0.71 [95% CI 0.55–0.92]). The Empagliflozin in patients with Chronic Kidney Disease (EMPA-KIDNEY) trial was a randomized, parallel-group, double-blind, placebo-controlled, clinical trial to evaluate the effect of empagliflozin in patients with chronic kidney disease (80). Eligible patients were adults with an eGFR of at least 20 but less than 45 ml per minute per 1.73 m^2 , regardless of the level of albuminuria, or with an eGFR of at least 45 but

less than 90 ml per minute per 1.73 m^2 with a urinary albuminto-creatinine ratio of at least 200 at the screening visit. Patients with or without diabetes were eligible. The prespecified primary outcome was the first occurrence of progression of kidney disease or death from cardiovascular causes. The prespecified key secondary outcomes were a composite of hospitalization for heart failure or death from cardiovascular causes, hospitalization for any cause (including the first and any subsequent hospitalizations), and death from any cause. Progression of kidney disease or death from cardiovascular causes occurred in 13.1% in the empagliflozin group and in 16.9% in the placebo group (HR, 0.72; 95% confidence interval [CI], 0.64 to 0.82; P<0.001). This effect was consistent in all prespecified groups.

Cardiovascular effects

The BI 10773 (Empagliflozin) Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME) was a randomized, double blind trial that assessed the effect of empagliflozin, an SGLT2 inhibitor, versus placebo on cardiovascular outcomes in 7,020 patients with type 2 diabetes and existing cardiovascular disease. Study participants had a mean age of 63 years, 57% had diabetes for more than 10 years, and 99% had established cardiovascular disease. EMPA-REG OUTCOME showed that over a median follow-up of 3.1 years, treatment reduced the composite outcome of MI, stroke, and cardiovascular death by 14% (absolute rate 10.5% vs. 12.1% in the placebo group, HR in the empagliflozin group 0.86 [95% CI 0.74–0.99]; P 5 0.04 for superiority) and cardiovascular death by 38% (absolute rate 3.7% vs. 5.9%, HR 0.62 [95% CI 0.49–0.77]; P < 0.001) (57). The FDA added an indication for empagliflozin to reduce the risk of major adverse cardiovascular death in adults with type 2 diabetes and cardiovascular disease. Two large outcomes trials of the SGLT2 inhibitor canagliflozin have been conducted that separately assessed 1) the cardiovascular effects of treatment in patients at high risk for major adverse cardiovascular events and 2) the impact of canagliflozin therapy on cardiorenal outcomes in patients with diabetes-related chronic kidney disease (77). First, the Canagliflozin Cardiovascular Assessment Study (CANVAS) Program integrated data from two trials. The CANVAS trial that started in 2009 was partially unblinded prior to completion because of the need to file interim cardiovascular outcomes data for regulatory approval of the drug (78). Thereafter, the postapproval CANVAS-Renal (CANVAS-R) trial was started in 2014. Combining both of these trials, 10,142

participants with type 2 diabetes were randomized to canagliflozin or placebo and were followed for an average 3.6 years. The mean age of patients was 63 years, and 66% had a history of cardiovascular disease. The combined analysis of the two trials found that canagliflozin significantly reduced the composite outcome of cardiovascular death, MI, or stroke versus placebo (occurring in 26.9 vs. 31.5 participants per 1,000 patientyears; HR 0.86 [95% CI 0.75–0.97]). The specific estimates for canagliflozin versus placebo on the primary composite cardiovascular outcome were HR 0.88 (95% CI 0.75–1.03) for the CANVAS trial and 0.82 (0.66–1.01) for CANVAS-R, with no heterogeneity found between trials. Of note, there was an increased risk of lower-limb amputation with canagliflozin (6.3 vs. 3.4 participants per 1,000 patient-years; HR 1.97 [95% CI 1.41–2.75]) (58).

The Dapagliflozin Effect on Cardiovascular Events–Thrombosis in Myocardial Infarction 58 (DECLARE-TIMI 58) trial was another randomized, double-blind trial that assessed the effects of dapagliflozin versus placebo on cardiovascular and renal outcomes in 17,160 patients with type 2 diabetes and established ASCVD or multiple risk factors for atherosclerotic cardiovascular disease (76). Study participants had a mean age of 64 years, with 40% of study participants having established ASCVD at baseline—a characteristic of this trial that differs from other large cardiovascular trials where a majority of participants had established cardiovascular disease. DECLARE-TIMI 58 met the prespecified criteria for noninferiority to placebo with respect to major adverse cardiovascular events but did not show a lower rate of major adverse cardiovascular events when compared with placebo (8.8% in the dapagliflozin group and 9.4% in the placebo group; HR 0.93 [95% CI 0.84–1.03]; P 5 0.17). A lower rate of cardiovascular death or hospitalization for heart failure was noted (4.9% vs. 5.8%; HR 0.83 [95% CI 0.73–0.95]; P 5 0.005), which reflected a lower rate of hospitalization for heart failure (HR 0.73 [95% CI 0.61–0.88]). No difference was seen in cardiovascular death between groups. The Evaluation of Ertugliflozin Efficacy and Safety Cardiovascular Outcomes Trial (VERTIS CV) (81) was a randomized, double-blind trial that established the effects of ertugliflozin versus placebo on cardiovascular outcomes in 8,246 patients with type 2 diabetes and established ASCVD. Participants were assigned to the addition of 5 mg or 15 mg of ertugliflozin or to placebo once daily to background standard care. Study participants had a mean age of 64.4 years and a mean duration of diabetes of 13 years at baseline and were followed for a median of 3.0 years. VERTIS CV met the prespecified criteria for noninferiority of ertugliflozin to

placebo with respect to the primary outcome of major adverse cardiovascular events (11.9% in the pooled ertugliflozin group and 11.9% in the placebo group; HR 0.97 [95% CI 0.85–1.11]; P < 0.001). Ertugliflozin was not superior to placebo for the key secondary outcomes of death from cardiovascular causes or hospitalization for heart failure; death from cardiovascular causes; or the composite of death from renal causes, renal replacement therapy, or doubling of the serum creatinine level. The hazard ratio for a secondary outcome of hospitalization for heart failure (ertugliflozin vs. placebo) was 0.70 [95% CI 0.54–0.90], consistent with findings from other SGLT2 inhibitor cardiovascular outcomes trials. Sotagliflozin, an investigational SGLT1 and SGLT2 inhibitor that lowers glucose via delayed glucose absorption in the gut in addition to increasing urinary glucose excretion, has been evaluated in the Effect of Sotagliflozin on Cardiovascular and Renal Events in Patients With Type 2 Diabetes and Moderate Renal Impairment Who Are at Cardiovascular Risk (SCORED) trial (82). A total of 10,584 patients with type 2 diabetes, chronic kidney disease, and additional cardiovascular risk were enrolled in SCORED and randomized to sotagliflozin 200 mg once daily (uptitrated to 400 mg once daily if tolerated) or placebo. SCORED ended early due to a lack of funding; thus, changes to the prespecified primary end points were made prior to unblinding to accommodate a lower than anticipated number of end point events. The primary end point of the trial was the total number of deaths from cardiovascular causes, hospitalizations for heart failure, and urgent visits for heart failure. After a median of 16 months of follow-up, the rate of primary end point events was reduced with sotagliflozin (5.6 events per 100 patient-years in the sotagliflozin group and 7.5 events per 100 patientyears in the placebo group [HR 0.74 (95% CI 0.63–0.88); P < 0.001]). Sotagliflozin also reduced the risk of the secondary end point of total number of hospitalizations for heart failure and urgent visits for heart failure (3.5% in the sotagliflozin group and 5.1% in the placebo group; HR 0.67 [95% CI 0.55–0.82]; P < 0.001) but not the secondary end point of deaths from cardiovascular causes. No significant between-group differences were found for the outcome of all-cause mortality or for a composite renal outcome comprising the first occurrence of long-term dialysis, renal transplantation, or a sustained reduction in eGFR. In general, the adverse effects of sotagliflozin were similar to those seen with use of SGLT2 inhibitors, but they also included an increased rate of diarrhea potentially related to the inhibition of SGLT1.

Effects in patients with Heart Failure

As many as 50% of patients with type 2 diabetes may develop heart failure (83). These conditions, which are each associated with increased morbidity and mortality, commonly coincide and independently contribute to adverse outcomes (84). Strategies to mitigate these risks are needed, and the heart failure–related risks and benefits of glucose-lowering medications should be considered carefully when determining a regimen of care for patients with diabetes and either established heart failure or high risk for the development of heart failure.

Reduced incidence of heart failure has been observed with the use of SGLT2 inhibitors (76, 77). In the EMPA REG OUTCOME, the addition of empagliflozin to standard care led to a significant 35% reduction in hospitalization for heart failure compared with placebo(57). Although the majority of patients in the study did not have heart failure at baseline, this benefit was consistent in patients with and without a history of heart failure (85). Similarly, in CANVAS and DECLARE-TIMI 58, there were 33% and 27% reductions in hospitalization for heart failure, respectively, with SGLT2 inhibitor use versus placebo (58, 76). Additional data from the CREDENCE trial with canagliflozin showed a 39% reduction in hospitalization for heart failure, and 31% reduction in the composite of cardiovascular death or hospitalization for heart failure, in a diabetic kidney disease population with albuminuria (UACR of >300 to 5,000 mg/g) (77). These combined findings from four large outcomes trials of three different SGLT2 inhibitors are highly consistent and clearly indicate robust benefits of SGLT2 inhibitors in the prevention of heart failure hospitalizations. The EMPA-REG OUTCOME, CANVAS, DECLARE-TIMI 58, and CREDENCE trials suggested, but did not prove, that SGLT2 inhibitors would be beneficial in the treatment of patients with established heart failure. More recently, the placebo-controlled DAPA-HF trial evaluated the effects of dapagliflozin on the primary outcome of a composite of worsening heart failure or cardiovascular death in patients with New York Heart Association (NYHA) class II, III, or IV heart failure and an ejection fraction of 40% or less. Of the 4,744 trial participants, 45% had a history of type 2 diabetes. Over a median of 18.2 months, the group assigned to dapagliflozin treatment had a lower risk of the primary outcome (HR 0.74 [95% CI 0.65–0.85]), lower risk of first worsening heart failure event (HR 0.70 [95% CI 0.59–0.83]), and lower risk of cardiovascular death (HR 0.82 [95% CI 0.69–0.98]) compared with placebo. The effect of dapagliflozin on the primary outcome was

consistent regardless of the presence or absence of type 2 diabetes (86). Ongoing trials are assessing the effects of several SGLT2 inhibitors in heart failure patients with both reduced and preserved ejection fraction. EMPEROR-Reduced assessed the effects of empagliflozin 10 mg once daily versus placebo on a primary composite outcome of cardiovascular death or hospitalization for worsening heart failure in a population of 3,730 patients with NYHA class II, III, or IV heart failure and an ejection fraction of 40% or less (87). At baseline, 49.8% of participants had a history of diabetes. Over a median follow-up of 16 months, those in the empagliflozin-treated group had a reduced risk of the primary outcome (HR 0.75 [95% CI 0.65–0.86]; P < 0.001) and fewer total hospitalizations for heart failure (HR 0.70 [95% CI 0.58–0.85]; P < 0.001). The effect of empagliflozin on the primary outcome was consistent irrespective of diabetes diagnosis at baseline. The risk of a prespecified renal composite outcome (chronic dialysis, renal transplantation, or a sustained reduction in eGFR) was lower in the empagliflozin group than in the placebo group (1.6% in the empagliflozin group vs. 3.1% in the placebo group; HR 0.50 [95% CI 0.32–0.77]). Therefore, in patients with type 2 diabetes and established HFrEF, an SGLT2 inhibitor with proven benefit in this patient population is recommended to reduce the risk of worsening heart failure and cardiovascular death (88). The benefits seen in this patient population likely represent a class effect, and they appear unrelated to glucose lowering given comparable outcomes in HFrEF patients with and without diabetes. Additional data are accumulating regarding the effects of SGLT inhibition in patients hospitalized for acute decompensated heart failure and in heart failure patients with HFpEF. As an example, the investigational SGLT1 and SGLT2 inhibitor sotagliflozin has also been studied in the Effect of Sotagliflozin on Cardiovascular Events in Patients With Type 2 Diabetes Post Worsening Heart Failure (SOLOIST-WHF) trial (89). In SOLOIST-WHF, 1,222 patients with type 2 diabetes who were recently hospitalized for worsening heart failure were randomized to sotagliflozin 200 mg once daily (with uptitration to 400 mg once daily if tolerated) or placebo either before or within 3 days after hospital discharge. Patients were eligible if hospitalized for signs and symptoms of heart failure (including elevated natriuretic peptide levels) requiring treatment with intravenous diuretic therapy. Exclusion criteria included end-stage heart failure or recent acute coronary syndrome or intervention, or an eGFR < 0.001). No significant between-group differences were found in the rates of cardiovascular death or all-cause mortality. Both diarrhea (6.1% vs. 3.4%) and severe hypoglycemia (1.5% vs. 0.3%) were more

common with sotagliflozin than with placebo. The trial was originally also intended to evaluate the effects of SGLT inhibition in patients with HFpEF, and ultimately no evidence of heterogeneity of care treatment effect by ejection fraction was noted. However, the relatively small percentage of such patients enrolled (only 21% of participants had ejection fraction >50%) and the early termination of the trial limited the ability to determine the effects of sotagliflozin in HFpEF specifically. In the EMPEROR-Preserved Trial (90, 91), 5988 patients with class II-IV heart failure were randomly assigned to receive either empagliflozin (10 mg once daily) or placebo, in addition to usual therapy. The primary outcome was a composite of cardiovascular death or hospitalization for heart failure. Over a median of 26.2 months, a primary outcome event occurred in 415 of 2997 patients (13.8%) in the empagliflozin group and in 511 of 2991 patients (17.1%) in the placebo group (hazard ratio, 0.79; 95% confidence interval [CI], 0.69 to 0.90; P<0.001). This effect was mainly related to a lower risk of hospitalization for heart failure in the empagliflozin group. The effects of empagliflozin appeared consistent in patients with or without diabetes. The total number of hospitalizations for heart failure was lower in the empagliflozin group than in the placebo group (407 with empagliflozin and 541 with placebo; hazard ratio, 0.73; 95% CI, 0.61 to 0.88; P<0.001). Uncomplicated genital and urinary tract infections and hypotension were reported more frequently with empagliflozin. The Dapagliflozin evaluation to Improve the Lives of Patients with Preserved Ejection Fraction Heart Failure (DELIVER) trial (92), was a phase 3, internation, multicenter, double blind, randomized, controlled trial in patients with heart failure with an ejection fraction of 40% or more. The primary outcome was a composite of worsening heart failure, Secondary outcomes were the total number of worsening heart failure events and cardiovascular deaths, the change from baseline in the total symptom score on the Kansas City Cardiomyopathy Questionnaire at month 8, cardiovascular death, and death from any cause. After a median duration of follow-up of 2.3 years, the primary outcome occurred in 512 patients (16.4%) in the dapagliflozin group and in 610 patients (19.5%) in the placebo group (HR, 0.82; 95% CI 0.73-0.92; p<0.001). Similar results were obtained in the group of patients with an LVEF of 60% or more. Furthermore, the effect of dapagliflozin was consistent across all prespecified subgroups, including the presence or absence of diabetes.

Adverse Events

Effect on cancer

Several studies have suggested that persons with T2DM exhibit an increased cancer risk. Although the implicated mechanisms remain largely unknown, pathophysiological factors of T2DM such as obesity have been proposed to play a significant role in increased cancer risk (93, 94). Additionally, some antidiabetic drugs have been shown to affect certain cancers risk, although here too, the mechanisms are still unknown. In this context, metformin has been shown to decrease cancer risk in T2DM patients (95, 96), whereas pioglitazone has been associated with an increased bladder cancer risk, although no clear conclusions have been drawn (97, 98). Regarding SGLT2 inhibitors, concerns were raised from an FDA Advisory Committee in 2011 regarding the risk of bladder and breast cancer associated with dapagliflozin. Until November 2013, a total of 10 cases of bladder cancer among 6045 patients (0.17%) were noted in the dapagliflozin treatment group compared with 1 case of bladder cancer among 3512 patients (0.03%) in the placebo arms. All 10 cases of bladder cancer were reported within 2 years of starting dapagliflozin and (except one) showed hematuria within 6 months of treatment initiation. Additionally, the incidence rate remained stable during the first 2 years of dapagliflozin exposure, but no additional case was reported between 2 and 4 years of exposure. A biological heterogeneity of bladder cancer cases, from low grade to high grade and from noninvasive to widely metastatic, was evident that did not pointed to a single triggering cause. Additionally, until November 2013, an increased number of breast cancer cases were reported with dapagliflozin compared with placebo (12 vs. 3 cases; incidence rate 0.40 per 100 patient-years [95% confidence interval (CI) 0.21–0.70] in dapagliflozin and 0.19 [95% CI 0.04–0.56] in the control group). However, all female breast cancers were diagnosed within the first year of treatment and were heterogeneous in patient age, tumor type, stage, progesterone/estrogen receptor status, and HER2/neu status, evidence that also does not support a single triggering cause. Moreover, a more recent pooled analysis of 21 phase 2b/3 clinical trials of up to 208 weeks' duration showed that dapagliflozin administration was not associated with an increased cancer risk (99). It should be mentioned that exposure of mice and rats to dapagliflozin for up to 2 years at greater than a 100-fold human levels did not increase tumor incidence or urinary bladder proliferative/preneoplastic lesions

(100). Additionally, dapagliflozin administration in Sprague– Dawley rats up to 186-fold human exposure for 90 weeks in males and 105 weeks in females was not associated with any mammary tumor in the male rats, whereas in the female rats no dapagliflozin-associated increase in tumor incidence or in onset time was noticed (100). Regarding canagliflozin, a pooled analysis of eight phase 3 clinical trials showed that canagliflozin administration did not increase the overall incidence of bladder, breast, and renal cancers (101). In the CANVAS program, the event rate per 1000 patient-years of renal cell (0.2 vs. 0.6, p = 0.17), bladder (1.1 vs. 1.0, p = 0.74), and breast (2.6 vs. 3.1, p = 0.65) cancer did not significantly differ between placebo and canagliflozin group (58). In the EMPA-REG OUTCOME trial, among 7028 patients, bladder cancer rate was significantly elevated in the empagliflozin-treated group compared with the placebo group (nine bladder cancer cases versus no case, respectively). However, the cases are too low to draw a definite conclusion (57). In the DECLARE trial, (102), no difference was observed between dapagliflozin treated patients and placebo treated patients in the rate of cancer (any type) occurrence [481 patients (5.6%) vs 486 (5.7%) in the dapagliflozin and in the placebo group respectively, p=0.83). Although the rate of breast cancer did not differ between the groups, the rate of bladder cancer was significantly lower in the dapagliflozin arm [26 (0.3%) vs 45 (0.5%), p=0.02], although no conclusion can be drawn due to the low number of cases. A systematic review and meta-analysis showed that SGLT2 inhibitors did not increase the risk of cancer (103). In a meta-analysis of 46 clinical trials (n = 34,569 patients), overall cancer incidence was 1.78% in the SGLT2 inhibitor group versus 1.55% in the comparator group (odds ratio [OR] 1.14, 95% CI 0.96–1.36) (104). However, this nonsignificant result in the whole sample became statistically significant in obese participants (body mass index >30 kg/m2; OR 1.23; 95% CI 1.02–1.48). Furthermore, bladder cancer risk was significantly elevated in the SGLT2-treated group, particularly in the comparison of empagliflozin with comparators (again with a small number of cases) (104). On the other hand, canagliflozin was associated with a decreased risk of gastrointestinal cancers compared with other hypoglycemic agents (OR 0.15, 95% CI 0.04–0.6). No significant differences between SGLT2 inhibitors and comparators were observed in other types of cancer (104). Overall, the available data do not point to a causative role of SGLT2 inhibitors on malignancy risk. It should be mentioned that SGLTs, especially SGLT1, may play a role in cancer cell survival through increased glucose uptake (105). Additionally, SGLTs are located in many cancers, such as in pancreatic, prostate, and

gastrointestinal tract malignancies (105, 106). In this context, SGLT2 inhibitors that also possess anti-SGLT1 activity, such as canagliflozin, may protect from certain cancers through SGLT1 inhibition induced reduction in glucose uptake from cancer cells.

Diabetic ketoacidosis

Diabetic ketoacidosis (DKA) is a medical emergency and is most often identified in individuals with type 1 diabetes. Known precipitating factors include insulin omission and stressful conditions which necessitate higher insulin dose such as infection, reduced food/fluid intake, surgery, myocardial infarction, trauma, or alcohol abuse (107, 108). In such cases, DKA is the result of a relative or absolute insulin deficiency in relation with concomitant increases in counter-regulatory hormones (cortisol, epinephrine, glucagon, and growth hormone). The classic criteria for the diagnosis of DKA are an arterial pH <7.3, a serum bicarbonate level <15 mmol/l, a serum anion gap >12 mmol/l, and presence of ketones (109). Concerns have been raised regarding an increased risk of DKA in SGLT2 inhibitor-treated T2DM patients. In these cases, similarly with the classic DKA, an increased anion gap metabolic acidosis with ketonemia/ketonuria is observed (110, 111). Importantly, the SGLT2 inhibitor-induced DKA is not usually combined with profound hyperglycemia, but, on the other hand, in most cases, it is accompanied by serum glucose levels <200 mg/dl (euglycemic DKA) (112).Main metabolic alteration that contributes to SGLT2 inhibitor-induced DKA seems to be the imbalance between insulin and glucagon serum levels. In this context, a reduction in insulin dose or a glucosuria-mediated reduction in serum insulin levels in T2DM patients leads to increased lipolysis in the adipose tissue, which, along with a reduction in serum glucose levels, induces an increased fatty acid oxidation in myocytes and hepatocytes leading to increased production of acetyl-CoA, which then can be converted into ketones (acetoacetic and β-hydroxybutyric acid) due to the increased glucagon-to-insulin ratio seen in SGLT2 inhibitor-treated patients (113, 114). The SGLT2 inhibitor-induced increased glucagon levels are attributed to the SGLT2 inhibition-induced decreased serum glucose levels and to a direct inhibition of SGLT2 in the pancreatic α-cells, leading to increased glucagon secretion due to K+ –adenosine triphosphate (ATP) channel activation (115, 116, 117). Additionally, renal mechanisms may be involved in the accumulation of ketones in SGLT2 inhibitor treated patients, such as a SGLT2 inhibition-induced increase in renal tubule sodium concentration,

which induces increased renal reabsorption of ketones due to reabsorption of both Na+ and ketoacids in the collecting tubules (113). It should be mentioned that the above mechanisms explain the SGLT2 inhibitor-induced rise in ketone bodies (118, 119), which in most individuals is asymptomatic, but in a few, especially in the presence of predisposing factors, may lead to DKA. Clues for the diagnosis of SGLT2 inhibitor-induced DKA are slightly elevated blood glucose levels (in most cases <200 mg/dl), ketonemia consisting mainly of β-hydroxybutyric acid, while ketonuria is not a constant finding due to increased reabsorption of ketones, in patients with nonspecific symptoms such as nausea, vomiting, thirst, or abdominal pain, and predisposing factors for developing SGLT2-inhibitor-induced DKA such as a low reserve of insulin-secreting cells, a sudden decrease in insulin dose, reduced food intake, hypovolemia, acute illness, surgery, trauma, or alcohol abuse (120). In cases of DKA, SGLT2 inhibitors should be immediately discontinued. It should be mentioned that although many case reports have been published, the incidence of DKA in observational and randomized controlled trials (RCTs) is very low and generally not significantly icreased compared with placebo (57, 58, 121, 122, 123); for example, a meta-analysis of 10 RCTs (n = 13,134 participants) reported only 14 DKA events and showed a nonsignificant greater risk with SGLT2 inhibitors compared with the control groups (OR 1.71, 95% CI 0.56–5.20) (124). In the DECLARE trial, which included 8.574 patients treated with dapagliflozin, compared with 8569 patients treated with placebo, (102), the rate of ketoacidosis was higher in the dapagliflozin treated arm compared with placebo [27 patients (0.3%) vs 12 patients (0.1%) in the dapagliflozin and in the placebo group respectively, p=0.02). Another systematic review and meta-analysis included 81 trials comparing SGLT2 inhibitors with placebo or another hypoglycemic agent (125). Between these trials, data from eight trials reporting at least one event of ketoacidosis (n = 10,157 in SGLT2 inhibitors and n = 5,396 in comparator groups) showed no increased risk for ketoacidosis for SGLT2 inhibitors as a class (Mantel–Haenszel OR 1.14, 95% CI 0.45–2.88, p = 0.78) or as individual molecule. It should be mentioned that randomized trials include a rather selected population that may have lower risk of DKA which may have covered an increased DKA risk; alternatively, these results show that the risk of DKA is small when SGLT2 inhibitors are properly prescribed. It should be mentioned that the asymptomatic SGLT2-inhibitor-induced increased rise in ketone bodies may play a role in their cardioprotective and renoprotective effects. It has been proposed that the increase in serum levels of ketone bodies induces an enhanced

oxidation of ketones by the heart resulting in increased energy availability and improvement of cardiac contractility and oxygenation of kidney tissues (126, 127).

Genitourinary infections

T2DM patients exhibit increased risk of urinary tract infections (UTIs), due to multiple factors such as glucosuria, increased adherence of the bacteria to the uroepithelium, increased estrogen levels, and immune dysfunction of diabetic patients (128, 129, 130, 131, 132, 133, 134). Furthermore, T2DM patients have increased risk of genital fungal infections, balanitis–balanoposthitis in men, and vulvovaginitis in women (135). Candida species are the major pathogens implicated in vaginal fungal infections (136), among which the intrinsically resistant to many azoles Candida glabrata may be the main pathogen (137, 138). SGLT2 inhibitors increase urinary glucose output by blocking glucose reabsorption in the proximal tubule of the kidney; thus, they are expected to increase the rate of genitourinary infections. Indeed, all agents (canagliflozin, empagliflozin, and dapagliflozin) increase the risk of genitourinary infections (59, 139, 140). In CANVAS, an increase in male genitalia infection and female mycotic genital infection was noted in the canagliflozin-treated group (34.9 events per 1000 patient-years versus 10.8 events per 1000 patient-years, $p < 0.001$ for male infection and 68.8 events per 1000 patient-years vs. 17.5 events per 1000 patient-years, $p < 0.001$ for women infection). In contrast, no increase in UTIs was observed (40 events per 1000 patient-years with canagliflozin versus 37 events per 1000 patient-years with placebo, $p = 0.38$) (58). Similar results were found in the EMPA-REG OUTCOME trial (57). Specifically, genital infections (seen mainly in females) were increased in the empagliflozin-treated arm (6.4%) compared with the placebo arm (1.8%, $p < 0.05$), whereas the rate of UTIs was similar between empagliflozin and placebo (18.1% in the placebo group versus 18% in the empagliflozin group). Complicated UTIs were also similar in the placebo group (1.8%) and the empagliflozin-treated group (1.7%). In the DECLARE study, (102), the rate of genital infections was higher in patients treated with dapagliflozin compared with placebo [76 patients (0.9%) vs 9 patients (0.1%) in the dapagliflozin and in the placebo group respectively, $p<0.001$]. On the other hand, no difference was observed in the rate of UTI. Similar results were reported by other authors (141, 142) who showed no increased risk of UTIs in patients receiving SGLT2 inhibitors compared with placebo (relative risk [RR] 1.02, 95% CI 0.54–1.91), whereas Vasilakou et al. (143) reported that UTIs were more

common in patients receiving SGLT2 inhibitors compared with placebo or active comparators (OR 1.34, 95% CI 1.03–1.74). A systematic review and meta-analysis of 77 RCTs did not show a statistically significant difference in the incidence of UTIs with SGLT2 inhibitors combined versus controls (RR for SGLT2 inhibitors 1.05, 95% CI 0.98–1.12) (144), whereas an increased risk of genital infections was evident (raw event rate 4.7%, RR 3.30, 95% CI 2.24–4.74). However, in the same meta-analysis, dapagliflozin was associated with an increased risk of UTIs (RR 1.34, 95% CI 1.11–1.63) (144). Other meta-analyses also reported that among SGLT2 inhibitors, only dapagliflozin is associated with increased risk of UTIs compared with placebo (OR 1.32, 95% CI 1.06–1.63) (52, 145). It should be mentioned that in the above studies, most events were reported within the first 24–26 weeks of treatment with a subsequent decrease of their incidence. Thus, available evidence suggests that SGLT2 inhibitors increase the risk of genital mycotic infections by four to five times, although usually these infections are mild to moderate, are adequately treated with standard medical treatment, and do not need the discontinuation of the drug (146). On the other hand, risk of UTIs is still unclear, since the large RCTs did not show an increased risk of UTIs, but relevant meta-analyses have found mixed results. Although severe UTIs including pyelonephritis and urosepsis have rarely been described, special attention is needed in male patients with urinary tract outlet obstruction since in these cases severe UTIs may be observed (147, 148).

Amputation risk

In the CANVAS study, an increased risk of amputation of toes, feet, and legs was noticed with canagliflozin (6.3 vs. 3.4 per 1,000 patient-years, $p < 0.001$, hazard ratio [HR] 1.97, 95% CI 1.41–2.75) with 71% of the affected participants having their highest amputation at the levels of toe or metatarsal. This risk was more pronounced in patients with a history of peripheral vascular disease or in patients who had a history of a previous amputation (58). On the other hand, in the EMPA-REG OUTCOME trial, the proportion of participants with lower limb amputations was similar in the placebo group (1.8%) and the empagliflozin-treated group (1.9%). Toe amputations occurred in 0.9% of participants in the placebo group and 1.3% of participants in the pooled empagliflozin group (57). Furthermore, in a pooled analysis of phase 1–3 clinical trials, the frequency of lower limb amputations was similar across all treatment groups (1.1% in the placebo group, 1.1% in the empagliflozin 10 mg group, and 1.1% in the

empagliflozin 25 mg group) (149). Thus, an increased risk of amputation is confined only with canagliflozin, and the current evidence does not suggest a class effect. The underlying pathogenetic mechanisms have not been clarified. Accordingly, no increased risk for amputation was evident by SGLT2 inhibitors in a recent analysis of published data from randomized trials (150). However, an analysis of reports in the FAERS up to 31 March 2017 showed that mainly canagliflozin was associated with an increased risk of amputations with a PRR of 5.33 (95% CI 4.04–7.04; $p < 0.0001$), but a marginally increased risk for empagliflozin was also observed (PRR 2.37, 95% CI 0.99–5.70,$p = 0.054$), while the PRR for dapagliflozin was 0.25 (95% CI 0.03–1.76, $p = 0.163$). The most common level of amputation was the toe, although there were reports of above-ankle leg or limp amputations (151). In the DECLARE study, (102), dapagliflozin administration did not result in an increased risk of amputation compared to placebo [123 cases with dapagliflozin (1.4%) compared with 113 cases with placebo (1.3%), p=0.53]. In a systematic review and meta-analysis by Heyward et al (152), no increased risk of lower extremity amputation was observed following the initiation of SGLT-2 inhibitor treatment. In conclusion it is still not clear whether SGLT-2 treatment or more specifically, canagliflozin treatment is associated with an increased risk of lower limb amputation.

Effect on fracture risk

In the CANVAS program, the rate of all fractures was higher with canagliflozin than with placebo (15.4 vs. 11.9 participants with fracture per 1000 patient-years; HR 1.26, 95% CI 1.04– 1.52). A similar trend of low-trauma fracture events (11.6 vs. 9.2 participants with fracture per 1000 patient-years; HR 1.23, 95% CI 0.99–1.52) was observed (58). The increase in fracture risk was observed within a few weeks after treatment initiation, especially in a subset of patients who were older and had a higher baseline cardiovascular risk, lower baseline eGFR, and higher baseline use of diuretics than the overall study population. However, a recent analysis of eight pooled non-CANVAS studies (n = 5867) showed that the incidence of fractures was similar with canagliflozin (1.7%) and non-canagliflozin (1.5%) treatments, whereas in CANVAS, the fractures that were balanced between the upper and lower limbs, were significantly increased with canagliflozin (4.0%) compared with placebo (2.6%) (153). Additionally, in the overall population (eight studies plus the CANVAS study), the incidence of fractures was higher with canagliflozin (2.7%) compared with non-canagliflozin (1.9%)

treatments, an effect that was driven by the increase of fractures in CANVAS. It is worth mentioning that the incidence of reported fall-related adverse events was significantly higher with canagliflozin in CANVAS, which included as stated above older patients with characteristics predisposing to volume depletion-related adverse events, but not significantly different in the pooled non-CANVAS studies and the overall population. Thus, the increase in fracture risk in the CANVAS may be mediated by the volume depletion related increase in falls (153). Although this may be the main mechanism of increased fracture risk, it has been shown that canagliflozin administration over 104 weeks is associated with a decrease in total hip bone mineral density (BMD) (−0.9% versus placebo for canagliflozin 100 mg, −1.2% versus placebo for canagliflozin 300 mg), but not at other sites. In the same study, canagliflozin was associated with an increase in collagen type 1 β-carboxy-telopeptide that was significantly correlated with a reduction in body weight, an increase in osteocalcin, and, in women, a decrease in estradiol (154). On the other hand, no increased risk of bone fractures was documented in the EMPA-REG OUTCOMES trial (57).

The placebo arm had a rate of bone fractures of 3.9% versus 3.8% in the empagliflozin arm. The incidence of bone fractures was similar across all groups in a pooled analysis of phase 1–3 empagliflozin trials (placebo 1.7%, empagliflozin 10 mg 1.6%, and empagliflozin 25 mg 1.4% per 100 patient-years) (149). Furthermore, dapagliflozin treatment was not associated with significant changes from baseline in procollagen type 1 N-terminal propeptide (P1NP), C-terminal cross-linking telopeptides of type I collagen (CTX), and BMD after 50 weeks of treatment (155). In a meta-analysis of 38 RCTs, SGLT2 inhibitors were not associated with an increased rate of fractures (1.59% in the SGLT2 inhibitor group versus 1.56% in the placebo group). In the DECLARE trial, dapagliflozin administration was not associated with an increased fracture risk compared with placebo [457 fractures (5.3%) in patients receiving dapagliflozin compared with 440 (5.1%) fractures in patients receiving placebo, p=0.59] (76). Furthermore, event rates were similar across all SGLT2 inhibitors compared with placebo (canagliflozin OR 1.15, 95% CI 0.71–1.88, dapagliflozin OR 0.68, 95% CI 0.37–1.25, and empagliflozin OR 0.93, 95% CI 0.74–1.18) (156). In a recent metaanalysis of 20 studies (n = 8286 patients), the pooled RR of bone fracture in patients receiving SGLT2 inhibitors versus placebo was 0.67 (95% CI 0.42–1.07). The pooled RR for each SGLT2 inhibitor individually was also not increased compared with placebo (RR for canagliflozin 0.66, 95% CI 0.37–1.19, RR for dapagliflozin 0.84, 95%

CI 0.22–3.18, and RR for empagliflozin 0.57, 95% CI 0.20–1.59) (157). In a systematic review and network meta-analysis, (158), canagliflozin and dapagliflozin was associated with a decrease in fracture risk, whereas, on the other hand, empagliflozin and ertugliflozin were associated with an increased risk of fracture risk. Finally, in a meta-analysis of randomized controlled trials, (159), SGLT-2 inhibitor administration was not associated with a statistically significant increased fracture risk (RR 1.07,95% CI 0.99-1.16). It has been speculated that the mild increase in phosphate levels with SGLT2 inhibitors (see in the electrolytes section) may play a significant role in bone homeostasis. It has been shown that the small increase in serum phosphate is followed by an increase in serum parathormone (PTH) and fibroblast growth factor 23 concentration, the latter of which may decrease vitamin D concentrations leading to decreased calcium absorption (74). PTH increases bone resorption and combined with the reduced calcium absorption may lead to a decreased bone mineral density.

Effect on hypoglycemia risk

SGLT2 inhibitors are not pathophysiologically associated with an increased risk of hypoglycemic events. The absence of hypoglycemia due to SGLT2 inhibitor administration is multifactorial. First, SGLT2 inhibitors improve insulin resistance leading to decreased insulin secretion by the β-cells of the pancreas. Second, the SGLT-1-mediated reabsorption of glucose in the proximal renal tubules is increased substantially, thus limiting excess glucosuria. Third, SGLT-2 inhibitors increase glucagon secretion by the α-cells of the pancreas due to both lower serum glucose levels and a direct effect of SGLT-2 inhibitors on the pancreatic α-cells. The resulting increased blood glucagon levels result in increased hepatic glucose production, thus minimizing the risk of hypoglycemia caused by these agents (116, 117). However, the risk of hypoglycemia is increased when SGLT2 inhibitors are coadministered with sulfonylureas but not when SGLT2 inhibitors are coadministered with metformin or insulin (103). In a meta-analysis, canagliflozin was associated with an increased risk of hypoglycemic events when compared with placebo and with other SGLT2 inhibitors (empagliflozin and dapagliflozin) (52). Another recent meta-analysis, showed that SGLT-2 inhibitors were not associated with an increased risk of severe hypoglycemia (RR 0.86, 95% CI 0.71-1.03) (159). Furthermore, in a meta-analysis of 33 RCTs, hypoglycemia was significantly higher in the SGLT2 group compared with the placebo group (1.11, 95% CI 1.03–1.2). Subgroup analysis showed that canagliflozin was

associated with a higher risk for hypoglycemia (1.53, 95% CI 1.12–2.03), but not empagliflozin (1.03, 95% CI 0.9–1.19) or dapagliflozin (1.07, 95% CI 0.95– 1.17) compared with placebo. Less hypoglycemic events were documented in the SGLT2 inhibitor group compared with the sulfonylurea group (0.16, 95% CI 0.11–0.22). No difference in hypoglycemia rate was evident in patients treated with SGLT2 inhibitors compared with patients treated with metformin (0.5, 95% CI 0.18–1.43) or DPP-4 inhibitors (1.00, 95% CI 0.49–2.02) (63). Thus, hypoglycemia risk is increased when SGLT2 inhibitors (especially canagliflozin) are combined with sulfonylureas, in which case a lower dose of sulfonylureas may be needed. In the DECLARE study, (76), patients treated with dapagliflozin experienced fewer major hypoglycemic events compared with the patients treated with placebo, [58 (0.7%) patients in the dapagliflozin group compared with 83 (1%) patients in the placebo group, p=0.02].

Effect on liver associated enzymes

SGLT2 inhibitors have been associated with a reduction of liver function tests (LFTs) (including alanine aminotransferase [ALT], aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transferase) (160). Canagliflozin administration was found to lead to a reduction of LFTs in both doses, while bilirubin was augmented only in the 300 mg dosage (160). Furthermore, in a metaanalysis of 18 RCTs, SGLT2 inhibitors reduced ALT levels compared with placebo or compared with other antidiabetic treatment (63). The improvement in LFTs is possibly related to the body weight reduction and improvement of nonalcoholic fatty liver disease, which is a common coexisting condition in patients with T2DM.

Effect on skin reactions

SGLT2 inhibitor administration has been occasionally associated with skin reactions. Skin and subcutaneous disorders mostly appear within 2 weeks of SGLT2 inhibitor administration and include serious generalized rash, drug eruption, urticaria, erythema, and eczema (161). The incidence of skin reactions is higher when ipragliflozin was administered possibly because ipragliflozin and its metabolites are more readily found in the skin as animal studies have suggested (161).

Effect on stroke risk

The diuretic effect of SGLT2 inhibitor treatment has been related with an increase in hematocrit due to hemoconcentration, although other mechanisms such as enhancement

of erythropoiesis owing to increased erythropoietin levels have been proposed (162). The SGLT2 inhibitor-induced increase in hematocrit (increase in viscosity) has been associated with a possible increase in stroke risk (163, 164). This assumption was mainly based on the results of the EMPA-REG OUTCOME trial that showed a nonsignificant increase in stroke risk (HR 1.18, 95% CI 0.89–1.56, p = 0.26 for fatal or nonfatal stroke; 1.24, 95% CI 0.92– 1.67, p = 0.16 for nonfatal stroke) (57). Additionally, a meta-analysis of 57 studies with SGLT2 inhibitors showed an increase in nonfatal stroke incidence (RR 1.30, 95%CI 1.00–1.68, p = 0.049) (103). However, no increase in stroke risk was shown in the CANVAS (HR 0.87, 95% CI 0.69–1.09) (58) and in a meta-analysis of 21 dapagliflozin clinical trials (HR 0.99, 95% CI 0.536–1.864) (165). Moreover, a modified intention-to-treat analysis of the EMPAREG OUTCOME study revealed that the numeric difference in stroke between empagliflozin and placebo was primarily due to a difference in patients with a first event >90 days after last intake of study drug (18 patients in the empagliflozin group versus 3 on placebo), whereas no difference in stroke incidence was observed when events during treatment or ≤90 days after last dose of drug were analyzed (HR 1.08, 95% CI 0.81–1.45, p = 0.60). Indeed, no increased stroke risk was shown in patients with the largest increases in hematocrit (166). In the DECLARE study, dapagliflozin was not associated with an increased stroke risk compared with placebo [235 patients in the dapagliflozin group (2.7%) compared with 231 patients in the placebo group (2.7%), RR 1.01, 95% CI 0.84-1.21] (76). In a meta-analysis of randomized controlled trials, SGLT-2 inhibitors were associated with a lower risk of total stroke in patients with type 2 diabetes and impaired renal function (hazard ratio, HR 0.90, 95% CI: 0.74-1.09, p = 0.29, I^2 = 58%) (167). Finally, in a large meta-analysis of 13 placebo controlled randomized trials, the administration of SGLT-2 inhibitors resulted in a decrease of the incidence of atrial fibrillation, while no effect on stroke risk was found (168). Thus, although theoretically the SGLT-2 inhibitor-induced increase in viscosity may be associated with an elevated stroke risk, the available data are not conclusive.

Effect on Fournier's gangrene

In August 2018, the FDA issued a warning stating that cases of Fournier's gangrene have been reported in patients with DM2 using SGLT-2 inhibitors. Fournier's gangrene is a bacterial infection of the skin and surrounding tissues (including blood vessels, nerves, muscles and fat) of the perineum. Treatment consists of broad spectrum

antibiotic coverage and aggressive surgical debridement (169). Early diagnosis and prompt initiation of treatment is crucial as mortality rates average 20-40% even with aggressive treatment (170). Fadini et al, reported 47 cases of Fournier's gangrene associated with the use of SGLT-2 inhibitors (171). In a recent review of case reports and of spontaneous post-marketing cases in the FDA Adverse Event Reporting System (FAERS), 500 cases of Fournier's gangrene associated with the use of SGLT-2 inhibitors were identified (172). Although no direct causal relationship can be confirmed, clinicians should be alert to the possibility of the development of Fournie's gangrene in diabetic patients receiving SGLT-2 inhibitors, especially in patients with local predisposing factors, until further studies are carried out.

SGLT-2 inhibitors; Dapagliflozin

Mechanism of action

Dapagliflozin is a highly potent (K_i: 0.55 nM) and highly selective and reversible inhibitor of SGLT-2 (42). Dapagliflozin administration leads to inhibition of SGLT-2 in the proximal renal tubule thus reducing glucose reabsorption from the glomerular filtrate with a concomitant reduction in sodium reabsorption leading to urinary glucose excretion and osmotic diuresis (173). Therefore, delivery of sodium to the distal tubule is increased which in turn increases the tubuloglomerular feedback and reduce intraglomerular pressure. This, combined with osmotic diuresis, leads to a reduction in volume overload, reduced blood pressure and lower preload and afterload which may have beneficial effects on cardiac remodeling and preserve renal function (174). Other effects include an increase in hematocrit and reduction in body weight as already stated. Dapagliflozin improves both fasting and post-prandial plasma glucose levels by reducing renal glucose reabsorption leading to urinary glucose excretion. This effect is observed after the first dose, continues over the 24 hour dosing interval and is sustained (175). The amount of glucose removed through this mechanism is dependent upon the blood glucose concentration and the glomerular filtration rate (GFR) (175). In this regard, dapagliflozin does not increase the risk of hypoglycemia in patients with normal blood glucose and/or low GFR as the amount of filtered glucose is small and can be reabsorbed by SGLT-1 and unblocked SGLT-2 transporters. SGLT-2 is selectively expressed in the kidney. Dapagliflozin does not inhibit other glucose transporters important for glucose transport into peripheral tissues and is >1400 times more selective

for SGLT-2 versus SGLT-1, the major transporter in the gut responsible for glucose absorption (42).

Metabolic consequenses

The metabolic consequenses of SGLT-2 inhibition follow the primary action of glycosuria and natriuresis. Firstly, the extracellular space is partially emptied out of glucose, with a fall of serum glucose in the fasting and in the postprandial phase as well (175). In response to decreasing glucose levels, insulin levels and secretion also decline, while on the other hand glucagon levels rise due to predominantly decreasing inhibition of α cell activity by intraislet insulin (176). This paracrine effect may be enhanced though direct α cell SGLT-2 inhibition by dapagliflozin (115). Secondly, due to a reduced insulin to glucagon ratio (the prime controller of hepatic glucose metabolism) (177), fasting endogenous glucose production is augmented and insulin suppression of post-meam endogenous glucose production is blunted (116, 117). Thirdly, the relative hypoinsulinemia and reduced insulin to glucagon ratio result in increased lipolysis rate and increased free fatty acids (FFA) which enter the circulation and access tissues down a concentration gradient. The extra FFA delivery to the liver coupled with the increased glucagon to insulin ratio lead to the production of ketone bodies. Ketones (in particular, β-hydroxybutyrate) are then exported from the liver, which lacks the rate-limiting enzyme for their oxidation, and are taken up from the bloodstream by most tissues through the monocarboxylic acid transporters (178). Furthermore, due to glycosuria and reduced insulin levels, tissues are glucose deprived, thus a compensatory increase in lipid oxidation is noted along with a concomitant rise in plasma β-hydroxybutyrate levels (119, 179). Uric acid levels have been shown to decrease following the administration of dapagliflozin, or of any SGLT-2 inhibitor in a dose-dependent manner through an increase in renal clearance (180). While insulin jointly enhances the renal reabsorption of sodium and uric acid in humans (181), evidence suggests that the increased concentration of glucose in the lumen produced by SGLT-2 inhibition stimulates uric acid excretion mediated by GLUT9 isoform 2 and inhibits uric acid reabsorption mediated by GLUT9 isoform 2 in the collecting duct (182). Little is known about what other metabolites change as a consequence of chronic glycosuria. Lactate levels have been shown to consistently decrease, particularly postprandially, following SGLT-2 inhibition, presumably as a result of enhanced liver uptake, reduced tissue glucose disposal (119), and increased renal clearance (175). Branched-chain

amino acids, a signature of insulin resistance might also be affected, either directly (e.g., via urinary excretion) or indirectly (through the improved insulin sensitivity).

Clinical efficacy and safety

Dapagliflozin administration was proven effective at reducing HbA1c levels since the first studies carried out. In a study of patients receiving high doses of insulin or insulin sensitizers, dapagliflozin decreased HbA1c levels by 0.7% compared with placebo (183). Furthermore, dapagliflozin decreased body weight by 4.5 kg, with no increased adverse events. Similar results were obtained in another study in both early stage and late stage diabetes regarding HbA1c levels and weight loss (184). In a phase 3, randomized controlled trial in type 2 diabetes patients on metformin monotherapy, dapagliflozin was compared with placebo. Administration of 10 mg once daily dapagliflozin led to a mean reduction of HbA1c level of 0.84% compared with placebo without increased hypoglycemic events (185). Similar results were reported in another study, where dapagliflozin decreased HbA1c levels by 0.78% compared with placebo, in patients receiving metformin monotherapy. This decrease was sustained during the 102 weeks of the study (186). Increased rates of genital infections were reported with dapagliflozin, as expected. Similar results were reported in a study in patients on sulfonylurea monotherapy. 10 mg of Dapagliflozin led to HbA1c reduction of 0.82% compared with placebo, body weight reduction without increased hypoglycemic events (187). In patients receiving insulin therapy, dapagliflozin led to a reduction of HbA1c levels by 0.57% compared with placebo, a reduction of insulin dose and body weight, while these effects were sustained for at least 6 months (188). Same results were reported in another randomized controlled trial in Japanese patients with type 2 diabetes (189). Patients on insulin therapy were randomized to either dapagliflozin or placebo (as part of double blind treatment) for 16 weeks and then to dapagliflozin (as part of open label treatment) for another 36 weeks. HbA1c levels decreased by -0.62% at 16 weeks and -0.74% at 52 weeks, in the dapagliflozin group, vs -0.08% at 16 weeks and -0.83% at 52 weeks, in the placebo-dapagliflozin group. Body weight decreased at both times in the dapagliflozin group, and at 52 weeks in the placebo-dapagliflozin group. When compared with glipizide, a sulfonylurea, dapagliflozin did not reduce HbA1c levels, but led to weight reduction and fewer advese events, mainly hypoglycemic events (190). A somewhat smaller reduction of HbA1c levels were reported in drug

naïve type 2 diabetes patients (by 0.46% compared with placebo) in Japanese patients treated for 12 weeks with 10 mg of dapagliflozin (191). In a 24 week randomized controlled trial, dapagliflozin was compared with placebo, in patients with type 2 diabetes on metformin and sitagliptin dual treatment (139). Dapagliflozin decreased HbA1c levels by 0.5% compared with placebo, while also decreasing body weight by 2.3 kg compared with placebo. These effects were maintained through week 48 of the extension. Adverse events were balanced between the two groups through week 48, except for genital infections which were more frequent in patients receiving dapagliflozin. Another randomized controlled trial comparing dapagliflozin with placebo in patients receiving dual oral treatment with metformin and saxagliptin, resulted in greater HbA1c level reduction with dapagliflozin (-0.8%) compared with placebo (-0.1%) at 24 weeks (192). Adverse events were similar between groups, except for genital infections, which were more frequent with dapagliflozin. To summarize, dapagliflozin administration consistently leads to a reduction of HbA1c levels by 0.5-0.8% which is sustained. Furthermore, dapagliflozin leads to weight loss, without adding severe adverse events, except for mild genital infections.

Large Randomized Controlled Trials

DECLARE-TIMI-58

The DECLARE-TIMI 58 was a randomized, double blind, placebo-controlled trial of dapagliflozin in patients with type 2 diabetes and established atherosclerotic cardiovascular disease or multiple risk factors for atherosclerotic cardiovascular disease (193). Eligible patients were 40 years of age or older, with type 2 diabetes mellitus, HbA1c levels of at least 6.5% and less than 12% and a creatinine clearance of 60 ml or more per minute. Furthermore, patients also had multiple risk factors for atherosclerotic cardiovascular disease (which included, men >55 years old or women >60 years old with one or more traditional risk factors, including hypertension, dyslipidemia or use of tobacco) or had established atherosclerotic cardiovascular disease (defined as clinically evident ischemic heart disease, ischemic cerebrovascular disease or peripheral arterial disease). The primary safety outcome was MACE (cardiovascular death, myocardial infarction or ischemic stroke). The two primary efficacy outcomes were MACE and a composite of cardiovascular death or hospitalization for heart failure. Furthermore, two secondary efficacy outcomes were prespecified defined as a

sustained decrease of 40% or more in eGFR to less than 60 ml per minute per 1.73 m^2 of body surface area, new end-stage renal disease, or death from renal or cardiovascular causes. The other secondary outcome was death from any cause. A total of 17.160 participants completed the run in phase, including 6974 patients (40.6%) with established atherosclerotic cardiovascular disease and 10.186 (59.4%) with multiple risk factors.

In this trial, dapagliflozin administration met the prespecified criterion for noninferiority with respect to MACE (p<0.001 for noninferiority). Regarding efficacy, dapagliflozin led to a lower rate of CV death or heart failure hospitalization than placebo (4.9% vs 5.8%; HR 0.83; 95% CI, 0.73-0.93, p=0.005). This was mainly due to a lower rate of heart failure hospitalization in the dapagliflozin group (HR, 0.73; 95% CI, 0.61 to 0.88). Regarding cardiovascular death, there was no difference between the two groups. Dapagliflozin did not result in a lower rate of MACE than placebo (8.8% and 9.4% in the two groups, respectively; HR 0.93; 95% CI, 0.84-1.03; p=0.17). No difference was noted in the multiple risk factor population compared with the population with established atherosclerotic disease. Regarding safety, dapagliflozin led to fewer hypoglycemic events compared to placebo (p=0.02) and fewer acute kidney injury events compared to placebo (p=0.002). On the other hand, dapagliflozin led to a greater number of genital infections (p<0.001) and to increased diabetic ketoacidosis events (p=0.02) compared to placebo. No new safety concerns were identified in this study.

DAPA-HF

The Dapagliflozin and Prevention of Adverse Outcomes in Heart Failure (DAPA-HF) trial (194) was a prospective double blind, placebo controlled trial to evaluate the efficacy and safety of Dapagliflozin in patients with heart failure and a reduced ejection fraction, regardless of the presence or absence of diabetes. Patients were eligible if: they were at least 18 years old, with an ejection fraction of 40% or less and New York Heart Association (NYHA) class II, III and IV symptoms. Patients were also required to have a serum level of N-terminal pro-B-type natriuretic peptide (NT-proBNP) of at least 600 pg/ml. Patients with atrial fibrillation or atrial flutter were required to have an NT-proBNP level of at least 900 pg/ml. Furthermore, patients were required to receive standard heart failure therapy and standard drug treatment. The primary outcome was a composite of worsening heart failure or death from cardiovascular causes. An episode

of worsening heart failure was either an unplanned hospitalization or an urgent visit resulting in intravenous therapy for heart failure. A key secondary outcome was a composite of hospitalization for heart failure or cardiovascular death. Of the 8134 patients who underwent screening, 4744 were randomized to either dapagliflozin or placebo. After a median duration of 18.2 months, the primary composite outcome of worsening heart failure or death from cardiovascular causes occurred in 386 patients (16.3%) in the dapagliflozin group and in 502 patients (21.2%) in the placebo group (HR, 0.74; 95% CI 0.65 to 0.85; p<0.001). Event rates for all three components of the composite outcome favored dapagliflozin, with a number need to treat to prevent one primary event of 21. The effect of dapagliflozin was consistent across prespecified subgroups, including patients without diabetes at baseline.

DELIVER

The Dapagliflozin evaluation to Improve the Lives of Patients with Preserved Ejection Fraction Heart Failure (DELIVER) trial (92), was a phase 3, internation, multicenter, double blind, randomized, controlled trial in patients with heart failure with an ejection fraction of 40% or more. Eligibility criteria included: adults of 40 years of age or older, stabilized heart failure, with or without type 2 diabetes, with a LVEF of 40% or more, with evidence of structural heart disease and an elevated natriuretic peptide level. The primary outcome was a composite of worsening heart failure, which was defined as either an unplanned hospitalization for heart failure or an urgent visit for heart failure, or cardiovascular death. Secondary outcomes were the total number of worsening heart failure events and cardiovascular deaths, the change from baseline in the total symptom score on the Kansas City Cardiomyopathy Questionnaire (KCCQ; scores range from 0 to 100, with higher scores indicating fewer symptoms and physical limitations) at month 8, cardiovascular death, and death from any cause. A total of 10418 patients were screened, while 6263 patients were randomly assigned to receive either dapagliflozin or placebo. After a median duration of follow-up of 2.3 years, the primary outcome occurred in 512 patients (16.4%) in the dapagliflozin group and in 610 patients (19.5%) in the placebo group (HR, 0.82; 95% CI 0.73-0.92; p<0.001). Similar results were obtained in the group of patients with an LVEF of 60% or more. Furthermore, the effect of dapagliflozin was consistent across all prespecified subgroups, including the presence or absence of diabetes. No new safety signals were reported in this trial as well.

DAPA-CKD

The Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease (DAPA-CKD) (195), was a randomized, double blind, placebo-controlled clinical trial. Adults with or without type 2 diabetes who had an estimated glomerular filtration rate (GFR) of 25 to 75 ml per minute per 1.73 m2 of body-surface area and a urinary albumin-to-creatinine ratio (with albumin measured in milligrams and creatinine measured in grams) of 200 to 5000 were eligible for participation. All the participants were required to be receiving a stable dose of an ACE inhibitor or ARB for at least 4 weeks before screening. Patients were randomly assigned to receive dapagliflozin 10 mg once daily or matching placebo. The primary composite outcome, assessed in a time-to-event analysis, was the first occurrence of any of the following: a decline of at least 50% in the estimated GFR (confirmed by a second serum creatinine measurement after ≥28 days), the onset of end-stage kidney disease (defined as maintenance dialysis for ≥28 days, kidney transplantation, or an estimated GFR of <15 ml per minute per 1.73 m2 confirmed by a second measurement after ≥28 days), or death from renal or cardiovascular causes. Secondary outcomes (also assessed in time-to-event analyses) were, in hierarchical order, the composite kidney outcome of a sustained decline in the estimated GFR of at least 50%, end-stage kidney disease, or death from renal causes; a composite cardiovascular outcome defined as hospitalization for heart failure or death from cardiovascular causes; and death from any cause. An independent committee whose members were unaware of the trial-group assignments adjudicated all primary and secondary outcomes, except for a sustained decline in the estimated GFR of at least 50% and a sustained estimated GFR of less than 15 ml per minute per 1.73 m2. 7517 patients were screened, of whom 4094 received either dapagliflozin or matching placebo. After a median follow-up of 2.4 years, the primary outcome of a sustained decline in the eGFR of at least 50%, end-stage kidney disease, or death from renal or cardiovascular causes occurred in 197 patients (9.2%) in the dapagliflozin group and in 312 patients (14.5%) in the placebo group (HR, 0.61; 95% CI 0.51-0.72; p<0.001). The number of patients needed to be treated with dapagliflozin to prevent one primary outcome was 19. Results were consistent across prespecified subgroups including in patients with or without type 2 diabetes. Furthermore, dapagliflozin resulted in lower number of deaths compared with placebo (HR, 0.69, 95% CI, 0.53-0.88; p=0.004). There were no new safety concerns in this trial.

Insulin Degludec

Mechanism of action

Insulin Degludec (IDeg) is a new basal insulin which forms bi-hexamers in presence of phenol and zinc (196, 197). Following a subcutaneous injection, the molecules form poly-hexamers which gradually breakdown and are gradually absorbed. After entering the circulation, insulin molecules bind with plasma proteins thus maintaining a relatively steady plasma concentration of insulin (197). The duration of action of IDeg is over 42 hours, with a plasma half-life of 25 hours (198, 199). Compared with Insulin glargine (IGlar), IDeg has been shown to have better steady state concentration and increased hypoglycemic effect (200, 201, 202, 203, 204, 205). The BEGIN program, compared the safety and efficacy of IDeg with IGlar in patients with type 1 and type 2 diabetes (206). These non inferiority phase 3 clinical trials showed that patients receiving IDeg had fewer episodes of nocturnal hypoglycemia along with lower fasting glucose levels compared with patients that received IGlar (207, 208, 209, 210).

Clinical Trials

The SWITCH 2 which was a randomized controlled trial showed that patients who received IDeg compared with IGlar U100 for 32 weeks, had significantly fewer hypoglycemic episodes (185.6 versus 265.4 episodes per 100 patient-years, $p<0.001$) (211). A metanalysis of 7 clinical phase 3a randomized controlled trials compared the effect in fasting glucose levels and nocturnal hypoglycemias in patients receiving either IDeg or IGlar. 2 Studies included 957 patients with type 1 diabetes, while 5 studies included 3360 patients with type 2 diabetes. Fasting glucose levels were lower in patients receiving IDeg in all examined studies, while this reached statistical significance in 3 studies. Furthermore, IDeg was associated with a lower incidence of nocturnal hypoglycemia, independent of the type of diabetes or concurrent treatments (208). Another metanalysis included 18 randomized clinical trials which compared IDeg with IGlar, in 16791 patients with diabetes. IDeg was associated with significantly decreased risk of hypoglycemia (ERR=0.81, 95% CI 0.72-0.92, p=0.001). Furthermore, a decrease in the risk of nocturnal hypoglycemia was found (ERR=0.71, 95% CI 0.63-0.80, $p<0.001$). However, the risk of serious hypoglycemia decreased significantly only in patients with type 2 diabetes (ERR=0.65, 95% CI 0.52-0.89, p=0.005), but not in patients with type 1 diabetes. Median fasting glucose levels were lower in patients who

received IDeg (p=0.001). Regarding other adverse events, cardiovascular events and all cause mortality, no significant difference was detected between IDeg and IGlar (212).

The CONCLUDE trial, was a head-to-head randomized clinical trial which was designed to examine the risk of hypoglycemia and evaluate the safety of IDeg 200U/ml compared with IGlar 300 U/ml in patients with type 2 diabetes who were already on treatment with basal insulin. Of the 1609 randomized patients, 733 of 805 (91.1%) of patients who received IDeg completed the trial and 734 of 804 (91.3%) of the patients who received IGlar completed the trial. Regarding the primary endpoint, IDeg did not result in significantly lower risk of symptomatic hypoglycemia compared with IGlar U300 (RR=0.88, 95% CI 0.73-1.06). However, a lower risk of nocturnal hypoglycemia (RR=0.63, 95% CI 0.45-0.84) and serious hypoglycemia (RR=0.20, 95% CI 0.07-0.57) were evident with IDeg compared with IGlar U300 (213).

The DEVOTE clinical trial included 6509 patients with type 2 diabetes and established cardiovascular disease and/or chronic kidney disease. Compared with IGlar, IDeg was non inferior regarding cardiovascular safety and hypoglycemic efficacy (214, 215, 216).

Metabolomics

Metabonomics and Metabolomics are terms that appeared in the medical literature during the past 3 decades and are used interchangeably, although their meaning is not the same. The most often used definition of metabonomics is: “the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification’’ (217). A similar definition is used for the term metabolomics: “The study of the quantitative complement of metabolites in a biological system and changes in metabolite concentrations or fluxes related to genetic or environmental perturbations. Studies are typically holistic in nature though targeted studies are also encompassed in the term metabolomics’’ (218). In broad terms, both definitions describe the metabolome, which is defined as the collection of the endogenous small molecule metabolites (<1500 Daltons) (219). Older technologies such as genomics (the study of the human genome), transcriptomics (the study of gene transcripts) and proteomics (the study of proteins) are similar to the term metabolomics. Studying the human metabolome provides a distinct advantage over these older technologies, since it allows us to take a precise snapshot of the organism’s current

metabolic state. The metabolome is influenced by both intrinsic and extrinsic factors, such as, diet, medications, lifestyle, gender and age. In this regard, metabolomics can provide useful information for various processes in a single measurement as compared with the above mentioned older technologies (220).

At present, the methods used for the study of metabolome are: Nuclear Magnetic Resonance (NMR), gas-chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS) and high performance liquid chromatography (HPLC) (221, 222, 223).

Type 2 Diabetes (T2D), is the most common metabolic disease worldwide and it is estimated that by the year 2030, more than 500 million persons will be affected (1). Diabetes is characterized by an elevated blood glucose concentration (224) and represents a considerable burden to both the patient and the health system since its natural history is characterized by the development of various microvascular and macrovascular complications. Microvascular complications include: diabetic nephropathy, retinopathy and neuropathy. On the other hand, macrovascular complications include: coronary artery disease, stroke and peripheral artery disease. Macrovascular complications are the leading cause of death in patients with diabetes whereas the microvascular ones contribute significantly to the increased morbidity that characterizes this patient population (225). Type 2 diabetes is not a static condition but rather, a continuous one. The natural history of the disease begins years before the development of hyperglycemia and is characterized by peripheral insulin resistance and a progressive decline in the production of insulin by pancreatic beta cells. The fist clinical sign of disturbed carbohydrate metabolism are small increases in serum glucose concentrations that do not fulfill the criteria for the diagnosis of diabetes. This condition, termed prediabetes, is defined by increased fasting glucose (blood glucose levels 100-125 mg/dL, or impaired fasting glucose-IFG) or by an abnormal response to oral glucose tolerance test (two-hour glucose levels 140-199 mg/dL, or impaired glucose tolerance-IGT) (224). Interestingly, macrovascular and microvascular damage in people with T2D, starts before the diagnosis of diabetes is evident. Indeed, in the initial phase of T2D, which is characterized by hyperinsulinemia and normoglycemia, macrovascular disease is already underway. In addition, even with prompt diagnosis,

the complications of diabetes usually follow a downhill course, since the available therapeutic tools are far from ideal. For these reasons, the design of research methods that allow the characterization and the understanding of the metabolic perturbations that characterize the course of diabetes and the pathogenesis of its complications is of paramount importance for both diagnostic and therapeutic reasons. Here, we review the role and the results of metabolomic research in individuals with prediabetes and diabetes.

Metabolomics in the prediction of type 2 diabetes (Table 4)

Changes in carbohydrate metabolism

Altered sugar metabolism is the hallmark of diabetes. Indeed, metabolomic studies have identified several carbohydrate disturbances in patients with various stages of the disease (226). For example, plasma hexose sugars are positively associated with T2D and glucose is the main hexose elevated. Elevation of other carbohydrates are also evident, such as fructose, mannitol and sorbitol (227, 228). In another study, an inverse correlation between deoxyhexose sugars (anhydroglucitol, deoxy-galactose, deoxy-glucose) and T2D was found (229). Furthermore, in the same study, a sugar alcohol comprising 1-*O*-β-D-glucopyranosyl-D-mannitol and/or maltitol was negatively related to T2D (229). Elevated levels of various carbohydrates (such as glucose, mannose, fructose and hexose) were found 3-7 years before the diagnosis of T2D was evident (230, 231). Increased hexose concentrations may indicate the disturbance of all 6-carbon carbohydrate metabolism which is found in prediabetic conditions (230). In another study, increased levels of glucose, d-galactose, gluconate and decreased levels of glycerol were predictive of diabetes development (232). Galactose is a 4 carbon epimer of glucose and can be rapidly converted to glucose through the Leloir pathway (233). Increased galactose metabolism may lead to long-term, gradual increases in serum glucose and may contribute to insulin resistance (232).

Changes in amino-acid metabolism

Disturbances in amino acid levels, particularly in branched-chain amino acids (BCAA) and in aromatic amino acids (AAA), have long been recognized as a marker of obesity-induced insulin resistance (234). Elevated levels of the three BCAAs (leucine, isoleucine, valine) have been found up to 13.7 years ahead of T2D clinical manifestation (235, 236). Elevated levels of BCAAs have also been found in

prediabetic states (226, 237). In a recent study, elevated levels of BCAAs in T2D patients were correlated with carotid intima media thickness (238). Urine BCAAs levels have also been found elevated in insulin resistant states (239). While BCAA are consistently elevated in prediabetes and in T2D, it remains unclear whether this is simply the result of increased proteolysis due to insulin resistance, or due to increased intake and absorption of these molecules. However, it is now recognized that increased BCAA levels may play a direct pathophysiological role in the development of T2D. Two theories have been suggested to explain the role of BCAA in T2D. The first theory is that BCAA may cause insulin resistance through mammalian target of rapamycin (mTORC1) (237) and S6 kinase 1 (S6K1) activation (240, 241). This activation, in turn, results in serine phosphorylation of insulin receptor substrate 1 and 2 (IRS1 and IRS2) and in turn results in increased insulin resistance. Mice lacking S6K1, maintained proper glycemic control while on a high fat diet (242). Furthermore, selective inhibition of S6K1 resulted in increased insulin sensitivity in vitro and in mice (243). On the other hand, chronic inhibition of mTORC1 by rapamycin resulted in increased insulin resistance (243). A second theory suggests that, toxic BCAA metabolites lead to pancreatic β-cell dysfunction and eventually, apoptosis, which contributes to the onset of T2D (244).

Aromatic amino acids (phenylalanine, tyrosine, tryptophan) also predict the development of T2D and are found increased 12 years prior to the onset of diabetes (230, 235). Phenylalanine and tyrosine were strongly predictive of T2D, while tryptophan was also associated, but to a lesser extend (236). Alanine has also been associated with hyperglycemia and T2D (245, 246). Furthermore, alanine was associated with a higher risk for future diabetes development (247). Alanine serves as a hepatic substrate for gluconeogenesis and is also a stimulator of glucagon secretion, both of which result in elevated glucose levels (248, 249). On the other hand, other amino acids tend to be decreased in insulin resistance and T2D. Decreased levels of glycine were associated with insulin resistance (245) and may predict both IGT and T2D development (250). Glutamine is also found to be low in insulin resistant states (251). Interestingly, high glutamine levels or a high glutamine/glutamate ratio are associated with a lower risk of diabetes development even after adjusting for BMI and BCAA levels (245). Glutamine was also inversely associated with 6-year glucose levels in women (252). In order to test the hypothesis that glutamine ameliorates glucose

tolerance, Cheng et al (245) performed a dietary intervention in mice. Three groups of C57Bl6 mice were each fed a different diet. One group was fed with glutamine plus standard chow, a second group was fed with glutamate with standard chow and a third group was fed with standard chow alone. After the 8-week diet intervention, an intraperitoneal glucose tolerance test was performed. Compared with mice in the control and glutamate groups, mice in the glutamine group had the lowest plasma glucose levels measured at each time point following glucose administration. Glutamine may exert its anti-diabetic effects through increased insulin sensitivity in skeletal muscle, as was shown in a study in obese rats. Furthermore, in a study in persons with type 1 diabetes, glutamine supplementation increased the rates of post exercise nighttime hypoglycemia (253). Amino acid metabolites are also disturbed in insulin resistant and diabetic states. The branched chain keto-acid 3-methyl-2-oxovalerate strongly predicts IFG. Similarly, in the same study, other branched keto-acids such as 4-methyl-2-oxopentanoate and 3-methyl-2-oxobutyrate (3-MOB) were also elevated in IFG and T2D (226, 254). On the other hand, α-hydroxybutyric acid (which is a product of methionine and threonine metabolism) was found to be a selective and reproducible marker of IGT (254). In this study, the top metabolites associated with IGT were: a-hydroxybutyric acid (a-HB), linoleoyl-glycerophosphocholine (L-GPC), X-12063, oleic acid, β-hydroxybutyrate (β-HB), and glycine. On the other hand, IFG was associated with: a-ketobutyrate (a-KB), 3-MOB, 2-aminoadipic acid (2-AAA), 4-methyl-2-oxobutyric acid (4-MOP), and 3-hydroxyisobutyric acid (3-HIB). 2-AAA is a product of lysine degradation and appears in plasma as a result of whole tissue or protein degradation. Normoglycemic individuals with increased levels of 2-AAA had a 4-fold higher odds of developing diabetes over a 12-year period (255). The authors also investigated the role of 2-AAA regarding insulin resistance. They found that 2-AAA stimulates insulin secretion. Thus, the elevated levels of 2-AAA in prediabetes might act as a compensating mechanism to lower glucose levels.

Products of the intestinal microbiota such as creatine, 1-palmitoleoylglycerol (16:1), urate, 2-hydroxybutyrate/2-hydroxyisobutyrate, xanthine, xanthurenate, kynurenate, 3-(4-hydroxyphenyl)lactate, 1-oleoylglycerol (18:1), 1-myristoylglycerol (14:0), dimethylglycine, and 2-hydroxyhippurate (salicylurate) were significantly associated with an increased risk of type 2 diabetes (256). These metabolites have been related to

decreased insulin secretion, increased insulin resistance or both through various mechanisms.

Changes in lipid metabolism

Obesity is a major risk factor for developing insulin resistance and T2D. Insulin resistance and insulin depletion lead to reduced fatty acid synthesis in adipose tissue and increased lipolysis. Free fatty acids are then released into the bloodstream leading to ectopic accumulation of fat in the liver or the muscles (257). Ectopic fat accumulation in the liver, the muscles and pancreatic β-cells is thought to be a decisive factor in the development of T2D (258, 259, 260). Interestingly, triglycerides containing fatty acids with increased carbon atoms and double bonds were associated with a lower risk for insulin resistance and T2D development, compared with triglycerides rich in saturated fatty acids with a low number of carbon atoms (261). However, other studies have shown that, lipids with longer chain length (adrenate and arachidonate, C22:4 and C20:4, respectively) are elevated in IFG (226). Furthermore, lipids with short chain length, such as heptanoate, pelargonate, and 5-dodecenoate (C7:0, C9:0 and C12:1, respectively, are decreased in T2D (226). Increased levels of diacylphosphatidylcholines were found in prediabetes and were predictive of T2D (250, 262). Lysophosphatidylcholines and sphingomyelins were negatively associated with obesity and T2D (263, 264). Lysophosphatidylcholines may exert anti-diabetic and anti-inflammatory effects through peroxisome-proliferator receptor delta agonism (PPAR-δ) (265). PPAR-δ agonism is known to reduce insulin resistance, inflammation and endoplasmic reticulum stress (266). In this regard, low levels of lysophosphatidylcholines may play a role in the development of T2D. A recent study reported that decreased levels of phosphatidylcholines containing odd chain fatty acids (C19:1, C17) were associated with an increased risk of developing T2D in 7 years (267).

Acylcarnitines have also been studied as biomarkers for the prediction of T2D. In obese humans, various acylcarnitines have been found to be elevated (237). Studies have shown that various acylcarnitines are increased during both prediabetic states, as well as in overt diabetes (268, 269). In a recent study by Sun L et al (270), increased levels of acylcarnitines, particularly long-chain acylcarnitines, were predictors of T2D development in community living persons over a 6-year period. Interestingly, increased levels of acylcarnitines are found in offsprings of patients with T2D after a lipid

tolerance test. The authors suggest that a lipid tolerance test may be a valuable tool in assessing persons with increased likelihood of developing T2D (271). Furthermore, in mice lacking the acyl-CoA dehydrogenase 10 (ACAD10) gene, long chain acyl carnitine levels were increased. ACAD10 deficient mice exhibited abnormal glucose tolerance and higher insulin levels and the authors concluded that ACAD10 polymorphisms in humans may predict T2D development (272).

Branched fatty acid esters of hydroxy fatty acids (FAHFAs) are found elevated in obese yet insulin sensitive mice overexpressing the GLUT4 glucose transporter in adipose tissue (273). In the same study, FAHFA isomers (PAHSAs), were decreased in insulin resistant humans and mice. Interestingly, PAHSAs stimulate glucose mediated insulin secretion and glucagon like peptide-1 secretion in mice thus marking them as suitable candidates in the treatment of T2D.

Metabolomics in diabetes complications (Table 5)

Metabolomics in diabetic nephropathy

Diabetic kidney disease, or diabetic nephropathy, is the leading cause of end stage renal disease (ESRD) nowadays (274). Chronic kidney disease (CKD) due to diabetes affects roughly 20-40% of persons with diabetes (274). CKD is diagnosed by the presence of elevated urine excretion of albumin (albuminuria), by an estimated glomerular filtration rate (eGFR) <60 ml/min/1.73m^2 or when other manifestations of kidney disease are evident (275). Hyperfiltration is the first step in diabetic nephropathy and is accompanied by increased glucose reabsorption and hypertrophy of the proximal tubular cells. In turn this leads to mesangial expansion and glomerular basement membrane thickening, and finally to fibrosis (276). CKD is associated with increased risk of cardiovascular mortality and is considered to be a coronary artery disease equivalent (277).

Plasma accumulation of uremic solutes, is thought to be the hallmark of progression to ESRD (278, 279). Uremic solutes are metabolic end products, which normally are removed from the body via the kidneys. However, in impaired kidney function, these metabolites accumulate in plasma. Whether these metabolites are simply markers of impaired kidney function, or they have a direct role in the pathophysiology of CKD is still unclear (280). A study in 80 persons with type 2 diabetes who attended the Joslin Clinic with normal baseline kidney function, revealed alterations in plasma metabolites between progressors to ESRD and non-progressors (280). Compounds synthesized by

the microbial flora in the colon were shown to be elevated in progressors. These include phenyl compounds, such as p-cresol sulfate and phenylacetylglutamine which were already shown to be upregulated in uremic states (281, 282). Others, include derivatives of amino acids such as, phenol sulfate, indoleacetate and 3-indoxyl sulfate, which were elevated in plasma of progressors compared to non-progressors, although, not statistically significant. These compounds may be toxic to the endothelium and thus contribute to increased cardiovascular risk of these patients (283). Probiotic treatment in patients undergoing hemodialysis decreased p-cresol levels, although the study did not examine cardiovascular endpoints (284). Plasma concentrations of several nucleotide derivatives that are considered to be uremic solutes were also strongly associated with the progression to ESRD. Elevated concentration of pseudouridine in plasma was the strongest and most statistically significant predictor of progression. In another study (285), pseudouridine and N6-Carbamoylthreonyladenosine were associated with a eGFR <60 ml/min/1,73m^2. Uric acid is a metabolite of purine metabolism and increased plasma concentrations were associated with progression to ESRD (280). Regarding amino acids, plasma histidine was significantly lower in persons with type 2 diabetes who transitioned from microalbuminuria to macroalbuminuria (286). Furthermore, C-glycosyltryptophan levels were higher in progressors compared with non-progressors and was highly correlated with progressors to ESRD (280, 285). Interestingly, metabolites derived from tryptophan may promote oxidative stress, leukocyte activation, and inflammation in endothelial and vascular smooth muscle cells. All these may contribute to increased cardiovascular disease burden in CKD patients (287). Finally, acylcarnitines have been found to be increased in CKD patients (280, 285), while increased plasma butenoylcarnitine levels was associated with higher rates of transition from microalbuminuria to macroalbuminuria (286). Acylcarnitines are filtered through the kidney and about 75% are excreted into urine. Elevated acylcarnitines levels may reflect a role of proximal tubular dysfunction and michondrial dysfunction in the development of CKD in diabetes (276, 280). Urine samples may also be of significance in diabetic kidney disease. Indeed, Sharma et al found that 13 metabolites were significantly reduced in persons with diabetic kidney disease compared with healthy controls, while 12 of them remained statistically significant when compared with persons with diabetes alone (288). These metabolites were mostly water-soluble organic anions and as such their urine concentrations are regulated by organic anion transporters (OATs) found in proximal renal tubules.

Analysis of this data indicated that these metabolites were linked to mitochondrial metabolism, and that mitochondrial metabolism was suppressed in patients with diabetic nephropathy. In the FinnDiane study (289), most urine metabolites that discriminated diabetic kidney disease from normal kidney function were acyl-carnitines, acyl-glycines and metabolites related to tryptophan metabolism. Atresentan, an endothelin A receptor antagonist, was found to stabilize urinary metabolites in diabetic kidney disease, while they declined with placebo treatment (290). The authors hypothesized that treatment with atresentan may attenuate mitochondrial dysfunction in diabetic kidney disease. In a recent study, which compared the serum and urine metabolome of patients with DM with patients with diabetic nephropathy, the authors showed that there were significant alterations between the two groups (291). Notably, serum fumaric acid, a Krebs Cycle intermediate, was increased in patients with diabetic nephropathy, indicating continuous oxidative stress in progressive diabetic nephropathy. Interestingly increased urinary 1-xylonate-2 was found in patients with diabetic nephropathy. Hyperoxaluria is a metabolic disorder with oxalate crystal deposition in various organs, especially the kidney. The increased urinary levels of 1-xylonate-2 may worsen kidney damage in diabetic nephropathy and might serve as a therapeutic target.

Metabolomics in diabetic neuropathy

Neuropathy is the most common complication of diabetes, affecting approximately 50% of patients with DM during the course of the disease (292). Typical symptoms of diabetic neuropathy include numbness, pain, burning sensation, tingling and gait abnormalities. Neuropathy is also associated with depression, ulcerations on pressure points and amputation (293). The pathophysiology of diabetic neuropathy is complex and involves mitochondrial dysfunction, lipid oxidation and nitrosative stress (276). In a mouse model of type 2 diabetes, four of five measured glycolytic metabolites were decreased in sural and sciatic nerves. Furthermore, citrate and isocitrate were also decreased, although no other tricarboxylic cycle intermediates were changed (294). In streptozotocin-induced diabetic rats several metabolites were found to be altered in the sciatic nerve, the 4/5 dorsal root ganglia and the trigeminal ganglia (295). All tissues showed increases in glucose, fructose, sorbitol and decreases in myo-inositol and scyllo-inositol which is characteristic of the polyol pathway activation. Lipids on the other hand, were dysregulated mainly in the sciatic nerve. Acyl carnitines such as

palmitoylcarnitine and linoleycarnitine were increased while short chain triacylglycerols were decreased in the sciatic nerve, while they remained unchanged in both the dorsal root ganglia and the trigeminal ganglia. The authors hypothesize that lipid changes in diabetic neuropathy are found mainly in the peripheral nervous system and are more severe. The results suggest that diabetes affects mainly Schwann cells which are abundant in the peripheral nervous system. Nitrosative stress may also play a significant role in the development of diabetic neuropathy through activation of the poly-ADP ribose polymerase (PARP) pathway. 3-nitrotyrosine was found to be increased in the sciatic nerve and the spinal cord in streptozotocin-induced diabetic rats (296). PARP inhibition was found to ameliorate nerve conduction and axonal atrophy showing promising efficacy in diabetic neuropathy.

Metabolomics in diabetic retinopathy

Diabetic retinopathy is a devastating complication and is the leading cause of blindness worldwide and affects the majority of diabetic patients after 20 years of disease. Diabetic retinopathy begins as non-proliferative retinopathy, which is characterized by weakening of the blood-retinal barrier, thickening of the vascular basement membrane, and the death of pericytes, the contractile cells which surround retinal endothelial cells. Proliferative retinopathy is the next step, during which fragile new blood vessels grow and eventually begin to hemorrhage into the macula. The combination of fibrous neovascularization and macular swelling results in vision loss and may even lead to retinal detachment (297, 298). NMR-based metabolomics from vitreous fluid in patients with proliferative diabetic retinopathy revealed decreased galactitol levels. Decreased galactitol levels may reflect a trend towards sorbitol generation during hyperglycaemia (299). In the same study, increased levels of lactate were identified which may reflect hypoxic conditions and anaerobic metabolism in the diabetic eye. Furthermore, decreased levels of ascorbic acid were also found. Finally, lactate had a sensitivity of 86% and a specificity of 81% in identifying patients with proliferative diabetic retinopathy. In another study using MS-based metabolomics in vitreous, fluid samples from patients with proliferative diabetic retinopathy (300) revealed increased levels of multiple acylcarnitines and increased levels of arginine, proline, ornithine and citrulline. Arginine is metabolized by two pathways, the arginase pathway which produces ornithine and urea and the nitric oxide synthase pathway which produces citrulline and nitric oxide. Increased activity of the arginase pathway compared with

decreased activity of the nitric oxide synthase pathway may cause increased proline levels along with decreased nitric oxide levels which can lead to endothelial dysfunction and worsening retinal damage.

Metabolomics in type 2 diabetes therapy (Table 6)

Type 2 diabetes is treated with several oral and injectable drugs. Major drug classes include: biguanides (mainly metformin), sulfonylureas, dipeptidyl-peptidase 4 inhibitors (DPP4 inhibitors), thiazolidinediones (mainly pioglitazone), sodium-glucose co transporter 2 inhibitors (SGLT-2 inhibitors), glucagon like peptide 1 (GLP-1) agonists and insulin. Each drug class has its own pros and cons and the choice of the correct regimen for each patient with type 2 diabetes is challenging. Metabolomics studies have been carried out to assess the impact of antidiabetic therapy in persons with type 2 diabetes, and can be of help in individualizing antidiabetic therapy. In 25 persons with either IFG or T2DM, metformin and pioglitazone was used by Irving et al (301). In this study, several serum amino acids were reduced by this drug combination such as phenylalanine, tyrosine, glutamate, arginine, citrulline, aspartate, lysine, α-aminoadipic acid and ethanolamine. On the other hand, no BCAA alterations were reported. Furthermore, increased concentrations of serine and glycine were found after therapy. To compare whether these changes were due to increased insulin sensitivity or due to a direct drug effect, a 7-hour insulin infusion was carried out. The insulin infusion resulted in metabolic changes similar to those described above and thus the authors concluded that the metabolic alterations found in these patients were due to enhanced insulin action. In a study by Walford et al (302), 2 days of twice daily metformin 500 mg in insulin resistant adults, which reduced both insulin and glucose serum values, was found to increase BCAA concentrations significantly. On the other hand, one dose of glipizide 5 mg (a sulfonylurea), which decreased glucose and increased insulin, resulted in decreased levels of BCAAs in insulin sensitive adults only. Thiazolidinedione treatment was found to increase the expression of genes related to BCAA catabolism, such as branched chain amino acid transaminase 2 (BCAT2) and branched chain keto-acid dehydrogenase alpha (BCKDHa) resulting in increased catabolism of BCAA (303). Another agent which was found to lower BCAA in patients with T2DM was sitagliptin, a DPP4 inhibitor. In a study by Muscelli et al (304), sitagliptin treatment was associated with a selective reduction of both BCAA and a-

hydroxybutyrate (which is a product of amino acid degradation and a marker of insulin resistance) in response to meal. Another study in persons with T2DM who were treated with metformin for 3 months revealed increased levels of trimethylamine-N-oxide (TMAO) and 3-hydroxybutyrate. Elevated TMAO levels may be associated with disturbance of gut microbiota caused by metformin. Additionally, decreased levels of lipoproteins, unsaturated fatty acids and lysophasphatidylcholine were found. Reduction of lysophosphatidylcholine levels may suggest an attenuation of oxidative stress in metformin treated patients (305). In patients with T2DM and coronary heart disease, 4 months of rosiglitazone treatment (a PPAR-γ agonist) resulted in decreased lactate levels and increased glutamine levels due to increased insulin sensitivity. Furthermore, lactate levels were inversely correlated to myocardial glucose uptake (306). Urine metabolomics of persons with T2DM treated with sulfonylureas revealed decreased levels of urinary acyl-carnitines, hippurate and xanthine. On the other hand, increased urinary levels of citrate, aromatic amino acids (such as tryptophan and phenylalanine) and uric acid were observed (307). Acyl-carnitines are mainly derived from beta oxidation in mitochondria and are then excreted in the urine. In insulin resistant states, increased beta oxidation is observed, resulting in increased levels of urinary acyl-carnitines. Sulfonylurea treatment resulted in decreased excretion of acyl-carnitines suggesting a downregulation of beta oxidation. Furthermore, the increased citrate levels indicate increased aerobic glycolysis in the sulfonylurea treatment group. In a study by Buganova et al, the effect of liraglutide (a GLP-1 agonist) in urine metabolome of diabetic mice was studied (308). A two week treatment of liraglutide resulted in increased glycine metabolites in the urine compared with no treatment which may indicate increased insulin sensitivity. Hexanoylglycine was also found to be increased, which may suggest an upregulation of beta oxidation following liraglutide treatment, since hexanoylglycine originates from the conjugation of glycine with hexanoyl-coA, which is a beta oxidation intermediate. Furthermore, lower taurine levels were reported. Increased urinary taurine levels may indicate liver damage, thus the lower taurine levels may be associated with improvement in liver steatosis, a condition common in insulin resistant states. Finally, increased levels of 3-indoxyl sulfate were reported which may be associated with changes in the gut microflora. Similar results were reported in mice treated with vildagliptin (a DPP4 inhibitor), although vildagliptin treatment did not lower urinary taurine levels (309). The administration of an SGLT-2 inhibitor, dapagliflozin, in persons with diabetic kidney

disease with albuminuria, resulted in an increase of 9 urine metabolites (of a panel of 13 metabolites) which have been implicated to be predictors of mitochondrial function in diabetic kidney disease (310). The authors concluded that the administration of dapagliflozin improved the mitochondrial function of diabetic kidneys which could contribute to the known renoprotective effects of SGLT-2 inhibitors.

Conclusion

Diabetes prevalence is increasing worldwide and bears a substantial cost to both the patients and healthcare services. Its complications, both microvascular and macrovascular, pose a serious threat for the patient's health and quality of life. Although a number of treatment options are available to physicians, patients with diabetes continue to experience not only crippling disabilities such as blindness, neuropathy and chronic kidney disease but also increased cardiovascular mortality. Thus, there is an unmet need for early detection, before the disease becomes evident in order to plan an effective strategy for disease prevention.

Metabolomic studies are a relatively new concept that might be able to provide us with new biomarkers that will allow us to predict which patients will eventually develop overt diabetes and also will provide new information regarding diabetes pathophysiology.

Metabolomic studies have already identified metabolites which are deranged years before type 2 diabetes develops. Metabolite disturbances such as increased BCAAs, AAAs, a-hydroxybutyrate and others have already shown promising results in detecting persons with increased risk of developing type 2 diabetes. Other studies have also identified metabolites which could prove useful in detecting patients at increased risk for developing microvascular complications (such as diabetic kidney disease, diabetic retinopathy and diabetic neuropathy). Furthermore, metabolomic studies may be of help in assessing which patient might benefit more from each treatment option and thus enable the physician to choose the right treatment for each patient individually.

Table 4. Metabolomics in Type 2 DM		
Substance	Findings	Pathophysiological role
Hexoses	• Increased levels in PD/DM2	• Increased glucose production
Galactose	• Increased levels in PD	• Increased glucose production
BCAAs	• Increased levels in PD/DM2 • Prediction of DM2 development	• Pancreatic β-cell dysfunction • Increased insulin resistance
AAAs	• Increased levels in PD/DM2	• Not known
Alanine	• Increased risk of DM2 development	• Gluconeogenesis substrate • Glucagon secretion
Glycine	• Decreased levels in PD/DM2	• Not known
Glutamine	• Decreased levels in PD/DM2 • High glutamine/glutamate ratio is associated with high risk for DM2 development	• Increased insulin sensitivity in skeletal muscle
α-hydroxybutyrate (methionine and threonine metabolism)	• Increased levels in IGT/DM2	• Not known
2-amino-adipic acid (lysine degradation)	• Increased levels in IFG • High levels correlate with increased risk for DM2 development	• Increased insulin secretion (compensatory mechanism?)
Triglycerides with long chain fatty acids	• Lower risk for DM2	• Not known
Lysophosphatidylcholines	• Decreased levels in DM2	• PPAR-β/δ activation

Sphingomyelins	• Decreased levels in DM2	• PPAR-β/δ activation
Long chain acylcarnitines	• Increased levels in DM2 • Associated with increased risk of DM2	• Not known

Table 5. Metabolomics in Diabetic Kidney Disease

Substance	Findings
Phenyl compounds (Microbial flora compounds)	• Increased in DKD
Pseudouridine	• Increased in progressors to macroalbuminuria
Histidine	• Decreased in progressors to macroalbuminuria
Glycosyltryptophan	• Increased in progressors to macroalbuminuria
AcylCarnitines	• Increased in progressors to macroalbuminuria

Table 6. Metabolomics in Type 2 Diabetes Treatment

Drug	Plasma/Urine	Results
Metformin	Plasma	• Increased: BCAA, T-MAO, 3-hydroxybutyrate • Decreased: Lysophospatidylcholines
Glipizide	Plasma	• Decreased: BCAA
Thiazolidinediones	Plasma	• Decreased: BCAA, lactate • Increased glycine
Sitagliptin	Plasma	• Decreased: BCAA, a-hydroxybutyrate
Liraglutide	Urine	• Increased: glycine metabolites, 3-indoxyl-sulfate • Decreased: taurine
Vildagliptin	Urine	• Increased: glycine metabolites, 3-indoxyl-sulfate

Abreviations: BCAA: Branched-chain amino-acids; T-MAO: Trimethylamine-N-oxide.

Materials and methods

Subjects

The study was undertaken at the Outpatient Diabetes Unit of "University Hospital of Ioannina" in Ioannina, Greece. All subjects gave their written informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Hospital Scientific and Bioethics Committee. Patients with type 2 Diabetes Mellitus treated with metformin monotherapy and an HbA1c>7% were eligible for the study. In all participants, a full medical history was recorded including race, age, family history of diabetes mellitus, medication use and smoking habits. In addition, a detailed clinical examination including weight, height, BMI and blood pressure was performed. Patients with type 1 diabetes, eGFR <30 ml/min/m^2 and liver cirrhosis were excluded. Participants who fulfilled all inclusion criteria and did not fulfil any of the exclusion criteria were treated with either oral dapagliflozin 10 mg qd or subcutaneous insulin degludec for 3 months. In order to show that dapagliflozin altered the metabolomic profile irrespective of the reduction of glucose levels, insulin was selected as a comparator.

Fifty patients with type 2 diabetes mellitus were included in the dapagliflozin arm and received 10 mg qd and 30 patients receiving insulin degludec for 3 months were included. Insulin was titrated every 3 days to a target fasting plasma glucose levels of 100-120 mg/dl.

Samples

Venous fasting blood samples were drawn into EDTA-containing tubes (for whole blood samples) and in Vacutainer tubes for serum samples. The serum was separated by centrifugation at 1500× g for 15 min and an aliquot was stored at −80 °C until NMR analysis. Urine samples were collected and centrifuged at 1000 g for 10min and stored at −80°C until NMR analysis. Biochemical parameters were measured directly by standard laboratory methods. Urinary α1-microglobulin and immunoglobulin G levels were measured by immunonephelometry on a BN ProSpec System (Siemens, Marburg, Germany).

Determination of Biochemical Parameters

The serum levels of glucose and lipid parameters were measured on an AU5400 Clinical Chemistry analyzer (Beckman, Hamburg, Germany) by standard procedures. LDL-cholesterol was calculated by the Friedewald formula. HbA1c was measured in ion exchange HPLC system (Variant II, Bio-Rad Laboratories, Hercules, CA, USA).

^{1}H NMR Spectroscopy of Macromolecules-Free Serum

Serum samples contain a large amount of macromolecules (proteins and lipoproteins), which prevent the detection and quantification of low abundant and small metabolites in NMR spectroscopy. For this reason, we removed macromolecules from serum samples using the centrifugal filter devices Amicon Ultra-2 mL, 3-kDa cutoff (Merck KGaA, Darmstadt, Germany). Filter devices were first rinsed three times each with 500 µL distilled water (40 °C) followed by centrifugation (4000× g, 25 °C for 30 min) to remove glycerol preservatives. Then, serum samples (800 µL) were transferred to centrifugal filter tubes and centrifuged for 60 min at 4000× g. An aliquot of the filtrated serum samples (400 µL) was diluted with 200 µL of phosphate buffer (0.2 M Na2HPO4/0.2 M NaH2PO4, pH 7.4) and sodium 3-trimethylsilyl-(2,2,3,3-H4)-1-propionate (TSP) and transferred to 5 mm NMR tubes with a final concentration 0.456 mmol/L of TSP for the NMR measurements.

^{1}H NMR spectra of the serum samples were acquired on a 11.7 T Bruker Avance DRX NMR spectrometer (NMR Center, University of Ioannina) at a proton frequency of 500.13 MHz and at a constant temperature of 300 K. A Bruker standard 1D Nuclear Overhauser Enhancement Spectroscopy (NOESY) presaturation pulse sequence RD-90°-t1-90°-tm-90°-FID, with a mixing time of 0.1 s, an acquisition time of 3.28 s and a relaxation delay of 4 s was used for all NMR experiments to suppress the water signal. For each sample, the ^{1}H NMR spectrum was collected with 128 scans into 64K computer data points with a spectral width of 10.000 Hz. The free induction decays (FIDs) were multiplied with an exponential line broadening factor of 0.3 Hz prior to Fourier transformation. The phase and baseline of 1H NMR spectra were manually corrected by applying a simple polynomial curve fit with the Topspin software package version 4.0.6 (Bruker Biospin, Rheinstetten, Germany) and the chemical shifts were referenced to TSP (δ = 0.00 ppm). The identification of serum metabolites was based

on the available databases such as the Human Metabolome Database (HMDB; http://www.hmdb.ca (accessed on 16 November 2022)), the Biological Magnetic Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu (accessed on 16 November 2022)), the spectral reference libraries of Chenomx NMR Suite 8.4 (Edmonton, AB, Canada) software, J-res 2D experiments, and the existing NMR-based metabolomics literature.

Targeted Metabolite Profiling

For identification and quantification of the metabolites, processed NMR spectra of serum and urine samples were imported into Chenomx NMR Suite software (version 8.4, Chenomx, Edmonton, AB, Canada). The 500 MHz spectral reference library from Chenomx and the above-mentioned TSP of known concentration as an internal standard were used for the calculation of the quantitative values of metabolites expressed in micromoles per liter (μM).

Statistical Analysis

Data entry and analysis were performed with SPSS software (version 23.0; IBM Corp., Armonk, NY, USA). All data are expressed as mean value ± SD. Group comparison was performed using independent samples t-test and p value < 0.05 was considered to indicate statistical significance. Check for normality was done using the Kolmogorov-Smirnov test. The mean concentration of the various metabolic parameters before and after the administration of dapagliflozin was compared using Student's paired *t*-test for normally distributed values and Wilcoxon matched pairs test for values deviating from normal distribution. The Bonferroni correction was applied to account for multiple comparisons. Two-way repeated measures analysis of variance was used for the comparison of the effects of dapagliflozin and insulin degludec on the concentrations of the various metabolites. The correlations between these changes were assessed using linear regression analysis after log-transformation of the values that did not follow normal distribution. Independent *t*-test was used for the comparison of the percentage changes in the concentrations of urine metabolites in dapagliflozin and insulin groups.

Untargeted Metabolite Profiling

Serum metabolite concentrations data was normalized to reduce systematic variation (introduced by experimental conditions, sample preparation, and instrumental settings)

and Pareto scaled using Metaboanalyst v.6.0. Principal component analysis (PCA) and partial least squares discriminant analysis discriminant analysis (PLS-DA) were used to construct pattern recognition models. PCA was used to obtain a general overview on samples and highlight possible clusters, trends, or outliers followed by PLS-DA analysis to eliminate the uncorrelated systematic variation and describe the maximum separation based on class membership. The results of PLS-DA analysis displayed by scores (detection observations lying outside the 0.95 Hotteling's T2 ellipse, grouping trend, or separation) and loading coefficient plots (contribution of NMR spectral regions or variables, corresponding to metabolites. to the grouping trend or separation seen in the scores plot). PLS-DA models were assessed by goodness-of-fit parameters R^2 (R^2X and R^2Y) and Q^2, related, respectively, to the explained and predicted variance. Cross-validated coefficient of variation analysis of variance (CV-ANOVA) and permutation tests were used to assess the significance and validity of the resulting PLS-DA models, respectively (311). Finally, model validation was performed by constructing new PLS-DA models with 80% of randomly selected samples considered as a training set, whereas the remaining 20% of samples, named as a test set, was used to predict their class membership.

Results

Table 7. Demographics and baseline clinical characteristics

Patient's demographics and baseline clinical characteristics are shown in **table 7**. No significant between group difference is noted.

	Dapagliflozin (n=50)	**Insulin Degludec (n=30)**	**P (for group comparison)**
Sex (male/female) %	60/40	50/50	NS
Age (years)	60.4±8.4	63±7.7	NS
Diabetes duration (years)	4.2±1.4	5.7±2.3	NS
Weight (kg)	91.6±17.2	87.8±9.2	NS
BMI (kg/m^2)	32.5±5.5	29.7±1.1	NS
SBP (mmHg)	139.9±18	137±16	NS
DBP (mmHg)	82.1±12.2	79±10	NS

Abbreviations: BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure

Effect on Blood pressure and Weight after 3 months of treatment

Dapagliflozin administration led to a significant decrease in weight and BMI values. Furthermore, both systolic and diastolic blood pressure values were decreased following dapagliflozin administration. On the other hand, insulin administration did not alter any of the aforementioned values. Compared with insulin, dapagliflozin led to a significant decrease in weight, BMI, systolic and diastolic blood pressure **(table 8)**.

Table 8. Effect on Blood Pressure and Weight

Indice	**Start**	**After 3 months of treatment**	**Difference, absolute value**	**Change per cent**	**P (for the comparison with starting values)**	**P (for group comparison of difference)**	**P (for group comparison of starting values)**
Weight (kg)							
Group D	91.6±17.2	89.5±18.3	-2.1	-2.29%	<0.001	<0.01	NS
Group I	87.8±9.2	88.1±8.9	+0.3	+0.3%	NS		

BMI (kg/m^2)							
Group D	32.5±5.5	31.7±5.8	-0.8	-2.46%	<0.001	<0.01	NS
Group I	29.7±1.1	29.9±1.3	+0.2	+0.7%	NS		
Systolic BP (mmHg)							
Group D	139.9±18	128±14.9	-11.9	-8.5%	<0.001	<0.001	NS
Group I	137±16	136±13	-1	-0.7%	NS		
Diastolic BP (mmHg)							
Group D	82.1±12.2	73.8±14.9	-8.3	-10.1%	<0.001	<0.001	NS
Group I	79±10	80±8.3	+1	+1.3%	NS		

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; BMI: Body Mass Index; BP: Blood Pressure

Effect on serum glycemic indices

As expected, both dapagliflozin and insulin decreased significantly glucose and HbA1c levels. HOMA-IR was significantly decreased by dapagliflozin administration **(table 9)**.

Table 9. Effect on serum glycemic indices

Indice	Start	After 3 months of treatment	Difference, absolute value	Change, per cent	P (for the comparison with starting values)	P (for group comparison)	P (for starting values)
Glucose (mg/dl)							
Group D	170.4±54.2	141.6±35.3	-28.8	-16.5%	<0.001	NS	0.013
Group I	199.4±55.5	171.3±76.9	-28.1	-14.1%	0.04		
Insulin (µU/ml)							
Group D	15.3±16.6	15±19.8	-0.3	-2%	NS		
Group I							
HbA1c							
Group D	8.06±1.3	7.36±0.9	-0.7	-8.7%	<0.001	NS	0.006
Group I	9.2±2	8.2±1.2	-1	-10.9%	0.003		
HOMA-IR							
Group D	6.5±7.6	4.8±6.4	-1.7	-26.2%	0.023		
Group I							
HOMA-β							
Group D	61.5±74.1	75.5±103.5	+14	+22.8%	NS		
Group I							

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; HbA1c: Glycated Hemoglobin; HOMA-IR: Homeostatic Model Assessment-Insulin Resistance; HOMA-β: Homeostatic Model Assessment-β cell secretion

Effect on serum lipid indices

No significant changes were observed following dapagliflozin or IDeg administration on serum lipid indices, except for HDL-C levels which were increased significantly following dapagliflozin administration **(table 10)**.

Table 10. Effect on serum lipid indices

Indice	Start	After 3 months of treatment	Difference, absolute value	Change, per cent	P (for the comparison with starting values)	P (for group comparison)	P (for starting values)
T-CHOL (mg/dl)							
Group D	187.9±49.7	182.1±38.1	-5.8	-3.1%	NS	NS	NS
Group I	186.6±40.8	180.7±36.5	-5.9	-3.2%	NS		
TRGs (mg/dl)							
Group D	164.1±88.3	153.6±59	-10.5	-6.4%	NS	NS	NS
Group I	169±106.3	151.5±112.1	-17.5	-10.4%	NS		
HDL-C (mg/dl)							
Group D	44.1±9.2	46.3±10.6	+2.2	+5%	0.048	NS	NS
Group I	46.9±9.8	46.8±12.5	-0.1	-0.2%	NS		
LDL-C (mg/dl)							
Group D	109.3±40.9	101.7±31.3	-8.2	-7.5%	NS	NS	NS
Group I	105.9±33.8	104.3±26.7	-1.6	-1.5%	NS		
ApoA1 (mg/dl)							
Group D	148.3±20.8	148±21.2	-0.3	-0.2%	NS	NS	NS
Group I	136.9±37.1	140.1±42.1	+3.2	+2.3%	NS		
ApoB (mg/dl)							
Group D	85.5±27.7	85.3±17.8	-0.2	-0.2%	NS	NS	NS
Group I	87.3±28.7	83.1±23.9	-4.2	-4.8%	NS		
Lp(a)							
Group D	21.9±32.8	23.7±37.2	+1.8	+8.2%	NS	NS	NS
Group I	21.4±31.7	22±33	+0.6	+2.8%	NS		

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; T-CHOL: Total Cholesterol; TRGs: Triglycerides; HDL-C: High Density Lipoprotein Cholesterol; LDL-C Low Density Lipoprotein Cholesterol; ApoA1: Apolipoprotein A1; ApoB: Apolipoprotein B; Lp(a): Lipoprotein (a)

Effect on renal function and electrolytes

Dapagliflozin decreased serum urate levels, while on the other hand, serum magnesium and serum phosphate levels were increased following dapagliflozin administration. IDeg did not affect serum electrolytes or renal function **(table 11)**.

Table 11. Effect on renal function and electrolytes

Indice	**Start**	**After 3 months of treatment**	**Difference, absolute value**	**Change, per cent**	**P (for the comparison with starting values)**	**P (for group comparison)**	**P (for starting values)**
Urea (mg/dl)							
Group D	35.8±9.8	37.5±11.8	+1.9	+5.3%	NS	NS	0.003
Group I	47.2±18.1	45.8±15.9	-1.4	-3%	NS		
Creatinine (mg/dl)							
Group D	0.8±0.2	0.9±0.2	+1	+12.5%	0.013	NS	0.004
Group I	1±0.3	1±0.3	0	0%	NS		
eGFR (ml/min/1.73m^2)							
Group D	87.8±15	86.1±15.1	-1.7	-1.9%	NS	NS	NS
Group I	83.7±15.7	81.1±17.1	-2.6	-3.1%	NS		
UA (mg/dl)							
Group D	5.3±1.5	4.7±1.1	-0.6	-11.3%	<0.001	<0.05	NS
Group I	5.3±2.2	5±1.2	-0.3	-5.7%	NS		
Na^+(mmol/l)							
Group D	138.4±2.1	139.3±2.3	+0.9	+0.7%	0.005	NS	NS
Group I	137.6±2.4	138.7±1.9	+1.1	+0.8%	NS		
K^+ (mmol/l)							

Group D	4.5±0.4	4.5±0.4	0	0%	NS	NS	NS
Group I	4.5±0.5	4.7±0.4	+0.2	+4.4%	NS		
TCA (mg/dl)							
Group D	9.5±0.3	9.6±0.3	+0.1	+1.1%	0.04	0.017	NS
Group I	9.6±0.3	9.5±0.4	-0.1	-1%	NS		
Mg^{2+} (mg/dl)							
Group D	1.7±0.2	1.8±0.2	+0.1	+5.9%	0.003	<0.05	0.001
Group I	1.5±0.2	1.5±0.2	0	0	NS		
Cl^- (meq/l)							
Group D	101.9±1.9	102.1±2.3	+0.2	+0.2%	NS	NS	NS
Group I	101.6±3.3	102.1±3.6	+0.5	+0.5%	NS		
PO (mg/dl)							
Group D	3.5±0.6	3.8±0.5	+0.3	+8.6%	0.001	<0.05	0.006
Group I	3.1±0.5	3.2±0.7	+0.1	+3.2%	NS		

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; eGFR: estimated Glomerular Filtration Rate; UA: Uric Acid; TCA: Total Calcium; PO: Phosphate

Effect on other serum indices

Regarding other serum indices, dapagliflozin increased significantly hemoglobulin levels as expected. On the other hand, IDeg was not associated with significant changes in the shown indices **(table 12)**.

Table 12. Effect on other serum indices

Indice	Start	After 3 months of treatment	Difference, absolute value	Change, per cent	P (for the comparison with starting values)	P (for group comparison)	P (for starting values)
Hb (mg/dl)							
Group D	14.3±1.4	14.9±1.4	+0.6	+4.2%	<0.001	0.001	NS
Group I	13.9±1.4	13.9±1.4	0	0%	NS		
Hct (%)							
Group D	42.6±3.7	44.7±3.9	+2.1	+4.92%	<0.001	<0.001	NS

Group I	42.1±3.4	42.1±3.8	0	0%	NS		
ALT (IU/ml) **Group D** **Group I**	30.2±19 21.3±17.7	27.3±11.7 17.5±6.5	-2.9 -3.8	-9.6% -17.8%	NS NS	NS	0.027
ALP (IU/ml) **Group D** **Group I**	69.6±27 63.6±15.3	68.9±21.1 65.7±27.4	-0.7 +2.1	-1% +3.3%	NS NS	NS	NS

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; Hb: Hemoglobin; Hct: Hematocrit; ALT: Alanine transaminase; ALP: Alkaline Phosphatase

Effect on urine indices

Dapagliflozin increased urinary excretion of urate significantly. On the other hand, no other significant changes were observed following either dapagliflozin or insulin administration **(table 13)**.

Table 13. Effect on urine indices

Indice	Start	After 3 months of treatment	Difference, absolute value	Change, per cent	P (for the comparison with starting values)	P (for group comparison)	P (for group comparison of starting values)
FEUA (%) **Group D** **Group I**	7.2±2.5 8.9±6.7	8.5±3.6 7.2±2.3	+1.3 -1.7	+11.3% -19.1%	0.03 NS	NS	NS
$FENa^+$ (%) **Group D** **Group I**	0.6±0.4 0.7±0.4	0.6±0.3 0.7±0.4	0 0	0% 0%	NS NS	NS	NS
FEK^+ (%) **Group D** **Group I**	9.2±4.1 12.1±5.2	10.5±4.3 13.9±5.3	+1.3 +1.8	+14.1% +14.9%	NS NS	NS	NS
FETCA (%) **Group D** **Group I**	1±0.7 0.9±0.7	1±0.9 0.9±0.6	0 0	0% 0%	NS NS	NS	NS

$FEMg^{2+}$ (%)							
Group D	2.2±1.3	2.4±1.4	+0.2	+9.1%	NS	NS	0.013
Group I	2±1.1	2±1.1	0	0	NS		
$FECl^{-}$ (%)							
Group D	1±0.5	1.1±0.5	+0.1	+10%	NS	NS	NS
Group I	1.3±0.8	1.3±0.7	0	0%	NS		
FEPO (%)							
Group D	11.7±4.5	11±5.5	-0.7	-6%	NS	NS	NS
Group I	8.6±4.2	9.7±4.8	+1.1	+12.8%	NS		

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; FE: Fractional Excretion; UA: Uric Acid; TCA: Total Calcium; PO: Phosphate

Serum untargeted metabolic profiling

For the untargeted analysis, the data set consisted of the concentrations of the serum metabolites derived from the spectra of 50 patients with type 2 diabetes before and after receiving dapagliflozin for 3 months and 30 patients with type 2 diabetes before and after insulin degludec administration for 3 months. Prior to multivariate analysis, metabolite concentration data were normalized on Metaboanalyst v.6.0 to improve the overall consistency of the experimental data. An unsupervised (PCA) was first applied to obtain a data overview for the detection of any group trend or potential outliers (located outside the 95% confidence region of the model), (data not shown). The supervised multivariate method PLS-DA was applied in the dapagliflozin group and a good separation with a degree of overlap between the groups was observed in the score plots of the PLS-DA model (**Figure 1**). The most influential metabolites on group separation are shown on **Figure 2** (VIP scores >1). The separation is estimated by the two quality parameters, R2 = 0.62 for the explained variation and Q2 = 0.2 for the predictive capability of the resulting model (**Figure 3a**). In addition, permutation tests (n = 1000 repeats) were performed, and the observed statistical p value (p=0.008) also confirms the validity of the model (**Figure 3b**). Pathway analysis revealed that significant changes in taurine, tryptophan and threonine metabolism were identified (**Figure 4**). Other pathways were less influenced by dapagliflozin administration.

The results in the untargeted analysis were modestly in line with those found in the targeted analysis. The observed discrepancies were mainly attributed to the high values of SD in the targeted analysis.

Figure 1. Scores plot in dapagliflozin group

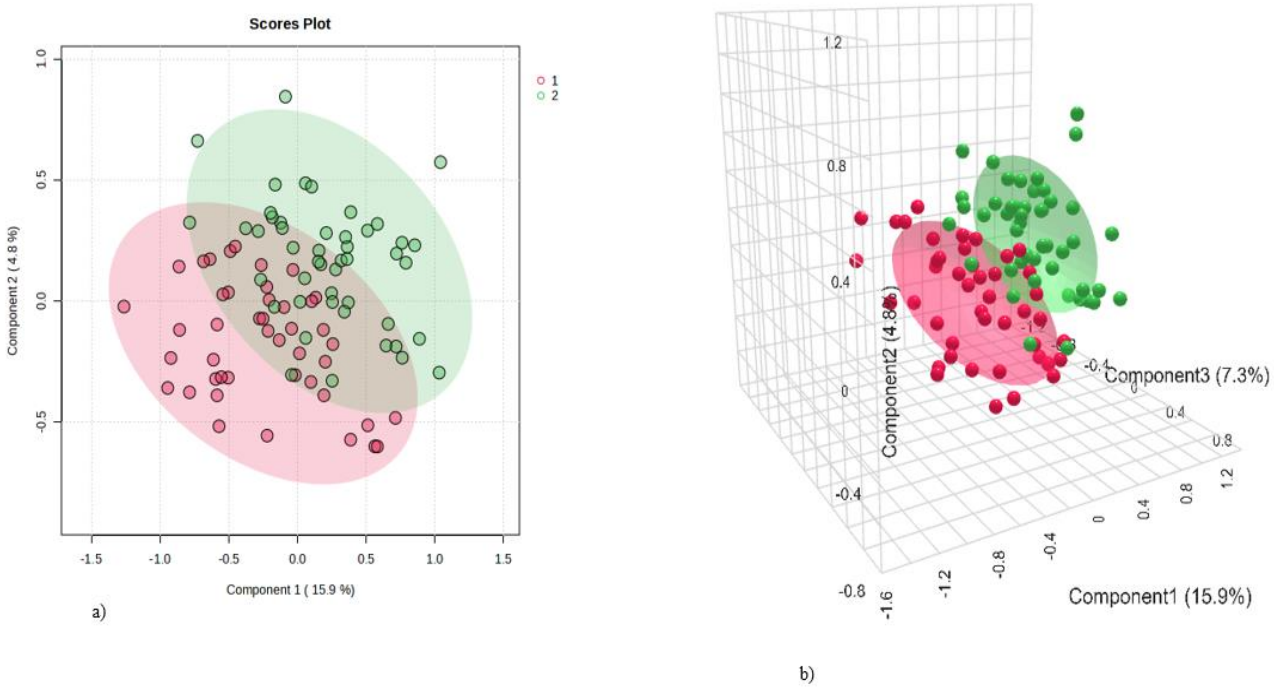


Figure 1: The PLS-DA multivariate analysis obtained for the 50 patients before (red dots and circle) and after treatment (green dots and circle). a) 2-D and b) 3-D PLS-DA Score Plot. Group separation is clearly shown in both 2-D and 3-D figures.

Figure 2. Relative influence of metabolites in group separation in dapagliflozin group

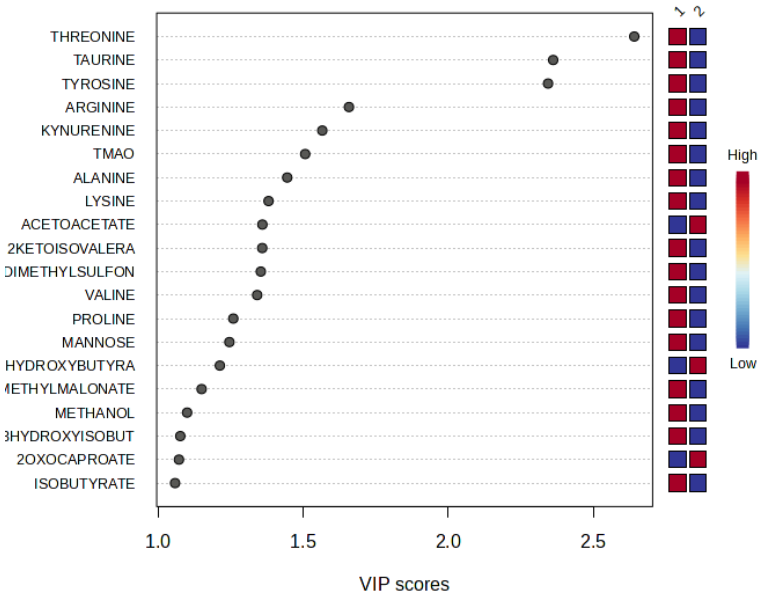
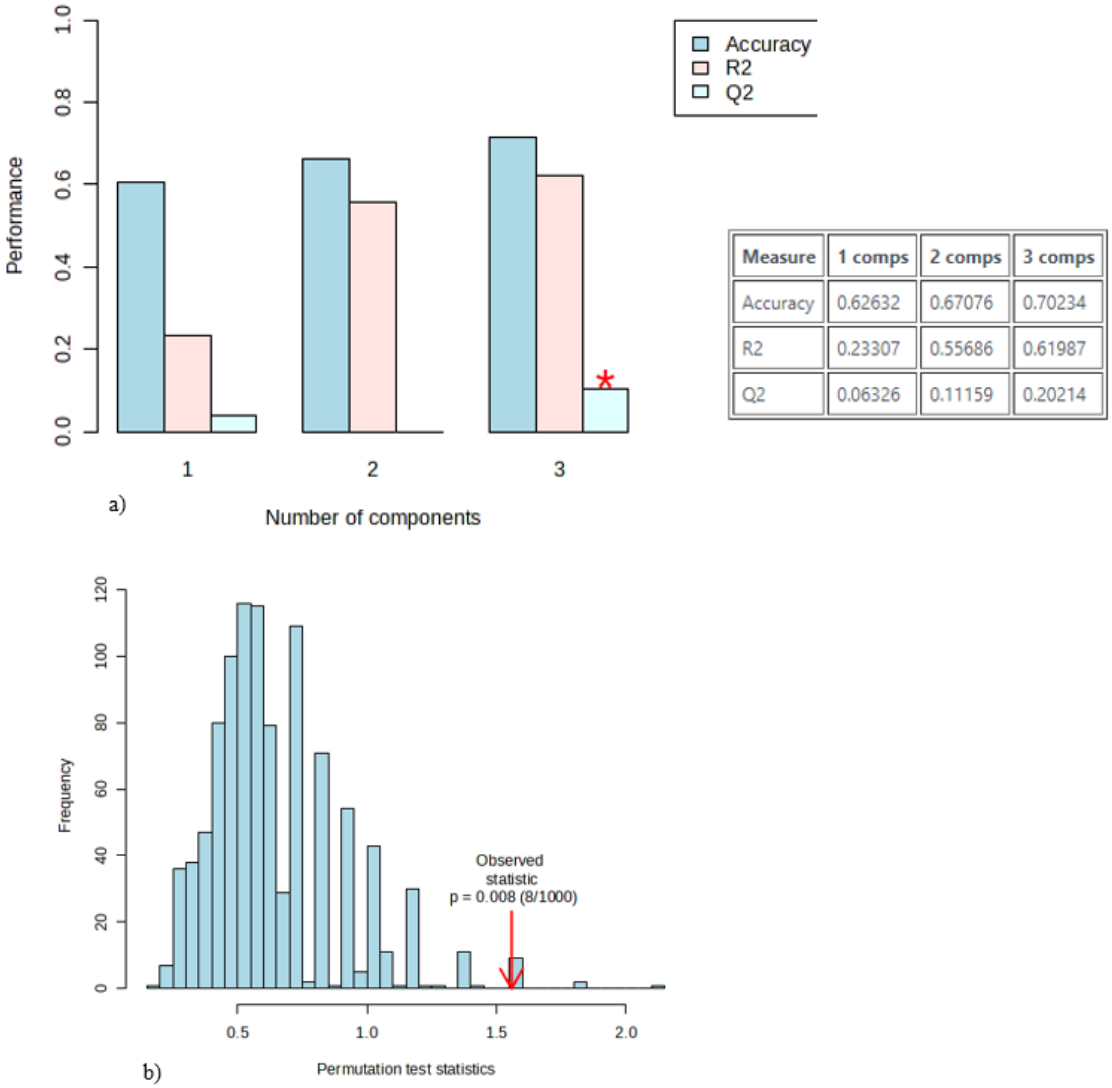


Figure 2: Relative influence of the shown metabolites on group separation (VIP scores>1).

Figure 3. PLS-DA cross validation and permutation tests in dapagliflozin group



Measure	1 comps	2 comps	3 comps
Accuracy	0.62632	0.67076	0.70234
R2	0.23307	0.55686	0.61987
Q2	0.06326	0.11159	0.20214

Figure 3a: PLS-DA cross validation quality parameters.

Figure 3b: Permutation tests (n=1000 repeats) which confirms the validity of the model.

Figure 4: Pathway analysis in dapagliflozin group

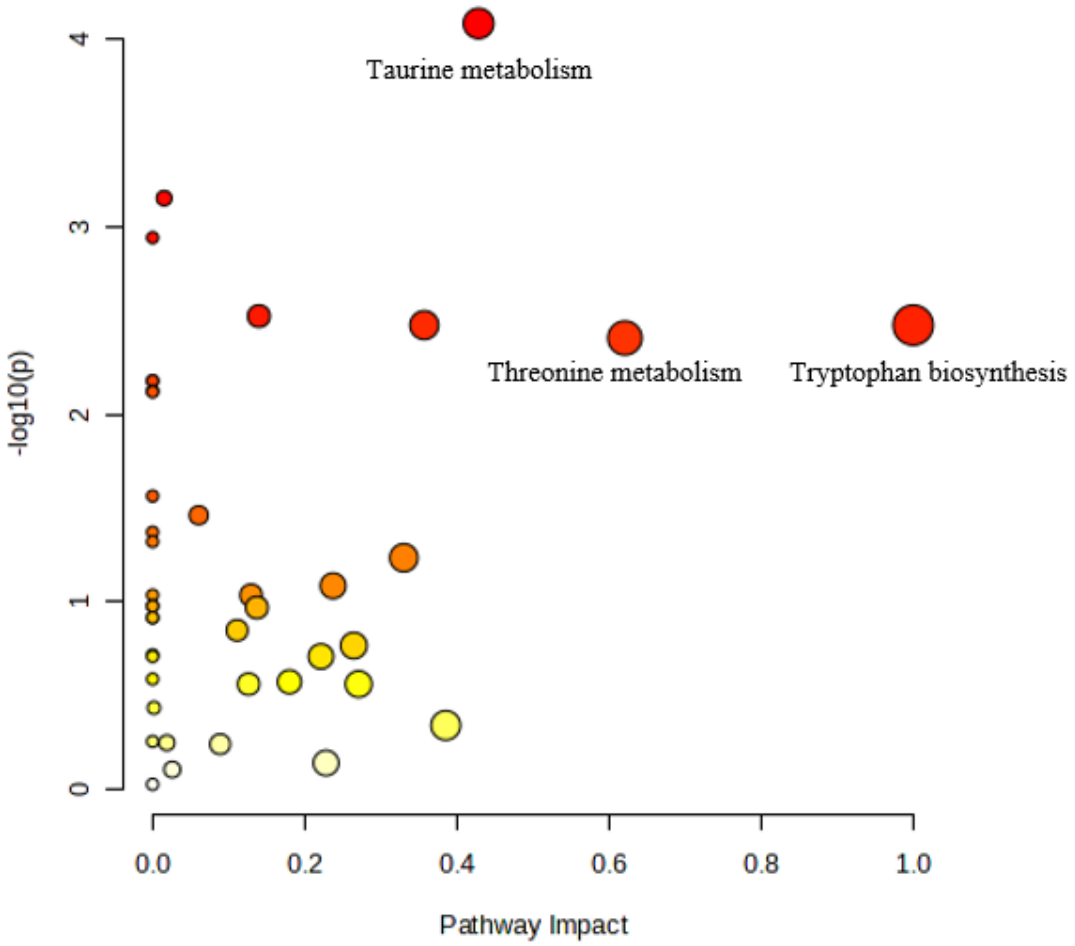


Figure 4: Pathway analysis of patients treated with dapagliflozin showing that taurine metabolism, threonine metabolism and tryptophan biosynthesis are significantly altered by dapagliflozin.

Furthermore, untargeted metabolomic analysis of patients treated with insulin degludec, failed to show significant changes in the metabolome. The supervised multivariate method PLS-DA was applied and a poor separation between the groups was observed in the score plots of the PLS-DA model **(Figure 5a)**. Indeed, permutation analysis showed that insulin degludec did not significantly affect serum metabolome composition **(Figure 5b).**

Figure 5. Scores plot and permutation tests in insulin group

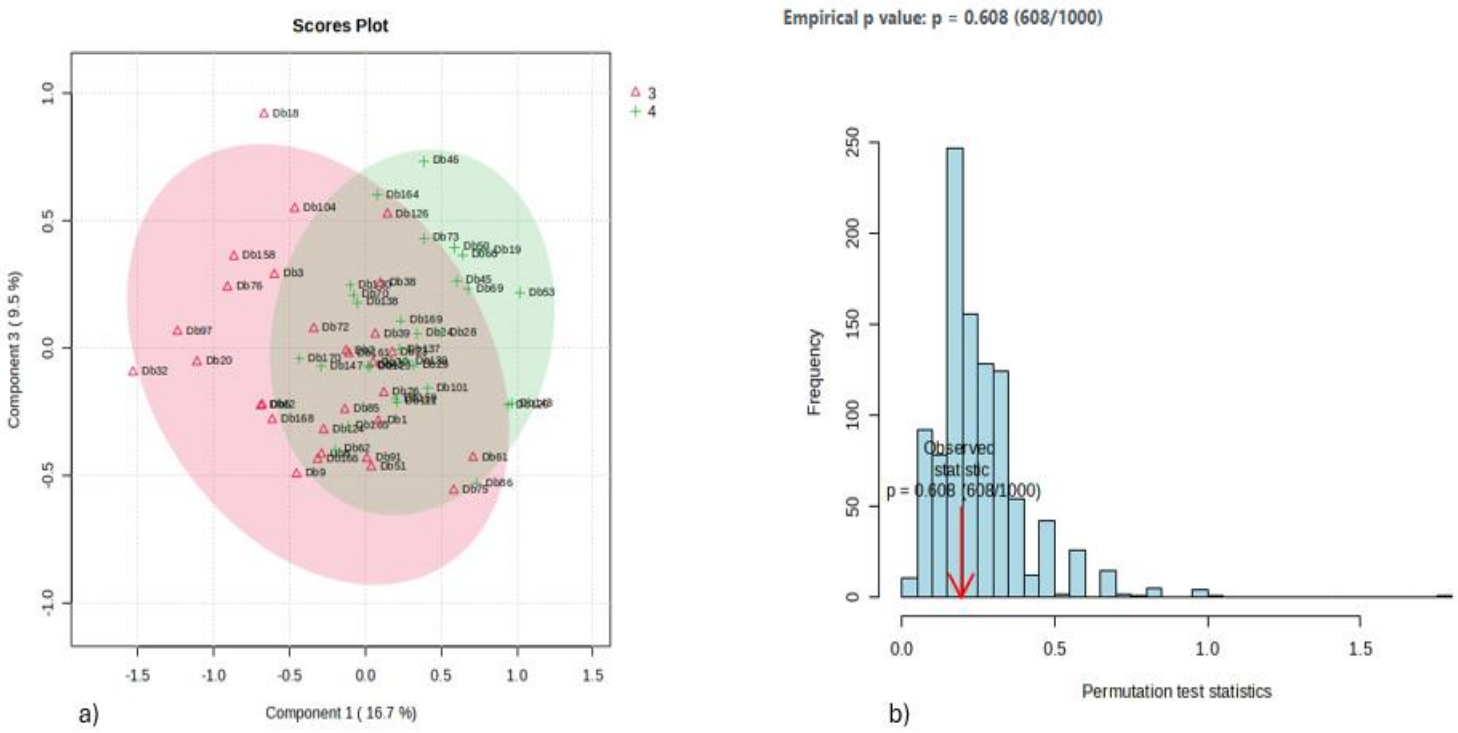


Figure 5: a) Scores plot of patients that received insulin degludec showing poop separation between pre and post insulin administration. **b)** Permutation test of patients receiving insulin showing that pre and post insulin groups do not separate significantly.

Urine untargeted metabolic profiling

For the untargeted analysis the data set consisted of the 1-dimensional ^{1}H-NMR spectra of the urine derived from all patients before and after the administration of dapagliflozin and insulin degludec. PCA was initially applied to obtain a general overview. No remarkable outliers were observed in the scores plot whereas a separation trend appeared between diabetic patients before and after treatment with dapagliflozin. In the OPLS-DA scores plot of the untargeted metabolic profile, the groups before and after the administration of dapagliflozin were well separated with a small degree of overlap, with the samples of diabetic patients before treatment placed on the right half of the plot, and those after treatment, on the left half. The separation between the 2 groups assessed by the following quality parameters of the resulting OPLS-DA model: R^2X = 0.819, R^2Y = 0.627, and Q^2Y = 0.362, and the CV-ANOVA *P*-value was <0.001. As

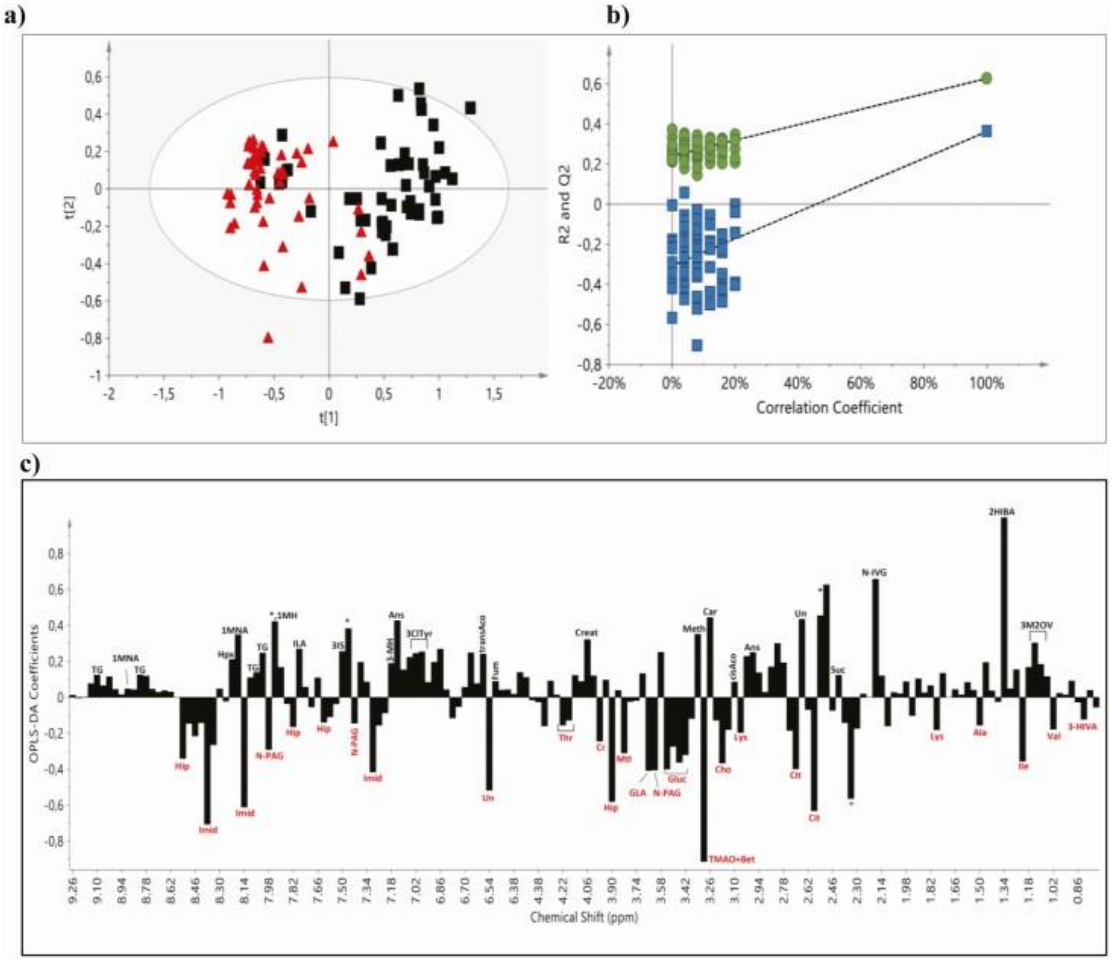
shown in , the OPLS-DA model had a R^2Y intercept of 0.242 and a Q^2Y intercept value of −0.306, indicating that the resulting statistical model is valid.

The OPLS-DA loading coefficient plot, which depicts the main spectral regions contributing to group discrimination, yielded a rather large number of metabolites (**Figure 6C**). In addition to marked glycosuria, posttreatment samples presented higher levels of hippurate, citrate, trimethylamine N-oxide (TMAO) and betaine, isoleucine, choline, gluconate, and N-phenylacetylglycine, as well as lower levels of 2-hydroxyisobutyrate, carnitine, N-isovaleroylglycine, trigonelline, methanol, and anserine as compared to those before treatment. Higher excretion of mannitol, creatine, and valine and lower excretion of 3-methyl-2-oxovalerate, 3-methylhistidine, trans-aconitate, and hypoxanthine made a smaller contribution to the group separation.

To test the reliability of the OPLS-DA model between diabetic patients before and after treatment with dapagliflozin, validation was carried out. The corresponding training (40 patients before/40 patients after treatment) and test (10 patients before/10 patients after treatment) sets were randomly selected, and the validation was repeated 3 times with a new random selection of equally numbered sets each time. The average classification rate was 86.67% for patients before treatment (first repeat: 9 out of 10; second repeat: 9 out of 10; and third repeat: 8 out of 10) and 90% for those after treatment (first repeat: 8 out of 10; second repeat: 9 out of 10; and third repeat: 10 out of 10.

PCA was applied and no distinct grouping was identified between the patients before and after the administration of insulin degludec in the scores plot. OPLS-DA scores also did not show significant separation between the 2 groups with low goodness-of-fit parameters ($R^2X = 0.937$, $R^2Y = 0.414$, and $Q^2Y = -0.109$, and the CV-ANOVA *P*-value was >0.05).

Figure 6.



(A) OPLS-DA and validation **(B)**of the corresponding OPLS-DA models via permutations tests (n = 100) of the urine metabolic data from patients with type 2 diabetes mellitus before (black squares) and 3 months after (red triangles) administration of dapagliflozin. The regression line of Q^2 (goodness of prediction, blue squares) with intercept at −0.306 and R^2 (goodness of fit, green circles) regression line intercept at 0.242 indicate that the resulting OPLS-DA model has higher R^2 (goodness of fit) and Q^2 (goodness of prediction) values in the validation test than the permutated models generated. **(C)** The corresponding OPLS-DA regression coefficient plot of the urine metabolites, upper (black): higher before and down (red): higher after treatment. Abbreviations: 1MH, 1-methylhistidine; 1MNA, 1-methylnicotinamide; 2HIBA, 2-hydroxyisobutyrate; 3ClTyr, 3-chlorotyrosine; 3HIVA, 3-hydroxyisovalerate; 3IS, 3-indoxylsulfate; 3M2OV, 3-methyl-2-oxovalerate; 3MH, 3-methylhistidine; Ala, alanine; Ans, anserine; Bet, betaine; Car, carnitine; Cho, choline; cisAco, cis-aconitate; Cit, citrate; Cr, creatine; Creat, creatinine; Fum, fumarate; GLA, gluconate; Gluc, glucose; Hip, hippurate; Hpx, hypoxanthine; ILA, indole-3-lactate; Ile, isoleucine; Imid, imidazole; Lys, lysine; Mtl, mannitol; Meth, methanol; N-IVG, N-isovaleroylglycine; N-PAG, N-phenylacetylglycine; Suc, succinate; TG, trigonelline; Thr, threonine; TMAO, trimethylamine N-oxide; transAco, trans-aconitate; Val, valine; *, drug metabolites; Un, unknown.

Serum targeted metabolic profiling (table 14)

64 metabolites were identified in the 1H NMR spectra of patients before and after dapagliflozin administration and quantified using the Chenomx NMR Suite 8.4 software. The concentration values for each group are summarized in the subsequent tables. Glucose and Creatinine levels were shown earlier and will not be shown here.

The results show that 8 of the 62 shown metabolites were significantly altered by the administration of dapagliflozin. Compared with baseline values, dapagliflozin administration led to significantly elevated levels of the ketone bodies 3-hydroxybutyrate, acetoacetate and acetone, of the aromatic amino acid tryptophan and of the organic acid citrate. Significantly lower in patients treated with dapagliflozin were the levels of threonine which is involved in one-carbon metabolism, of taurine and of mannose.

Table 14. Serum targeted metabolic profiling

Indice	Start	After 3 months of treatment	Difference, absolute value	Change, per cent	P (for the comparison with starting values)	P (for the comparison between groups)	P (for starting values)
2-aminobutyrate (µM)							
Group D	18.8±8.4	20±6.6	+1.2	+6.4%	NS	-	-
Group I	-	-	-	-	-		
2-hydroxybutyrate (µM)							
Group D	91.3±33.5	94.4±25.5	+3.1	+3.4%	NS	0.006	NS
Group I	98.9±38.1	76.8±23.9	-22.1	-22.3%	0.006		
2-Hydroxyisovalerate							
Group D	11.2±4.6	11.2±4.3	0	0	NS	NS	NS
Group I	12.5±6.6	12.1±6.3	-0.4	-3.2%	NS		

2-Keto-isovalerate (µM)							
Group D	10.2±1.5	10.2±1.9	0	0	NS	-	-
Group I	-	-	-	-	-		
2-oxocaproate (µM)							
Group D	4.2±1.9	4.7±2.2	+0.5	+17.2%	NS	-	-
Group I	-	-	-	-	-		
2-oxoglutarate (µM)							
Group D	16.5±8	17.8±10	+1.3	+7.9%	NS	-	-
Group I	-	-	-	-	-		
2-oxoisocaproate (µM)							
Group D	15.4±3.8	16.2±4.1	+0.8	+5.2%	NS	NS	0.034
Group I	13.3±4.9	11.6±2.7	-1.7	-12.8%	NS		
3-hydroxy-butyrate (µM)							
Group D	78.8±44	101.8±62.6	+23	+29.2%	0.007	0.006	0.02
Group I	145.9±146.3	84.2±47.9	-61.7	-42.3%	0.036		
3-hydroxyisobutyrate (µM)							
Group D	10.4±3.6	10.1±2.9	-0.3	-2.9%	NS	NS	<0.001
Group I	21.6±7	19.3±7.6	-2.3	-10.6%	NS		
3-hydroxyisovalerate (µM)							
Group D	5.9±3.4	6.5±3.6	+0.6	+10.2%	NS	-	-
Group I	-	-	-	-	-		
3-methyl-2-oxovalerate (µM)							
Group D	14.2±4.3	14.3±4.2	+0.1	+1%	NS	NS	0.003

Group I	19.3±8.1	18.6±7.5	-0.7	-3.6%	NS		
Acetate (µM)							
Group D	69.8±23.8	72.3±26.2	+2.5	+3.6%	NS	NS	<0.001
Group I	50.4±22.3	48.4±13.8	-2	-4%	NS		
Acetoacetate (µM)							
Group D	23.2±17.1	30.8±25.3	+7.6	+32.8%	0.016	0.010	0.004
Group I	52.3±42.4	32.7±14	-19.6	-37.5%	0.049		
Acetone (µM)							
Group D	38±29.2	50.8±44.7	+12.8	+33.7%	0.023	0.004	NS
Group I	29.8±19.1	20.1±8	-9.7	-32.6%	0.017		
Alanine (µM)							
Group D	576.9±121	585.6±125.8	+8.7	+1.5%	NS	NS	NS
Group I	567.2±182.7	559.7±116.2	-7.5	-1.3%	NS		
Arginine (µM)							
Group D	75.9±20.1	71.3±14.1	-4.6	-6.1%	NS	-	-
Group I	-	-	-	-	-		
Asparagine (µM)							
Group D	58.7±13.5	61.5±17.8	+2.8	+4.8%	NS	NS	NS
Group I	64±23.6	65.4±17.1	+1.4	+2.2%	NS		
Aspartate (µM)							
Group D	46.1±15.4	44.9±13	-1.2	-2.6%	NS	-	-
Group I	-	-	-	-	-		
Betaine (µM)							
Group D	77.4±38.8	69.9±30.1	-7.5	-9.7%	NS	NS	0.002
Group I	56.6±18.4	61.4±20.8	+4.8	+4.9%	NS		
Carnitine (µM)							
Group D	51.6±14.2	51.9±15.6	+0.3	+0.6%	NS	NS	0.004
Group I	41.6±14.6	44.1±12.7	+2.5	+6%	NS		
Choline (µM)							
GroupD	17.9±5.2	19.6±9.7	+1.7	+9.5%	NS	NS	NS
Group I	18.1±6.9	21.2±7.5	+3.1	+17.3%	NS		
Citrate (µM)							
Group D	182.6±45	195±41.8	+12.4	+6.8%	0.023	NS	0.009

Group I	214.4±60.4	217.7±57.7	+3.3	+1.5%	NS		
Creatine (µM)							
Group D	47.5±21.7	50.3±28.4	+2.8	+5.9%	NS	NS	NS
Group I	38.4±20.4	37.6±16.5	-0.8	-2.1%	NS		
Dimethylamine (µM)							
Group D	6.8±4.2	7.2±3.8	+0.4	+5.9%	NS	NS	<0.001
Group I	3.2±1.3	4.1±1.2	+0.9	+28.1%	0.001		
Dimethylsulfone (µM)							
Group D	11.3±6.6	9.9±5.4	-1.4	-12.4%	NS	-	-
Group I	-	-	-	-	-		
Ethylene glycol (µM)							
Group D	54.2±59.7	55.4±64.5	+1.2	+2.2%	NS	-	-
Group I	-	-	-	-	-		
Formate (µM)							
Group D	34.7±11.1	36.7±13.6	+2	+5.8%	NS	NS	NS
Group I	33.5±16.9	35.7±15.2	+2.2	+6.6%	NS		
Glutamate (µM)							
Group D	153.1±42.2	161.6±51.2	+8.5	+5.6%	NS	NS	NS
Group I	142.3±58.8	171±71.4	+28.7	+20.2%	NS		
Glutamine (µM)							
Group D	470.9±68	460.6±83.9	-10.3	-2.2%	NS	NS	NS
Group I	501.1±135.6	502.2±71.5	+1.1	+0.2%	NS		
Glycine (µM)							
Group D	400.2±106.3	415.6±114.3	+15.4	+3.8%	NS	NS	<0.001
Group I	283.7±87.2	312.9±64.8	+29.2	+10.3%	NS		
Histidine (µM)							
Group D	86±16.1	86.8±21.1	+0.8	+0.9%	NS	NS	NS
Group I	87.1±28.1	84.3±25.5	-2.8	-3.2%	NS		
Homocysteine (µM)							
Group D	40.8±10.6	43±11.5	+2.2	+5.4%	NS	-	-

Group I	-	-	-	-	-		
Hypoxanthine (µM)							
Group D	15.7±6	16.6±6.4	+0.9	+5.7%	NS	NS	NS
Group I	15.7±7.4	19.8±6.6	+4.1	+26.1%	0.027		
Inosine (µM)							
Group D	2.9±2.1	3.3±2.9	+0.4	+13.8%	NS	-	-
Group I	-	-	-	-	-		
Isobutyrate (µM)							
Group D	11.1±4.5	10.7±3	-0.4	-3.6%	NS	-	-
Group I	-	-	-	-	-		
Isoleucine (µM)							
Group D	103.3±19.7	106.1±25.3	+2.8	+2.7%	NS	NS	<0.001
Group I	73.8±24	69.6±22.8	-4.2	-5.7%	NS		
Kynurenine (µM)							
Group D	3±1.1	2.8±1	-0.2	-6.6%	NS	-	-
Group I	-	-	-	-	-		
Lactate (µM)							
Group D	3889.7±1142	3983.8±1173.8	+94.1	+2.4%	NS	NS	<0.001
Group I	2797.3±984	3187.8±914	+390.5	+14%	NS		
Leucine (µM)							
Group D	193±37.9	200.4±44.6	+7.4	+3.8%	NS	NS	<0.001
Group I	131.2±36	125.1±35.2	-6.1	-4.9%	NS		
Lysine (µM)							
Group D	211.4±39.1	209.9±36.8	-1.5	-0.7%	NS	NS	<0.001
Group I	165.1±42	172±40.9	+6.9	+4.2%	NS		
Malonate (µM)							
Group D	17.6±11.2	18.7±11.4	+1.1	+6.3%	NS	-	-
Group I	-	-	-	-	-		
Mannose (µM)							
Group D	122.4±49.1	105.7±35.5	-16.7	-13.6%	0.018	0.026	NS
Group I	135.2±53	130±49.6	-5.2	-3.8%	NS		
Methanol (µM)							

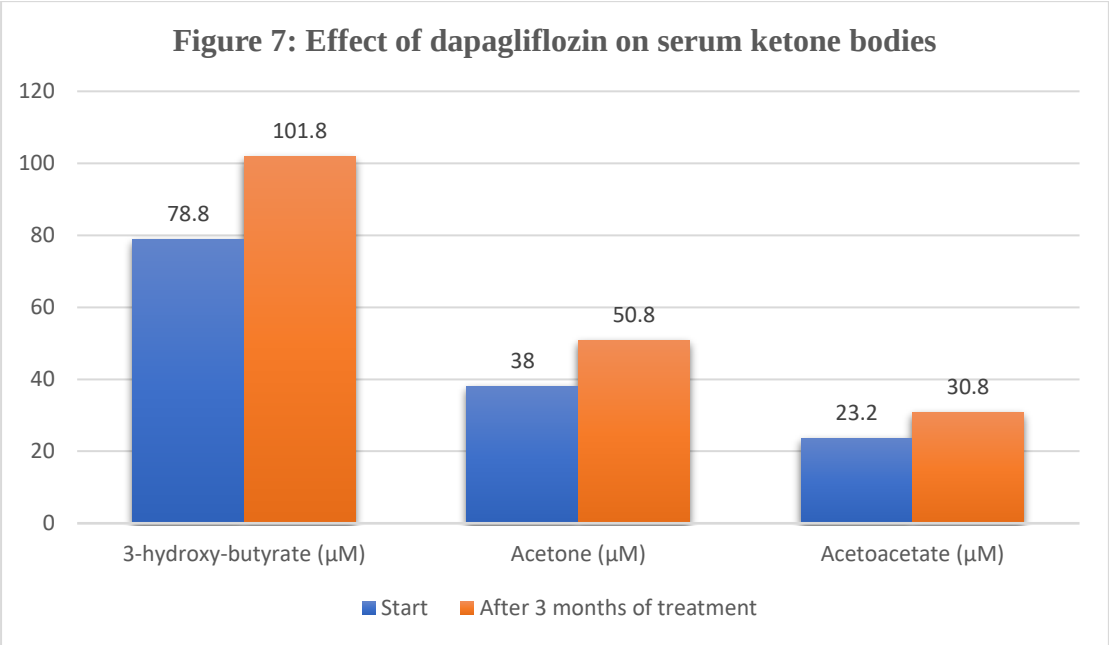
Group D	215.2±107.1	198.7±114.3	-16.5	-7.7%	NS	-	-
Group I	-	-	-	-	-		
Methionine (µM)							
Group D	39.8±8.1	40.1±9.3	+0.3	+0.8%	NS	NS	NS
Group I	36.1±11.6	36.3±8.3	+0.2	+0.6%	NS		
Methyl-malonate (µM)							
Group D	29.1±7.5	28.9±7.7	-0.2	-0.7%	NS	-	-
Group I	-	-	-	-	-		
Myo-Inositol (µM)							
Group D	54.2±9.3	53.1±11.4	-1.1	-2%	NS	-	-
Group I	-	-	-	-	-		
N-Methyl-hydantoin (µM)							
Group D	1.5±0.5	1.5±0.5	0	0	NS	-	-
Group I	-	-	-	-	-		
Dimethylglycine (µM)							
Group D	4.6±1.8	4.9±1.6	+0.3	+6.5%	NS	NS	NS
Group I	4.3±2.2	4.5±2.4	+0.2	+4.7%	NS		
O-Acetylcarnitine (µM)							
Group D	11.3±3.3	12.1±3.9	+0.8	+7.1%	NS	NS	NS
Group I	12.3±3.8	11.2±3.6	-1.1	-8.9%	NS		
Ornithine (µM)							
Group D	67.3±25.6	71.8±22.5	+4.5	+6.7%	NS	NS	NS
Group I	55.9±28.5	66.9±27	+11	+19.7%	NS		
Phenylalanine (µM)							
Group D	74.3±17.6	75±18.3	+0.7	+0.9%	NS	NS	0.04
Group I	83.6±22.1	85.9±26	+2.3	+2.8%	NS		
Proline (µM)							
Group D	234.2±91.1	229.7±75.2	-4.5	-1.9%	NS	NS	0.003

Group I	305.9±115.3	317.1±107.3	+11.2	+3.7%	NS		
Pyruvate (µM)							
Group D	104.8±50.5	101.4±47.7	-3.4	-3.2%	NS	NS	NS
Group I	97.6±52.6	87.9±33.7	-9.7	-9.9%	NS		
Sarcosine (µM)							
Group D	2.7±1.3	2.9±1.3	+0.2	+1.1%	NS	NS	0.011
Group I	3.7±1.7	3.8±1.2	+0.1	+2.7%	NS		
Serine (µM)							
Group D	214.5±28.4	217.3±34.9	+2.8	+1.3%	NS	NS	<0.001
Group I	107.6±41.1	123.1±32	+15.5	+14.4%	0.020		
Succinate (µM)							
Group D	11.2±4.6	12.1±6.8	+0.9	+8%	NS	NS	0.045
Group I	9.3±2.7	10.4±2.4	+1.1	+11.8%	NS		
Taurine (µM)							
Group D	173±35	156.8±32.8	-16.2	-9.4%	0.005	<0.001	NS
Group I	168.4±71.7	205.9±52.4	+37.5	+22.7%	0.012		
Threonine (µM)							
Group D	185.6±52.5	167.7±40.1	-17.9	-9.6%	0.015	0.036	NS
Group I	177.6±53.8	184.9±51.5	+7.3	+4.1%	NS		
TMAO (µM)							
Group D	32.7±14.3	30.2±14.7	-2.5	-7.6%	NS	NS	<0.001
Group I	55.7±26.3	44.7±18.4	-11	-19.7%	NS		
Tryptophan (µM)							
Group D	19.7±5.5	23.1±8.9	+3.4	+17.3%	0.015	0.039	NS
Group I	16.4±7.3	18.4±7.3	+2	+12.2%	NS		
Tyrosine (µM)							
Group D	89.8±19.6	85.2±19.9	-4.6	-5.1%	NS	NS	<0.001
Group I	67.9±23.6	65±22.4	-2.9	-4.3%	NS		
Valine (µM)							
Group D	356.2±64.1	358.7±76.7	+2.5	+0.7%	NS	NS	<0.001
Group I	241±65.1	233.9±51.7	-7.1	-2.9%	NS		

Abbreviations: Group D: Group Dapagliflozin; Group I: Group Insulin Degludec; TMAO: Trimethylamine-N-Oxide

Effect on serum ketone bodies

Dapagliflozin administration led to a significant increase in all serum ketone body levels by approximately 30% (3-hydroxy-butyrate, acetone, acetoacetate). On the other hand, insulin decreased ketone levels **(figure 7)**. The difference between the two groups was statistically significant in all ketones tested.



Effect on serum BCAAs and metabolites

On the other hand, serum BCAAs and their metabolites were not affected by dapagliflozin or insulin administration.

Effect on serum AAAs

Only serum tryptophan levels were significantly increased following dapagliflozin administration. Phenylalanine and tyrosine (the other two aromatic amino acids) were not altered. Insulin administration did not alter the levels of aromatic amino acids. Between group comparison showed that dapagliflozin significantly increased serum tryptophan levels compared with insulin.

Effect on other amino acids

Regarding other amino acids, only threonine levels were significantly decreased following dapagliflozin administration. Arginine levels were marginally decreased (albeit not significantly). Insulin administration led to significant increase of serum serine levels, while other amino acids were not significantly altered by insulin administration.

Effect on 1-C metabolism

Dapagliflozin administration led to a significant decrease of serum taurine levels. Other metabolites of one-carbon metabolism were not affected by dapagliflozin. On the other hand, insulin significantly decreased serum 2-hydroxybutyrate levels both compared to initial levels and to dapagliflozin. Furthermore, insulin significantly decreased serum hypoxanthine levels, although it did not reach statistical significance when compared with dapagliflozin administration.

Effect on other metabolites

Regarding other metabolites, dapagliflozin led to significant increase of citrate level and to a significand decrease of mannose levels. Other metabolites were not affected by dapagliflozin administration. Dimethylamine was the only metabolite significantly increased by insulin administration. No other metabolites were significantly affected by insulin.

Urine targeted metabolic profiling (table 15)

For the targeted analysis, 70 urine metabolites were quantified in the urine of the patients before and after dapagliflozin and insulin degludec treatment as well as of the control group. At baseline, patients with type 2 diabetes in both dapagliflozin and insulin groups had higher concentrations of 1-methylhistidine, 2-aminobutyrate, 2-hydroxy-3-methylvalerate, 3-chlorotyrosine, 3-hydroxybutyrate, 3-indoxylsulfate, 3-methyl-2-oxovalerate, 3-methyladipate, 3-methylhistidine, 4-hydroxybenzoate, acetoacetate, alanine, betaine, creatine, creatine phosphate, glucose, isoleucine, lactate, leucine, lysine, myoinositol, N-isovaleroylglycine, N-methylhydantoin, N-phenylacetylglycine, phenylalanine, tyrosine, and valerate compared to controls. The levels of butyrate, ethylmalonate, methanol, and sarcosine were significantly lower in these groups compared to those in the control group. We also observed differences in the urine concentrations of some metabolites between the dapagliflozin and insulin

groups at baseline. So, the concentrations of 1-methylnicotinamide, 2-hydroxy-3-methylvalerate, 2-hydroxyisobutyrate, 2-hydroxyisovalerate, 2-hydroxyvalerate, 3-hydroxybutyrate, 3-indoxylsulfate, 3-methyl-2-oxovalerate, phenylalanine, and valine were lower in the insulin group compared to the corresponding values in the dapagliflozin group, while that of 2-hydroxybutyrate was higher.

Our results show that 27 of the 70 quantified metabolites were significantly altered by dapagliflozin treatment: 2-aminobutyrate, 2-hydroxy-3-methylvalerate, 2-hydroxybutyrate, 2-hydroxyisovalerate, 3-hydroxybutyrate (+21.2%, $P < 0.0005$), 3-hydroxyisovalerate, acetoacetate (+18.5%, $P < 0.0005$), alanine, betaine (+54.9%, $P < 0.0001$), citrate (+18.4%, $P < 0.0005$), creatine, ethylmalonate, gluconate, glucose (+723.6%, $P < 0.0001$), hippurate, lactate, leucine (+22.2%, $P < 0.0005$), myo-inositol (+77.1%, $P < 0.0001$), N,N-dimethylglycine (+233.8%, $P < 0.0001$), N-methylhydantoin, sarcosine, trigonelline, and valine (+35.1%, $P < 0.0001$) were increased significantly. The metabolites whose concentration was significantly decreased after treatment were 3-chlorotyrosine (−30.5%, $P < 0.0001$), anserine, methanol, and N-isovaleroylglycine.

The results in the targeted analysis are nearly totally consistent with those found in the untargeted multivariate analysis. With both methods, all metabolites are altered to the same direction and most of them with similar significance. We observed discrepancy only in 2 metabolites, TMAO and N,N-dimethylglycine, due to the high values of SD.

The increases in the urinary excretion of the BCAAs were significantly correlated with the corresponding increase in the degree of glycosuria after dapagliflozin administration (Pearson correlation coefficients 0.346, 0.339, and 0.467 for leucine, isoleucine, and valine, respectively; $P < 0.05$ for all correlations). In addition, the baseline values of betaine and myo-inositol were highly correlated with urine concentration of glucose (Pearson correlation coefficients 0.511 and 0.658 for betaine and myo-inositol, respectively; $P < 0.001$ for all correlations) whereas the increase in the urine concentrations of these compounds following dapagliflozin administration was also significantly correlated with the drug-induced increase in glycosuria (Pearson correlation coefficients 0.341 and 0.397 for betaine and myo-inositol, respectively; $P < 0.001$ for all correlations).

Although insulin degludec improved glycemia to a degree similar to that observed with dapagliflozin, it had no effect on the levels of the majority of urine metabolites. In addition to a reduction in glycosuria, insulin degludec reduced the urine concentrations of 3-hydroxybutyrate, acetoacetate, betaine, lactate, and myo-inositol. Two-way analysis for time points revealed that all the statistically significant dapagliflozin-induced changes in urine metabolites were also different from the effect of insulin on the levels of these metabolites. In addition, although the changes in the concentrations of 3-methyl-2-oxovalerate (−18.4%), hypoxanthine (−18.9%), and N-phenylacetylglycine (+13.1%) after dapagliflozin treatment did not reach statistical significance, these alterations were found to be significantly different from those observed after insulin administration by 2-way analysis.

Table 15. Urine targeted metabolic profiling

	Dapagliflozin baseline values	**Insulin baseline values**	**% change, Dapagliflozin group**	***P***	**% change, Insulin group**	***P***	**2 way analysis (F)**	***P***
1-methylnicotinamide	2.72 ± 1.70	1.60 ± 0.49*	−6.6	NS	+15	NS	0.91	NS
1-methylhistidine	26.54 ± 14.14	13.18 ± 10.11	+2.4	NS	+17.7	NS	0.14	NS
2-aminobutyrate	6.46 ± 2.83	5.85 ± 4.34	+14.4	<0.0005	+6.6	NS	8.92	<0.05
2-hydroxy-3-methylvalerate	11.46 ± 6.34	5.56 ± 2.91*	+17.3	<0.0005	+10.2	NS	4.12	<0.05
2-hydroxybutyrate	1.20 ± 1.00	3.61 ± 1.64*	+30	<0.0005	+22.2	NS	3.71	<0.05
2-hydroxyisobutyrate	8.80 ± 3.08	4.31 ± 2.56*	−7.6	NS	+5.8	NS	1.61	NS
2-hydroxyisovalerate	4.72 ± 1.62	1.79 ± 1.22**	+14.5	<0.0005	−1.1	NS	4.37	<0.05
2-hydroxyvalerate	7.54 ± 6.29	3.33 ± 2.28*	+2.9	NS	+16.7	NS	0.06	NS

2-oxocaproate	2.7 ± 2.03	4.45 ± 3.72	+0.2	NS	−13.1	NS	0.71	NS
3-chlorotyrosine	24.17 ± 17.02	20.25 ± 19.27	−30.5	<0.0001	+4.9	NS	3.90	<0.05
3-hydroxybutyrate	46.00 ± 41.78	30.46 ± 20.02*	+21.2	<0.0005	−43.1	<0.0005	7.25	<0.05
3-hydroxyisobutyrate	12.34 ± 10.96	13.50 ± 12.91	+15.2	NS	−11.1	NS	2.28	NS
3-hydroxyisovalerate	4.78 ± 2.83	3.40 ± 2.53	+18	<0.0005	+5.3	NS	3.44	<0.05
3-indoxylsulfate	30.6 ± 17.61	21.17 ± 10.35*	−4.4	NS	+11.3	NS	0.69	NS
3-methyl-2-oxovalerate	12.23 ± 7.54	9.33 ± 4.88*	−18.4	NS	−10.6	NS	4.32	<0.05
3-methyladipate	7.32 ± 6.45	6.65 ± 4.34	+19.1	NS	+8.5	NS	0.92	NS
3-methylhistidine	27.16 ± 17.85	22.15 ± 19.22	−1.8	NS	+5.7	NS	0.54	NS
4-hydroxybenzoate	5.90 ± 5.76	5.50 ± 3.97	+7.9	NS	+40.1	NS	2.64	NS
Acetate	28.07 ± 9.25	20.86 ± 11.68	−1.3	NS	+23.5	NS	1.06	NS
Acetoacetate	43.68 ± 16.68	36.05 ± 18.06	+18.5	<0.0005	−45.2	<0.0005	36.68	<0.01
Acetone	3.35 ± 2.10	5.49 ± 4.72	+14	NS	−10.7	NS	1.05	NS
Alanine	44.63 ± 30.47	40.45 ± 24.93	+26.5	<0.0001	+13.1	NS	3.42	<0.05
Allantoin	8.55 ± 5.18	6.64 ± 5.58	+7.2	NS	+7.3	NS	0.25	NS
Anserine	9.76 ± 4.51	8.64 ± 6.32	−19.4	<0.0005	−8.3	NS	4.58	<0.05

Betaine	72.87 ± 48.04	76.92 ± 42.12	+54.9	<0.0001	−58.3	<0.0005	9.22	<0.01
Butyrate	1.69 ± 0.98	1.05 ± 0.84	+15.4	NS	+2.6	NS	1.20	NS
Carnitine	6.75 ± 5.85	6.73 ± 7.33	−15.9	NS	−10.4	NS	0.02	NS
Choline	6.69 ± 4.78	5.35 ± 4.08	+13.5	NS	+11.2	NS	0.07	NS
cis-Aconitate	16.56 ± 8.73	15.27 ± 7.84	−10.9	NS	+9.2	NS	3.02	NS
Citrate	357.30 ± 233.27	373.18 ± 273.63	+18.4	<0.0005	−10.5	NS	3.85	<0.05
Creatine	49.65 ± 31.64	50.69 ± 37.23	+40.3	<0.0001	+2.3	NS	4.22	<0.05
Creatine phosphate	155.92 ± 188.61	143 ± 127.89	+1.6	NS	−6.1	NS	0.04	NS
Cytosine	3.35 ± 2.59	6.34 ± 5.58	+1.2	NS	−9.6	NS	0.95	NS
Dimethylamine	24.67 ± 10.92	27.23 ± 14.85	+0.1	NS	+18.5	NS	0.68	NS
Ethylmalonate	1.64 ± 0.97	1.92 ± 1.34	+33.5	<0.0001	+4.6	NS	6.52	<0.01
Formate	22.92 ± 11.72	21.45 ± 21.31	−4.8	NS	+24.2	NS	0.56	NS
Fumarate	1.00 ± 0.72	1.23 ± 1.12	−2.0	NS	−14.6	NS	0.66	NS
Gluconate	45.55 ± 25.23	37.58 ± 26.88	+33.26	<0.0005	+7.2	NS	5.42	<0.05
Glucose	2273 ± 5551	1175 ± 217	+723.6	<0.0001	−68.1	<0.0005	31.18	<0.01
Glycine	121.35 ± 78.74	86.65 ± 76.54	−7.7	NS	+5.8	NS	0.05	NS

Hippurate	327.06 ± 259.02	300.01 ± 349.25	+33.3	<0.0005	+7.7	NS	6.33	<0.05
Hypoxanthine	5.55 ± 5.05	4.41 ± 4.76	−18.9	NS	+19.5	NS	5.88	<0.05
Indole-3-acetate	6.01 ± 4.11	9.35 ± 7.18	−3.3	NS	+8.2	NS	0.65	NS
Indole-3-lactate	13.03 ± 6.27	9.35 ± 8.42	−8.5	NS	+5.6	NS	0.58	NS
Isoleucine	6.46 ± 3.62	9.88 ± 6.43	+13.3	NS	−9.2	NS	2.85	NS
Kynurenate	2.86 ± 2.13	4.17 ± 2.84	+32.1	NS	+28.2	NS	0.74	NS
Lactate	12.93 ± 8.43	11.16 ± 7.02	+116	<0.0001	−25.2	<0.0005	9.82	<0.01
Leucine	11.27 ± 4.31	9.04 ± 9.03	+22.2	<0.0005	−8.5	NS	4.88	<0.05
Lysine	57.45 ± 17.51	46.83 ± 47.27	+7.2	NS	−7.3	NS	1.30	NS
Mannitol	72.21 ± 41.17	83.22 ± 117.08	+7.7	NS	−11.3	NS	0.23	NS
Methanol	23.31 ± 12.45	16.09 ± 13.63	−22.7	<0.0005	+18	NS	6.10	<0.05
Methylamine	5.52 ± 3.86	4.92 ± 4.42	−4.3	NS	−7.11	NS	0.10	NS
Myo-inositol	87.61 ± 71.31	82.88 ± 68.71	+77.1	<0.0001	−32.5	<0.0005	14.49	<0.01
N,N-dimethylglycine	4.47 ± 3.60	3.54 ± 3.11	+233.8	<0.0001	+1.4	NS	13.92	<0.01
N-isovaleroylglycine	3.39 ± 1.91	2.13 ± 1.54	−17.7	<0.0005	+13.6	NS	4.89	<0.05
N-methylhydantoin	20.83 ± 9.15	22.44 ± 21.34	+16	<0.0005	+2.9	NS	4.32	<0.05

N-phenylacetylglycine	49.64 ± 30.41	58.90 ± 43.36	+13.1	NS	−12.1	NS	4.81	<0.05
Phenylalanine	46.72 ± 39.10	30.19 ± 28.36*	−5.2	NS	+38.1	NS	2.25	NS
Pyruvate	5.56 ± 3.17	5.05 ± 3.20	−3.9	NS	−3.9	NS	0.06	NS
Sarcosine	1.66 ± 0.88	1.98 ± 1.53	+109	<0.0005	−14.1	NS	56.31	<0.01
Succinate	12.49 ± 5.89	13.99 ± 12.4*	+5.8	NS	−2.3	NS	0.03	NS
Threonine	36.61 ± 21.63	29.20 ± 22.91	+10.4	NS	+10.5	NS	0.01	NS
trans-Aconitate	4.24 ± 3,81	2.94 ± 1.91	−24.5	NS	+0.7	NS	2.41	NS
Trigonelline	35.69 ± 28.77	43.02 ± 19.15	+96.1	<0.0001	+7.6	NS	22.37	<0.01
Trimethylamine	3.79 ± 1.91	4.34 ± 4.13	−2.6	NS	+3.7	NS	0.22	NS
Trimethylamine N-oxide	36.38 ± 37.80	36.63 ± 28.06	−16.8	NS	+2.9	NS	0.47	NS
Tyrosine	19.11 ± 7.38	15.81 ± 14.69	+9.4	NS	−2.1	NS	0.29	NS
Valerate	20.96 ± 11.73	15.95 ± 13.00	−28.72	NS	+1.2	NS	1.99	NS
Valine	8.56 ± 4.10	3.11 ± 1.91**	+35.1	<0.0001	+19.2	NS	4.45	<0.05
Xanthurenate	18.17 ± 12.75	12.15 ± 28.24	+11.8	NS	+28.2	NS	1.23	NS

* $P < 0.0005$ and ** $P < 0.0001$ compared to dapagliflozin group baseline values.

Discussion

Effect on Blood pressure and Weight

In the present study we found that dapagliflozin decreased significantly body weight, BMI and systolic and diastolic blood pressure. On the other hand, insulin had a neutral effect on the aforementioned parameters. Furthermore, compared with insulin, dapagliflozin decreased significantly both systolic and diastolic blood pressure. As already mentioned, dapagliflozin induced glucosuria leads to increased caloric loss, while also reducing plasma volume by approximately 5-10% (45). Dapagliflozin administration inhibits SGLT-2 in the proximal renal tubule thus reducing glucose reabsorption from the glomerular filtrate with a concomitant reduction in sodium reabsorption leading to urinary glucose excretion and osmotic diuresis (173). Therefore, delivery of sodium to the distal tubule is increased which in turn increases the tubuloglomerular feedback and reduces intraglomerular pressure. This, combined with osmotic diuresis, leads to a reduction in volume overload and reduced blood pressure (174).

Effect on serum glycemic indices

Both dapagliflozin and insulin, as expected, reduced serum glucose and HbA1c levels, according to their mechanism of action. No significant difference was noted between the two groups. Dapagliflozin significantly decreased HOMA-IR index indicating reduced insulin resistance following dapagliflozin administration.

Effect on serum lipid indices

We found a small increase in HDL-C levels following dapagliflozin administration, whereas no change was observed in other lipid indices with either dapagliflozin, or insulin. The decrease of excreted insulin along with improvement in insulin sensitivity may explain the small increase in HDL-C levels following dapagliflozin administration (312).

Effect on serum renal function and electrolytes

We found that dapagliflozin increased significantly the levels of creatinine, sodium, phosphorus, magnesium and calcium whereas dapagliflozin decreased significantly the levels of uric acid. The increased concentration of glucose in the tubular lumen produced by SGLT-2 inhibition stimulates uric acid excretion mediated by GLUT9

isoform 2 and inhibits uric acid reabsorption mediated by GLUT9 isoform 2 in the collecting duct (182). These, lead to increased uric acid excretion and thus a reduction in serum uric acid levels. An initial, transient increase in serum creatinine levels is to be expected when starting SGLT-2 inhibitors due to the increased delivery of sodium to the distal tubule which in turn increases the tubuloglomerular feedback and reduce intraglomerular pressure (173). Serum phosphate levels increase due to the drug-induced reduction of proximal sodium transport which leads to an increased availability of sodium to be reabsorbed with phosphate via the Na+–PO4 3– co-transporters at the proximal tubules (74). Similar findings have been reported with serum magnesium, although hemoconcentration may also play a role in our findings.

Effect on other serum indices

Hemoconcetration due to plasma volume reduction as well as a direct effect towards increased erythropoietin production explain the increase in hemoglobin and hematocrit levels observed with dapagliflozin (175).

Effect on urine indices

As already mentioned, urine uric acid levels increase during dapagliflozin treatment. In line with these previous studies, we found that dapagliflozin increased significantly uric acid excretion.

Serum metabolomics

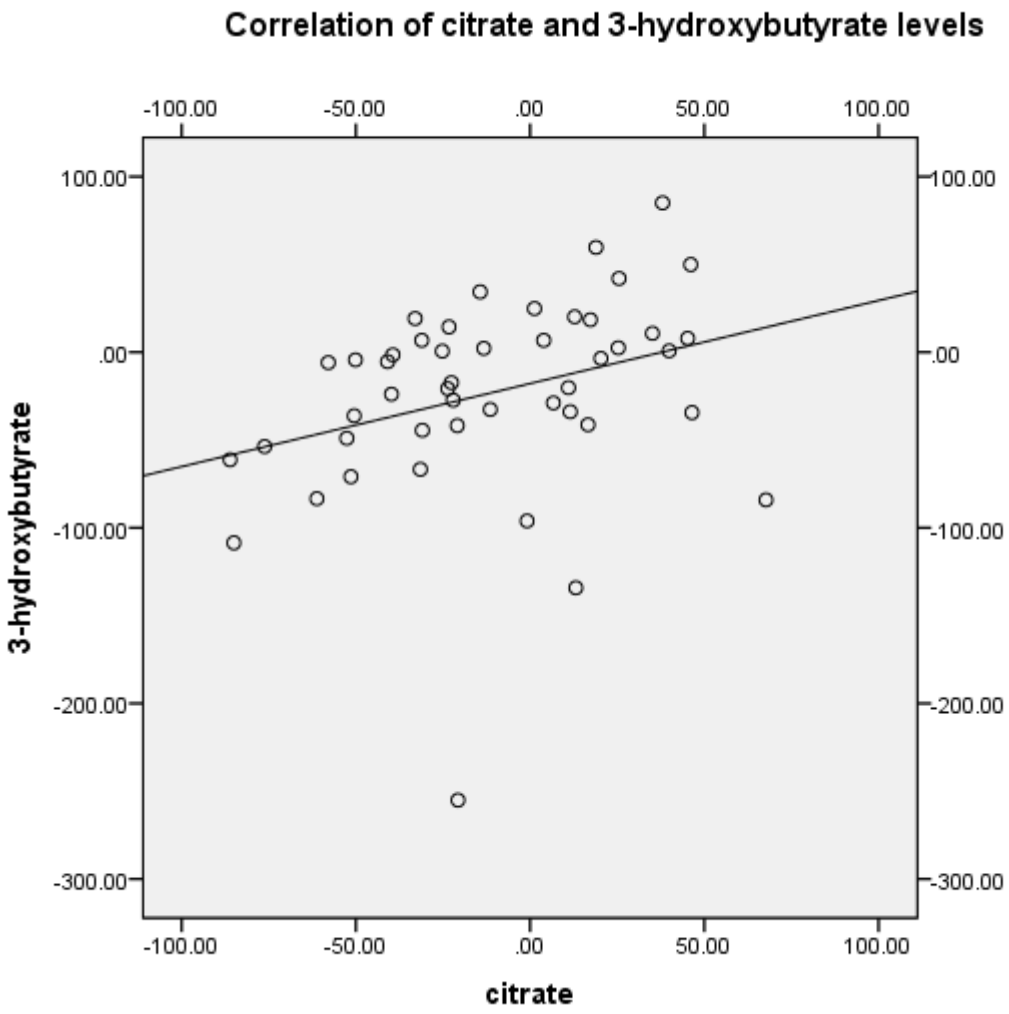
In the present study, we employed NMR-based metabolomic analysis to characterize the serum metabolic profile of patients with type 2 diabetes before and after receiving dapagliflozin for 3 months. The macromolecules-free-serum samples used, allowed enriched information based on the 64 quantified metabolites, a more reliable multivariate statistical analysis and, more importantly, a characterization of the affected metabolic pathways in these patients. Our results suggest that the metabolic pathways affected in this population are ketone body metabolism, one-carbon metabolism, energy production (citrate), taurine and mannose. In order to show that dapagliflozin altered the metabolomic profile irrespective of the reduction of glucose levels, a comparator group of patients that received insulin degludec, with the same level of glucose reduction, was used. Indeed, insulin administration did not affect the metabolites that were affected by dapagliflozin, except that insulin decreased serum ketone levels, as expected by its physiologic function.

In this study, we found that dapagliflozin increased serum ketone bodies (3-hydroxy-butyrate, acetone, acetoacetate) by approximately 30%. This is in line with previous studies which showed that SGLT-2 inhibitors increase modestly serum ketone bodies (126). Furthermore, we showed that dapagliflozin increases urine ketone levels (313). The increase observed in the serum may explain the increased levels which were found in the urine as well, even though no significant correlation was found between serum and urine ketone levels. Dapagliflozin inhibits glucose reabsorption in the kidneys leading to glucosuria. The fall of blood glucose in this way is sensed as a fasting state which in turn leads to decreased insulin secretion from pancreatic β-cells and increased glucagon secretion from pancreatic α-cells. Adding to that, there may be a direct effect of SGLT-2 inhibitors on the pancreatic α-cell to increase glucagon secretion. The aforementioned changes, lead to increased lipolysis in adipose tissue and increased free fatty acid flux to the liver (314). The increased free fatty acid flux and the reduced glucose utilization due to increased glucagon/insulin ratio lead to increased ketone body production in the liver (115, 315). The increased ketones observed may have a dual role in the cardiovascular and renal protective effects of SGLT-2 inhibitors. First, it has been proposed that ketones consist a more efficient fuel to the failing heart compared with free fatty acids and glucose (126). In a study in rat hearts, (316), empagliflozin led to a switch of metabolism from glucose utilization to ketone utilization irrespective of available substrate. Furthermore, it has been proposed that increased ketone levels lead to an adaptive response of cardiomyocytes, endothelial cells and other tissues that ultimately lead to the production of enzymes involved in ROS detoxification, DNA repair, proper protein folding during endoplasmic reticulum stress, autophagy and regeneration (317).

We have already found that dapagliflozin increased urine excretion of citrate. Furthermore, previous studies in diabetic and nondiabetic models of chronic kidney disease revealed significant disruption of mitochondrial function that parallels the evolution of the disease and is translated into abnormal renal excretion of citric acid cycle (tricarboxylic acid) intermediates (318, 319). Here, we also found that dapagliflozin significantly increased serum citrate levels, and that the increase in citrate levels, correlated significantly with the increase in 3-hydroxybutyrate levels (r^2=0.315, p=0.031), **figure 8**, and correlated strongly with the increase in acetoacetate levels (r^2=0.277, p=0.06). However, no correlation was found between serum and urine citrate

levels. In an animal model of HFpEF, dapagliflozin increased cardiac β-hydroxybutyrate levels in association with increased serum β-hydroxybutyrate levels. Citrate synthase is the crucial enzyme which catalyzes the condensation of oxaloacetate with acetyl CoA to form citrate. In this study, (315) citrate synthase activity was increased by dapagliflozin administration leading to improved ATP formation and reducing the acetyl-CoA pool. In accordance with the previous study, our finding that dapagliflozin increased serum citrate levels suggests that dapagliflozin improves whole body energy production.

Figure 8.

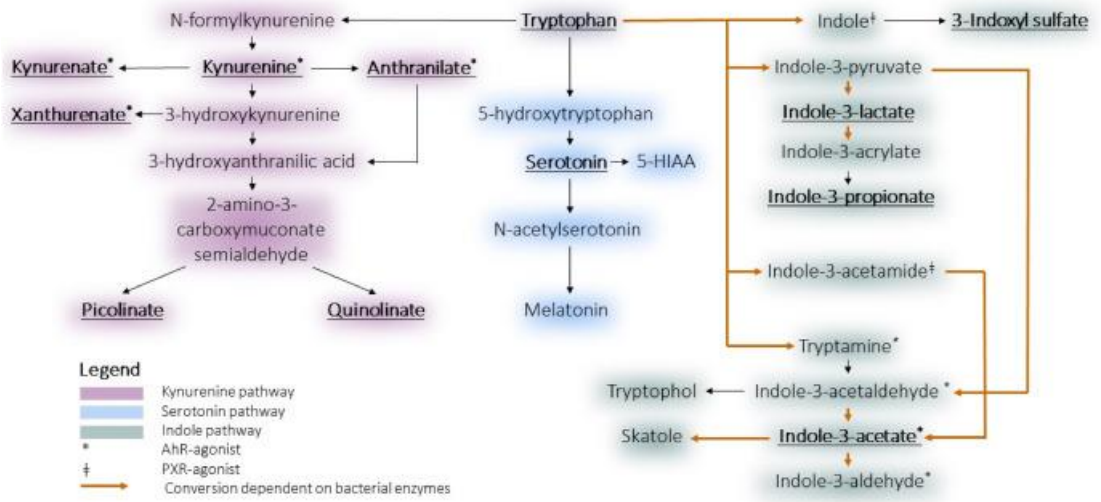


Tryptophan (Trp) is an alpha-amino acid and is one of 20 proteinogenic amino acids. It is classified as a non-polar, uncharged (at physiological pH) aromatic amino acid. Tryptophan is an essential amino acid. Tryptophan is the precursor of both serotonin

and melatonin. Metabolism of tryptophan into serotonin requires nutrients such as vitamin B6, niacin, and glutathione. Niacin (also known as vitamin B3) is an important metabolite of tryptophan. It is synthesized via kynurenine and quinolinic acids, which are products of tryptophan degradation (226, 230, 236). In mammals, Trp metabolism **(figure 9)** is predominantly divided into three major pathways: the kynurenine pathway (95%), the indole pathway (5%) and the serotonin/melatonin pathway (1–2%) (320). The rate-limiting step in the formation of tryptophan metabolites is the activity of the enzyme indoleamine 2,3-dioxygenase (IDO). IDO expression is strongly upregulated by proinflammatory molecules such as IFN-γ and interleukins, further increasing concentrations of downstream metabolites (321). IDO activity can be indirectly estimated as an increased [kynurenine]/[tryptophan]-ratio ([Kyn]/[Trp]-ratio), which is explained by a higher conversion rate of tryptophan into kynurenine (322). Previous studies have indicated that this increased conversion results in decreased levels of tryptophan (323, 324). Increased tryptophan levels have been correlated with increased risk of developing type 2 diabetes (236). In a recent study, higher tryptophan levels were associated with a lower incidence of stroke and ischemic heart disease over the ensuing 19.5 ± 5.9 years of follow-up after correction for cardiovascular risk factors and CRP (325), whereas elevated kynurenine levels, as well as a higher [Kyn]/[Trp]-ratio, were associated with a greater risk of developing CVD. Other studies have also reported that higher kynurenine levels and/or higher kynurenine/tryptophan ratio were associated with CVD (326, 327, 328). Furthermore, higher Kyn/Trp ratio has been correlated with poorer glycemic control in type 2 diabetes (329).

In our study we found that dapagliflozin administration increased serum tryptophan levels by 17.3%. Furthermore, we found that dapagliflozin decreased Kyn/Trp ratio ($p=0.025$). As the decreased Kyn/Trp ratio is an indication of decreased IDO activity, we speculate that dapagliflozin increased serum tryptophan levels by decreasing IDO activity. The decrease in IDO activity can be attributed to decreased proinflammatory molecules induced by dapagliflozin. If this can be attributed to the increased ketone body production and subsequent activation of anti-inflammatory genes should be further investigated.

Figure 9. Tryptophan metabolism



Threonine (Thr) is an essential amino acid in humans. In proteins, the threonine residue is susceptible to numerous posttranslational modifications. Threonine is metabolized in at least two ways. In many animals it is converted to pyruvate via threonine dehydrogenase. An intermediate in this pathway can undergo thiolysis with CoA to produce acetyl-CoA and glycine. In humans the gene for threonine dehydrogenase is an inactive pseudogene, so threonine is converted to alpha-ketobutyrate which is then converted to 2-hydroxybutyrate (330). In our study, dapagliflozin decreased serum levels of threonine, without significantly affecting serum levels of 2-hydroxybutyrate or 2-aminobutyrate which are known products of threonine catabolism. In our study, dapagliflozin led to increased urine levels of these catabolic products a finding indicating increased tubular secretion or decreased reabsorption or alternatively, increased renal synthesis of these compounds. Decreased levels of threonine may reflect increased urinary excretion due to SGLT-2 inhibition. Indeed, increased fractional excretion of Threonine was found (+41.2%, p=0.033) indicating increased urinary threonine loss which in turn leads to decreased serum threonine levels.

D-Mannose (also called Mannose or D-mannopyranose) is a hexose or a six-carbon sugar. It is also classified as an aldohexose. It is a fermentable monosaccharide and an isomer of glucose. Formally, D-Mannose is the 2-epimer of glucose and exists primarily as sweet-tasting alpha- (67%) or as a bitter-tasting beta- (33%) anomer of the pyranose form (331). Mannose is not an essential nutrient; it can be produced in the human body from glucose or converted into glucose. Mannose is ~5x as active as glucose in non-enzymatic glycation, which may explain why evolution did not favor it as a biological energy source (331). Dapagliflozin significantly decreased mannose levels, which may reflect the decrease in glucose and glycated hemoglobin levels. Indeed, the decrease in mannose levels correlated significantly with the decrease in glucose (r^2=0.297, p=0.04), while the decrease in mannose levels did not correlate with the decrease in HbA1c levels, although marginally (r^2=0.279, p=0.058). On the other hand, insulin administration did not significantly alter serum mannose level, even though serum glucose levels and HbA1c concentration were equally decreased by insulin administration. High mannose levels have recently been associated with increased insulin resistance and insulin secretion indexes (332). Indeed, in our study, the decrease in mannose levels correlated with the decrease in Homeostatic Model Assessment-Insulin Resistance index (HOMA-IR), r^2=0.503, p=0.002, indicating that the decrease of mannose levels can be explained by the improvement in insulin resistance by dapagliflozin administration. Increased insulin sensitivity has been reported after SGLT-2 inhibitor initiation (333). On the other hand, administration of insulin degludec does not improve insulin resistance, as such no reduction in mannose levels were found in the insulin treated group. High mannose levels have recently been associated with increased insulin resistance and insulin secretion indexes and higher mannose levels were associated with an increased risk of MACE independently of other traditional cardiovascular risk factors (HR=1.54; 95% CI=1.07-2.20) (332). In light of this, the reduction in mannose levels by dapagliflozin may add to the underlying mechanisms of cardiovascular protection associated with the use dapagliflozin.

In this study, we found that dapagliflozin administration decreased significantly serum taurine levels by 9.4%. Taurine is an intracellular, sulphur-containing amino acid that is not incorporated into any protein but is widely distributed in all animal tissues as a free amino acid involved in many important physiological functions (334). The level of taurine is regulated both by uptake, through the taurine transporter, and by

endogenous synthesis from methionine and cysteine. Apart from a central role in osmoregulation, taurine plays an important role in the modulation of insulin secretion, acting via partially unknown mechanisms (335). Taurine protects against the apoptosis of beta-cells and enhances their regeneration via its antioxidant and anti-inflammatory actions (336). The decrease of serum taurine levels may reflect increased urine excretion of taurine due to SGTL-2 inhibition. Increased urine excretion of taurine has been reported in patients with familial renal glucosuria (337). Although low levels of taurine have been linked to many diseases, including CNS diseases as taurine is the second most abundant inhibitory neurotransmitter (338, 339, 340), it is unlikely that the modest decrease of serum taurine levels by dapagliflozin is clinically significant.

Urine metabolomics

Regarding urine metabolomics, we show that patients with type 2 diabetes exhibit an altered urine metabolic profile characterized by changes in the concentrations of ketone bodies, osmolytes, amino acids, and various other metabolites. There were differences in the values of some metabolites between the patients with type 2 diabetes included in the dapagliflozin and insulin groups at baseline; however, these can be attributed to the lack of randomization. Although most of the clinical and conventional laboratory characteristics of the 2 groups were similar, residual confounding due to unmeasured factors cannot be excluded. Along with the expected increase in glycosuria, treatment with dapagliflozin resulted in significant changes in the renal excretion of amino acids and their derivatives, tricarboxylic acid cycle intermediates, amines, organic acids, and products of gut microbial origin.

In agreement with previous studies, we found a significant increase in the urine concentrations of ketone bodies (3-hydroxybutyrate and acetoacetate) as well as lactate following dapagliflozin administration (341). However, whether these changes represent drug-induced modifications in the tubular handling of these compounds, reflect shifts in the energy metabolism of renal cells, or result from the increased systemic production of these compounds remains indeterminate. Our finding of no correlation between serum and urine ketone levels may suggest that the increased urine levels of ketones is not attributed to the increased serum concentration. On the other hand, insulin significantly reduced the urine concentrations of these metabolites, a finding that can be attributed to parallel changes in the serum concentrations of these compounds and/or to changes in their renal metabolism and tubular handling.

The essential BCAAs leucine, isoleucine, and valine are important for tissue expansion and regeneration and are involved in various metabolic functions. BCAAs are found in abundance in dietary proteins, and it has been shown that a BCAA-rich diet correlates positively with metabolic health, including regulation of body weight, muscle protein synthesis, and glucose homeostasis. However, cross-sectional and prospective human studies have highlighted that increased fasting concentrations of circulating BCAAs and BCAA supplementation are associated with an increased risk for insulin resistance and type 2 diabetes (342). So, the effects of BCAA on human metabolism can be considered as pleiotropic, depending on host metabolic state. BCAA catabolism involves 2 steps: a reversible one—catalyzed by a branched-chain aminotransferase (BCAT), either cytosolic or mitochondrial, requiring pyridoxal to function as an amino group carrier by which the BCAA and 2-ketoglutarate produce a branched-chain keto acid and glutamate, and the irreversible mitochondrial process catalyzed by branched-chain keto acid dehydrogenase (BCKDH) leading to the formation of acetyl-CoA, propionyl-CoA, and 2-methyl-3-hydroxybutyryl-CoA (from leucine, valine, and isoleucine, respectively), which enter the tricarboxylic acid cycle leading to adenosine 5′-triphosphate formation (343). Impaired function of the BCAT and BCKDH enzymes has been observed in genetic disorders such as maple syrup urine disease or as a result of elevated concentrations of fatty acids, proinflammatory cytokines, or insulin. The resulting accumulation of branched-chain keto acids and other metabolites potentially contributes to the development of insulin resistance and decreases further the expression of BCAT and BCKDH in skeletal muscles, thus leading to the establishment of a vicious cycle that predisposes to the development of type 2 diabetes (343). Our results suggest that dapagliflozin increases the urine concentration of BCAAs. These effects presumably result from the decreased proximal reabsorption of these metabolites since dapagliflozin increased neither their serum levels nor eGFR. The mechanism and the clinical significance of this phenomenon remain indeterminate. However, previous studies have shown that proximal tubular cells in culture incorporate leucine more readily when they are incubated in media with high concentrations of glucose or sodium (344). This process possibly involves internalization of glucose and sodium through SGLT-2 and Na(+)/H(+) exchanger (NHE3) transporters (345, 346, 347). As a consequence, we propose that the inhibition of SGLT-2 and possibly NHE3 transporters by dapagliflozin is responsible for the increased renal excretion of BCAAs. Indeed, the percentage changes in the excretion rates of all 3 BCAAs showed a

significant correlation with the changes in glucose excretion. Since the incorporation of amino acids is an essential step for proximal tubular cell hypertrophy (an early manifestation of diabetic nephropathy), it can be assumed that the dapagliflozin-induced decrease in the reabsorption of BCAAs may contribute to the regression of proximal tubular hypertrophy (348).

In the present study, we identified 6 BCAA catabolism intermediates in the urine of patients with type 2 diabetes. More specifically, 2-hydroxy-3-methylvalerate, 2-hydroxyisovalerate, and 3-hydroxyisovalerate were significantly increased after dapagliflozin administration whereas 3-hydroxyisobutyrate fell short of statistical significance probably due to a large SD value. In addition, 2 toxic metabolites—namely, 3-methyl-2-oxovalerate and N-isovaleroylglycine (arising from the incomplete breakdown of isoleucine and leucine, respectively) that were found to be increased in patients with type 2 diabetes compared to controls—were significantly decreased. These changes possibly reflect an improvement in BCAA metabolism and are in line with those observed after empagliflozin administration (349).

Another finding of our study is the almost 2-fold increase in the urine concentrations of betaine and myo-inositol after dapagliflozin administration. These "compatible organic osmolytes" are present in high concentrations in renal medulla where they protect renal cells from hypertonicity (350). The increased urine levels of these molecules following dapagliflozin treatment may result from a decrease in their tubular reabsorption or may represent an adaptive response to the increased medullary tonicity that results from massive glycosuria and natriuresis. Both betaine and myo-inositol urine concentrations showed a significant correlation with glucose and sodium excretion at baseline. In addition, the increase in their urine concentrations following dapagliflozin administration significantly correlated with the changes in the concentration of glucose. The reduction in the urine concentrations of betaine and myo-inositol with insulin degludec, which reduced significantly the degree of glycosuria, is in line with this assumption.

In addition to their role as osmolytes, betaine and myo-inositol may also exert additional beneficial effects that possibly contribute to the renoprotective properties of dapagliflozin. Indeed, previous studies have shown that myo-inositol protects renal cells during their exposure to high glucose concentrations and reduces the fibrotic

changes that characterize the development of diabetic nephropathy (351). Furthermore, myo-inositol depletion has been shown to exert deleterious effects on tubular cells (352). This depletion may result from glucose toxicity since high glucose concentrations upregulate the myo-inositol-degrading enzyme myo-inositol oxygenase (353). In this perspective, the dapagliflozin-induced increase in urine myo-inositol may reflect the reduction in the exposure of tubular cells to high glucose concentrations and the restoration of cell metabolism. Similarly, previous studies suggest that betaine has important anti-inflammatory actions (354), reduces liver steatosis (355) and improves mitochondrial content and function in liver cells (356). Interestingly, a recent metabolomic study in patients with type 2 diabetes treated with dapagliflozin identified changes in urine metabolites consistent with an improvement in mitochondrial function (310). Whether increased renal concentrations of betaine contribute to this effect remains to be established.

N-methylhydantoin is an oxidative metabolite of creatinine. Previous studies in cell lines and animal models of kidney injury revealed that this compound exerts important antioxidant properties and can protect tubular cells during their exposure to various toxic insults (357, 358). We found that dapagliflozin induced a significant increase in the urine concentration of N-methylhydantoin. Since N-methylhydantoin is produced by the metabolism of creatinine by gut microbes it appears tempting to hypothesize that the previously described effect of the drug on gut microbiota (359) may contribute to the observed increase in the renal excretion of this compound. On the other hand, alterations in the renal handling of N-methylhydantoin may also play a role in these changes. The microbial decomposition of creatinine can also proceed via creatine as the first degradation product. Both N-methylhydantoin and creatine are further metabolized to sarcosine. Sarcosine can also be produced by choline that escapes microbial degradation and is oxidized to betaine, which is converted to N,N-dimethylglycine and then to sarcosine. This pathway is important for osmoregulation and as a source of methyl groups (360). In our study, the excretion of the previously mentioned metabolites N-methylhydantoin, creatine, betaine, sarcosine, and N,N-dimethylglycine was increased after dapagliflozin treatment, a finding suggesting that the drug may have a beneficial effect on gut microbiota metabolism as previously reported (307).

Methylglyoxal, a highly reactive dicarbonyl aldehyde, is a major precursor of nonenzymatic glycation of proteins and DNA, leading to the formation of advanced

glycation endproducts that can affect the function and structure of organs and tissues and has subsequently been implicated in the pathogenesis of type 2 diabetes and its complications (361). Methylglyoxal is a by-product of glycolysis but can also be produced by the catabolism of threonine (361). The principal threonine catabolic pathway in humans involves a glycine-independent serine/threonine dehydratase yielding 2-ketobutyrate, which is further catabolized to propionyl coenzyme A (CoA) and then to succinyl CoA or to 2-aminobutyrate and 2-hydroxybutyrate. Threonine can also be converted to 2-amino-3-ketobutyrate and then to glycine and acetyl-CoA. Alternatively, 2-amino-3-ketobutyrate can be decarboxylated nonenzymatically to aminoacetone and then to pyruvate or to methylglyoxal (362). In our study, succinate and pyruvate were not increased but 2-aminobutyrate and 2-hydroxybutyrate were significantly increased. These findings along with the decrease in the serum levels of threonine in our study following dapagliflozin treatment indicate an altered catabolism of threonine that does not favor the formation of methylglyoxal. In addition, the dapagliflozin-induced decrease in glucose utilization for energy production may further results in a decrease in the concentrations of methylglyoxal, which, in turn, may translate into a reduction in the complications of diabetes. However, how dapagliflozin affects threonine metabolism remains indeterminate.

The renal excretion of the compounds related to muscle metabolism, alanine and anserine, were also significantly altered by dapagliflozin administration. Alanine, a nonessential amino acid, is highly concentrated in muscles, functioning as key in glucose-alanine cycle between tissues and liver. Its biosynthesis occurs either from the conversion of pyruvate or the breakdown of DNA and the dipeptides carnosine and anserine. In our study, urine alanine concentration was significantly higher following dapagliflozin therapy. This change can be attributed either to the increased concentrations of this metabolite systemically or locally or to its decreased tubular reabsorption (363). In tubular cells alanine can be used either for ammoniagenesis, gluconeogenesis, or energy production (364, 365). Dapagliflozin, by inhibiting NHE3 exchangers in proximal tubular cells may decrease net acid excretion and thus the use of alanine as ammonia precursor. This, in turn, may result in increased concentration of this amino acid in tubular cells and increased urine leakage. On the other hand, the use of alanine as a substrate for gluconeogenesis in renal cells following dapagliflozin treatment has not been determined. Previous studies provided conflicting data on the

effect of SGLT-2 inhibitors on endogenous glucose production (119, 366, 367), whereas the role of the kidney in this process remains elusive. Although the clinical significance of the increased urine concentrations of alanine after dapagliflozin administrations is unknown, experimental studies suggest that alanine may protect tubular cells form hypoxic injury (368).

Trigonelline, N-methyl nicotinic acid or betaine nicotinate, is a product of niacin (vitamin B3) metabolism that is excreted in the urine. Experimental studies have shown that trigonelline ameliorates diabetic hypertensive nephropathy by suppression of oxidative stress in kidney and reduction in renal cell apoptosis and fibrosis (369). Trigonelline is also reported as a constituent found in tissues that correlates positively with lean mass quantity of which the physiological properties remain unexplored and as a metabolite that could reflect part of the metabolism of choline through betaine and glycine metabolic pathways (370). The statistically significant increased levels in our study after dapagliflozin treatment may indicate an improved metabolic status of our patients.

Proximal tubules have a very high content of mitochondria and are highly dependent on oxidative phosphorylation. Previous studies in diabetic and nondiabetic models of chronic kidney disease revealed significant disruption of mitochondrial function that parallels the evolution of the disease and is translated into abnormal renal excretion of citric acid cycle (tricarboxylic acid) intermediates (318, 319). In our patients, we observed a significant increase in the renal excretion of citrate following dapagliflozin treatment, a finding that may indicate a restoration of mitochondrial function and an improvement in energy metabolism in tubular cells. Adding to this, the finding that the increased urine citrate levels did not correlate with the increased serum citrate level may also indicate restoration of mitochondrial function in whole body metabolism. Other conditions that may increase citrate excretion like potassium depletion, acid-base abnormalities, hyperparathyroidism, or protein-rich diet are unlikely in our population. Hippurate is a metabolite normally found in human urine. It is synthesized in the kidney and liver from glycine and benzoic acid, secreted by the renal tubular cells, and excreted in the urine. Dapagliflozin altered hippurate excretion in the same direction as did for citrate. This covariation that has been also noted in certain renal disorders can be explained by the link between hippurate synthesis and mitochondrial function. The first step in the formation of hippurate from benzoate in the mitochondrial matrix requires

adenosine 5′-triphosphate. Thus, it is possible that impaired mitochondrial functioning may have contributed to the lower levels of hippurate excretion before treatment (371).

We also observed altered levels of a number of less studied metabolites such as ethylmalonate, nutrition-related gluconate, and 3-Chlorotyrosine. Ethylmalonate, also known as alpha-carboxybutyric acid, is a breakdown product of butyrate and member of the class of compounds known as branched fatty acids. Ethylmalonate may reflect metabolic processes involved in long-chain fatty acid metabolism (such as carnitine-dependent pathways) and related mitochondrial function, and it is mainly studied in cases of inherited metabolic disorders (372).

3-chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, was decreased after treatment in our study. It has been reported that increased levels of 3-chlorotyrosine predict chronic kidney disease severity and associated coronary artery disease (373).

Our study has limitations. The most important of them is probably the lack of a placebo arm. However, the inclusion of an insulin arm excludes the possibility that the dapagliflozin-induced changes in the serum and urine metabolome are the consequence of the improvement in glycemia, a conclusion that could not have been drawn had a placebo group been utilized. We understand that the mechanisms we propose for the observed dapagliflozin-induced changes in both serum and urine metabolome are speculative. However, since metabolomic studies usually detect changes in the concentrations of several metabolites, the complete characterization of the underlying mechanisms and the potential clinical consequences is extremely difficult. We tried to base our speculations on solid scientific evidence, but we believe that further studies are required to confirm and extend our observations.

Conclusion

In this study we sought to identify potential changes in the serum and urine metabolome in patients with type 2 diabetes receiving 3 months of dapagliflozin treatment.

A reduction in body weight, body mass index and blood pressure was achieved with dapagliflozin. Furthermore, dapagliflozin decreased serum glucose and HbA1c in line with previous studies. Serum electrolytes were also affected by dapagliflozin in line with previous results.

Regarding metabolomics, dapagliflozin treatment was associated with a minor change in serum metabolome. However, our results provide more evidence regarding the cardioprotective and nephroprotective properties of dapagliflozin. Further studies are needed to better understand the underlying mechanisms. Furthermore, dapagliflozin induced significant changes in the urine metabolome, which could enhance the nephroprotective properties of dapagliflozin.

ΠΕΡΙΛΗΨΗ ΣΤΗΝ ΕΛΛΗΝΙΚΗ

Ιστορικό και Στόχοι: Η επίπτωση του Σακχαρώδους Διαβήτης Τύπου 2 αυξάνεται παγκοσμίως και θεωρείται η επόμενη πανδημία. Οι ασθενείς με διαβήτη τύπου 2 διατρέχουν αυξημένο κίνδυνο μικροαγγειακών και μακροαγγειακών επιπλοκών, όπως στεφανιαία νόσο, εγκεφαλικό επεισόδιο, περιφερική αρτηριοπάθεια, χρόνια νεφρική νόσο και άλλες. Οι αναστολείς του συμμεταφορέα νατρίου-γλυκόζης 2 (SGLT-2), συμπεριλαμβανομένων της εμπαγλιφλοζίνης, της δαπαγλιφλοζίνης και της καναγλιφλοζίνης έχουν δείξει πολλά υποσχόμενα αποτελέσματα στη μείωση της συνολικής θνησιμότητας, των καρδιαγγειακών εκβάσεων καθώς και στην εξέλιξη της νεφρικής νόσου σε ασθενείς με σακχαρώδη διαβήτη τύπου 2. Επιπλέον, αυτοί οι παράγοντες έχουν πρόσφατα εγκριθεί για ασθενείς με καρδιακή ανεπάρκεια και χρόνια νεφρική νόσο, ανεξάρτητα από την παρουσία διαβήτη. Οι υποκείμενοι μηχανισμοί περιλαμβάνουν τη βελτίωση της ενεργειακής ομοιόστασης και τη μειωμένη αντίσταση στην ινσουλίνη, αν και οι ακριβείς μηχανισμοί δεν έχουν ακόμη διευκρινιστεί. Στόχος μας ήταν να εξετάσουμε τους υποκείμενους μηχανισμούς καρδιακής και νεφρικής προστασίας των αναστολέων SGLT-2 σε ασθενείς με διαβήτη τύπου 2 χρησιμοποιώντας μεταβολομική βασισμένη στο ^{1}H-NMR.

Υλικά και μέθοδοι: Ογδόντα ασθενείς με σακχαρώδη διαβήτη τύπου 2 σε μονοθεραπεία με μετφορμίνη και HbA1c > 7% συμπεριλήφθηκαν στη μελέτη. Πενήντα ασθενείς έλαβαν δαπαγλιφλοζίνη 10 mg ημερησίως και τριάντα ασθενείς έλαβαν ινσουλίνη degludec, η οποία τιτλοποιήθηκε κάθε 3 ημέρες σε ένα στόχο γλυκόζης νηστείας 100-120 mg/dl. Δείγματα ορού και ούρων ελήφθησαν πριν και μετά από 3 μήνες έναρξης της θεραπείας. Πραγματοποιήθηκε μεταβολομική με βάση το ^{1}H-NMR για δείγματα ορού και ούρων πριν και μετά την έναρξη της θεραπείας.

Αποτελέσματα: Ογδόντα ασθενείς συμπεριλήφθησαν στη μελέτη, 50 στο σκέλος της δαπαγλιφλοζίνης και 30 στο σκέλος της ινσουλίνης. Όλοι οι ασθενείς ολοκλήρωσαν με επιτυχία την περίοδο θεραπείας. Τα δημογραφικά στοιχεία, η διάρκεια του διαβήτη, το βάρος, ο δείκτης μάζας σώματος και η αρτηριακή πίεση ήταν παρόμοια μεταξύ των ομάδων. Η χορήγηση δαπαγλιφλοζίνης μείωσε το σωματικό βάρος και το δείκτη μάζας σώματος, τη συστολική και τη διαστολική αρτηριακή πίεση. Τα επίπεδα γλυκόζης και HbA1c μειώθηκαν και στις δύο ομάδες χωρίς διαφορά μεταξύ των ομάδων. Η δαπαγλιφλοζίνη αύξησε τα επίπεδα του φωσφόρου ορού, του μαγνησίου και της

αιμοσφαιρίνης στον ορό, ενώ τα επίπεδα του ουρικού οξέος μειώθηκαν. Το ουρικό οξύ στα ούρα αυξήθηκε μετά τη χορήγηση δαπαγλιφλοζίνης. Η μη στοχευμένη μεταβολομική παρουσίασε καλό διαχωρισμό στο σκέλος της δαπαγλιφλοζίνης πριν και μετά από 3 μήνες τόσο στον ορό όσο και στα ούρα, ενώ στο σκέλος της ινσουλίνης δεν παρουσιάστηκε διαφορά. Η στοχευμένη μεταβολομική αποκάλυψε ότι η δαπαγλιφλοζίνη προκάλεσε μια μικρή αλλά σημαντική αλλαγή στους μεταβολίτες του ορού σε σύγκριση με την ινσουλίνη, ενώ πιο εμφανής αλλαγή σημειώθηκε στη μεταβολομική των ούρων. Τα αυξημένα επίπεδα κετονών και η καλύτερη ενεργειακή ομοιόσταση μπορεί να ευθύνονται για τις προαναφερθείσες κλινικές επιδράσεις της χορήγησης δαπαγλιφλοζίνης.

Συμπέρασμα: Η χορήγηση δαπαγλιφλοζίνης οδήγησε σε αλλαγές στο μεταβολικό προφίλ του ορού και των ούρων που υποδεικνύουν βελτίωση της ενεργειακής ομοιόστασης σε ολόκληρο τον οργανισμό. Επιπλέον, βρέθηκαν αλλαγές που υποδεικνύουν μειωμένη φλεγμονώδη δραστηριότητα και μειωμένη αντίσταση στην ινσουλίνη μετά τη χορήγηση δαπαγλιφλοζίνης.

ΠΕΡΙΛΗΨΗ ΣΤΗΝ ΑΓΓΛΙΚΗ

Background and aims: Type 2 Diabetes Mellitus is increasing worldwide and is regarded as the next pandemic. Patients with type 2 diabetes are at an increased risk of microvascular and macrovascular complications including coronary heart disease, stroke, peripheral arteriopathy, chronic kidney disease and others. Inhibitors of Sodium-Glucose co-transporter 2 (SGLT-2), including empagliflozin, dapagliflozin and canagliflozin have shown promising results in reducing overall mortality, cardiovascular disease as well as progression of kidney disease in patients with type 2 diabetes mellitus. Furthermore, these agents have been recently approved for patients with heart failure and chronic kidney disease irrespective of the presence of diabetes. The underlying mechanisms include improvement in energy homeostasis and reduced insulin resistance, although the exact mechanisms have yet to be elucidated. We aimed to examine the underlying mechanisms of cardiac and renal protection of SGLT-2 inhibitors in patients with type 2 diabetes using ^{1}H-NMR based metabolomics.

Materials and methods: Eighty patients with type 2 diabetes mellitus on metformin monotherapy and HbA1c > 7% were included in the study. Fifty patients received dapagliflozin 10 mg daily and thirty patients received insulin degludec which was uptitrated every 3 days to a target fasting glucose of 100-120 mg/dl. Serum and urine samples were acquired prior to and after 3 months of treatment initiation. 1H-NMR based metabolomics was performed for serum and urine samples before and after treatment initiation.

Results: Eighty patients were included in the study, 50 on the dapagliflozin arm and 30 on the insulin arm. All patients completed successfully the treatment period. Demographics, diabetes duration, weight, body mass index and blood pressure were similar between groups. Dapagliflozin administration decreased body weight and BMI, systolic and diastolic blood pressure. Glucose and HbA1c levels decreased in both groups without between group difference. Dapagliflozin increased serum phosphate, serum magnesium and hemoglobin levels, while uric acid levels decreased. Urine uric acid was increased following dapagliflozin administration. Untargeted metabolomics showed good separation in the dapagliflozin arm before and after 3 months in both serum and urine, while in the insulin arm no separation was evident. Targeted metabolomics revealed that dapagliflozin induced a minor but significant change in serum metabolomics compared with insulin, while a more profound change was noted

in urine metabolomics. Increased ketone levels and better energy homeostasis may account for the aforementioned clinical effects of dapagliflozin administration.

Conclusion: Dapagliflozin administration led to changes in the serum and urine metabolome that indicate improvement in energy homeostasis at a whole body level. Furthermore, changes that indicate decreased inflammatory activity and reduced insulin resistance after dapagliflozin administration were found.

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