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AUTHOR(S):

Furuya, Futoshi; Fujita, Yoshihito; Matsuo, Naomi; Minamino, Hiroto; Oguri, Yasuo; Isomura, Nozomi; Ikeda, Kaori; ... Kondo, Aki; Mano, Fumika; Inagaki, Nobuya

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Liver autophagy-induced valine and leucine in plasma reflect the metabolic effect of sodium glucose co-transporter 2 inhibitor dapagliflozin



Futoshi Furuya, Yoshihito Fujita,^{a,*} Naomi Matsuo, Hiroto Minamino, Yasuo Oguri, Nozomi Isomura, Kaori Ikeda, Kohei Takesue, Ying Li, Aki Kondo, Fumika Mano, and Nobuya Inagaki*



Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Summary

Background Sodium glucose co-transporter 2 (SGLT2) inhibitors are anti-diabetic drugs for type 2 diabetes that lower blood glucose levels and body weight. It is of special interest that SGLT2 inhibitors also improve liver metabolism and fatty liver. Liver is an important organ in regulation of energy metabolism, but the metabolic action of SGLT2 inhibitors in liver remains unclear.

Methods We investigated the factors associated with the beneficial effects of dapagliflozin, a SGLT2 inhibitor, in the liver after confirming its glucose-lowering and weight loss effects using an obesity and diabetes mouse model. We also performed clinical study of patients with type 2 diabetes to explore candidate biomarkers that reflect the beneficial action of dapagliflozin in the liver.

Findings In animal study, dapagliflozin induced autophagy in the liver (LC3-II to LC3-I expression ratio: $P < 0.05$ vs. control), and valine and leucine levels were increased in plasma ($P < 0.01$ vs. control) as well as in liver ($P < 0.05$ vs. control). Thus, increased plasma valine and leucine levels are potential biomarkers for improved liver metabolism. Clinical study found that valine and leucine levels were markedly higher in patients treated with dapagliflozin (valine: $P < 0.05$ vs. control, leucine: $P < 0.01$ vs. control) than those not treated after one week intervention.

Interpretation Dapagliflozin improves liver metabolism via hepatic autophagy, and plasma valine and leucine levels may reflect its metabolic effect.

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Keywords: Sodium glucose co-transporter 2 (SGLT2) inhibitor; Diabetes; Liver; Autophagy; Branched chain amino acids (BCAAs); Biomarker

Introduction

The number of patients with type 2 diabetes is increasing worldwide; obesity often induces insulin resistance that leads to progression to diabetes.^{1,2} In addition, type 2 diabetes is a high-risk factor for cardiovascular disease (CAD) and non-alcoholic fatty liver

disease (NAFLD)/non-alcoholic steatohepatitis (NASH). Moreover, obesity itself is known to contribute to the pathogenesis of diabetes-related diseases such as CAD and NAFLD.^{3,4} Therefore, a weight loss effect is an important consideration in the selection of drug treatment for type 2 diabetes.

*Corresponding author. Department of Diabetes, Endocrinology and Nutrition, Graduate School of Medicine, Kyoto University, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.

E-mail addresses: yfujita9@kuhp.kyoto-u.ac.jp (Y. Fujita), inagaki@kuhp.kyoto-u.ac.jp (N. Inagaki).

^aLead contact.

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Research in context

Evidence before this study

Sodium glucose co-transporter 2 (SGLT2) inhibitors are anti-diabetic drugs approved for type 2 diabetes that lower blood glucose levels as well as body weight. Other pleiotropic effects of SGLT2 inhibitors have been identified in clinical trials, particularly protective effects of SGLT2 inhibitors on the liver, heart, and kidney. SGLT2 inhibitors have been reported to have beneficial effects on fatty liver, suggesting that they play an important role in liver metabolism. However, the mechanisms by which SGLT2 inhibitor influences metabolic change in the liver are not clear. Indeed, SGLT2 inhibitor treatment may induce hyperphagia in compensation for increased urine glucose excretion clinically, and it is difficult to predict who will respond to SGLT2 inhibitors and who will not in clinical practice. It is desirable to identify biomarkers that reflect their metabolic effects in liver and whole body under SGLT2 inhibitor administration.

Added value of this study

We found in this study that dapagliflozin activates hepatic autophagy, which increases the levels of amino acids including valine and leucine, branched chain amino acid (BCAAs), in liver. Valine and leucine are increased in plasma as well as in the liver and are therefore potential biomarkers for the beneficial action of dapagliflozin in liver metabolism. More importantly, we show that valine and leucine levels are markedly higher in patients treated with dapagliflozin than in those not treated after one week in clinical study.

Implications of all the available evidence

Our findings demonstrate further that dapagliflozin can induce the hepatic autophagy that is essential in generating its beneficial metabolic actions in liver. We also show that valine and leucine in plasma may be useful clinically as biomarkers reflecting the therapeutic effects of SGLT2 inhibitor in liver.

Sodium glucose co-transporter 2 (SGLT2) inhibitors are novel drugs for type 2 diabetes that prevent urinary reabsorption of glucose filtered by glomeruli and increase urinary glucose excretion.^{5,6} SGLT2 inhibitors lower blood glucose levels as well as body weight. Recently, clinical use has revealed pleiotropic effects of SGLT2 inhibitors including protective effects on the liver, heart and kidney, which were established in clinical trials including the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients-Removing Excess Glucose (EMPA-REG OUTCOME), the Canagliflozin cardiovascular Assessment Study (CANVAS), and the Dapagliflozin Effect on Cardiovascular Events-Thrombolysis in Myocardial Infarction 58 (DECLARE-TIMI 58) trials.^{7–10}

The liver plays an important role in glucose, lipid and energy metabolism, and many patients with diabetes have NAFLD/NASH. Some of these patients progress to hepatic cirrhosis and liver cancer.¹¹ No therapeutic approach for NAFLD/NASH is established at present. Some studies have reported that SGLT2 inhibitor improves fatty liver^{12,13}; it is thought that this effect is partly induced by the suppressive effect on weight gain and ectopic fat accumulation in the liver. However, the mechanisms by which SGLT2 inhibitor influences metabolic change in the liver are not clear.

As SGLT2 inhibitor administration to rodent models under ad libitum diet induces hyperphagia, which masks the metabolic effect of the drugs in whole body as well as in liver,¹⁴ we performed experiments under pair feeding using an obesity and diabetes mouse model. After confirming the glucose-lowering and weight loss effects of dapagliflozin, a SGLT2 inhibitor, we sought the factors involved in these effects by using metabolome analysis.¹⁵

We found that dapagliflozin induced autophagy in the liver, which may well underlie the beneficial metabolic effects of SGLT2 inhibitors in liver. We also explored candidate biomarkers that might reflect the beneficial actions of dapagliflozin in the liver using translational research: animal models as well as clinical study of patients with type 2 diabetes.

Research design and methods

Animal experiments

Six-week-old male KK-Ay mice were purchased from CLEA Japan, Inc. (MGI Cat# 6197468, RRID:MGI:6197468, Osaka, Japan). To minimize potential confounders, specific pathogen-free mice (SPF) were maintained individually after 1 week acclimation before study initiation under conditions of controlled temperature (25 °C ± 2 °C) on a 12:12-h light–dark cycle for 8 weeks with or without SGLT2 inhibitor treatment. Dapagliflozin was obtained from Med Chem Express. Dapagliflozin (5 mg/kg/day) in water or water alone as control was administered by oral gavage for 8 weeks and body weight was measured once a week. After 4 weeks, blood glucose levels were measured. After 8 weeks, liver triglycerides (TG), total cholesterol (T-CHO) and glycogen contents as well as plasma TG, non-esterified fatty acids (NEFA), T-CHO, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and total ketone body (T-KB) were measured. Insulin glargine (2–6 units/kg/day) was obtained from Sanofi, and was injected subcutaneously for 8 weeks to maintain similar blood glucose levels to those in the dapagliflozin group. As for fasting load test, mice were fasted for 1 day or 3 days. A total of 60 mice

were used for this study. The mice were randomly allocated to groups of 2 (control: $n = 6$, dapagliflozin: $n = 6$), 3 (control: $n = 6$, pair fed dapagliflozin: $n = 6$, ad-libitum fed dapagliflozin: $n = 6$), 3 (control: $n = 6$, dapagliflozin: $n = 6$, insulin glargine: $n = 6$), and 3 (fed: $n = 4$, 1 day fasting: $n = 4$, 3 days fasting: $n = 4$) by blood glucose levels and body weight. For the animal study, the primary endpoints were blood glucose levels and body weight of the two groups, the SGLT2 inhibitor intervention group and the non-intervention group. As differences in pre-intervention blood glucose levels and weight between the two groups might affect post-intervention group comparisons, blood glucose levels and body weight were included as factors in the stratified randomization. Sample size for each group was based on the previous findings in conditions having a significant difference on blood glucose levels after drug administration in comparison to a control group. The sample size for the animal study using KKAY mice is 5, which is based on preliminary experimental data under conditions resulting in significant difference in blood glucose levels after dapagliflozin administration in comparison with a control group, with one-sided $\alpha = 0.05$, power = 0.8, and effect size = 1.84. We set 6 KKAY mice for each group because we could obtain statistical power even with 10–20% dropouts. During the experiment, the health condition of the animals was monitored once a day. No adverse events were observed, and no sample was excluded from the analysis. The treatment and *in vivo* experiment were not blinded. Samples collected from mice were assigned a unique number to enable blinded analysis.

Intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT) and pyruvate tolerance test

IPGTTs were performed at week 4 of the study and glucose (1 g/kg) was injected after 24 h of fasting. Blood glucose levels were measured at 0, 30, 60, 90 and 120 min after injection. Blood samples were collected into heparinized tubes at 0, 30, 60, 90 and 120 min and centrifuged for the analysis of glucose-stimulated blood insulin. ITTs were performed at 5 weeks and regular insulin (1 unit/kg) was injected intraperitoneally after 6 h of fasting. PTTs were performed at 6 weeks and pyruvate (1 g/kg) was injected intraperitoneally after 24 h of fasting.

Tissue collection

On the last day of the study, the mice were euthanized under whole-body inhalable anesthesia. Liver, skeletal muscle, epididymal white adipose tissue (eWAT) and inguinal white adipose tissue (iWAT) were harvested and measured; some parts of liver, skeletal muscle and eWAT were flash frozen in liquid nitrogen for RNA or protein extraction.

Histological analysis

Liver and eWAT samples were fixed in 10% neutral buffered formalin before processing through paraffin. The paraffin sections of the liver and eWAT were stained with hematoxylin and eosin. Pancreas was fixed in Bouin's solution and transferred into 70% ethanol before being processed through paraffin. Embedded tissues were sliced and deparaffinized with a series of xylene and ethanol. Mouse anti-glucagon (1:200 dilution; Abcam Cat# ab10988, RRID:AB_297642) and rabbit anti-insulin antibody (1:200 dilution; Abcam Cat# ab181547, RRID:AB_2716761) were used for immunostaining. Images were taken using optical microscopy with FSX100 and FSX-BSW software (Olympus Life Science).

Quantitative RT-PCR

Total RNA was extracted from the liver using RNeasy Mini Kit (Qiagen) and eWAT using TRIzol reagent (Invitrogen) as previously described.¹⁶ The mouse sequences of forward and reverse primers to detect glucose 6-phosphate (*G6Pase*), phosphoenolpyruvate carboxykinase (*PEPCK*), carnitine palmitoyl transferase 1b (*Cpt1b*), sterol regulatory element binding protein 1c (*Srebp-1c*), stearoyl-CoA desaturase 1 (*Scd1*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*), adipose tissue triglyceride lipase (*Atgl*), hormone sensitivity lipase (*Hsl*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an inner control are shown in [Supplementary Table 1](#). SYBR Green PCR Master Mix (Applied Biosystems) was prepared for the quantitative RT-PCR run. The signals of the products were standardized against GAPDH signals after confirming that there was little variation in the expression levels of GAPDH in the representative experimental conditions of this study.

Immunoblot analysis

Immunoblotting was performed as described previously.¹⁷ Isolated liver and skeletal muscle tissues were homogenized in lysis buffer. Cell lysates were heated at 100 °C for 5 min and subjected to electrophoresis on 8–15% (vol/vol) sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes. Primary antibodies used were anti-LC3 (1:1000 dilution; Cell Signaling Technology Cat# 12741, RRID:AB_2617131), anti-p62 (1:1000 dilution; Cell Signaling Technology Cat# 5114, RRID:AB_10624872), anti-phospho-AMPK α (1:1000 dilution; Cell Signaling Technology Cat# 2531, RRID:AB_330330), anti-AMPK α (1:1000 dilution; Cell Signaling Technology Cat# 2532, RRID:AB_330331), anti-phospho-p70 S6 kinase (1:1000 dilution; Cell Signaling Technology Cat# 9205, RRID:AB_330944), anti-p70 S6 kinase (1:1000 dilution; Cell Signaling Technology Cat# 9202, RRID:AB_331676),

anti-GLUT4 (1:1000 dilution; Cell Signaling Technology Cat# 2213, RRID:AB_823508), anti-PEPCK (1:900 dilution; Abcam Cat# ab70358, RRID:AB_1925305), anti-G6Pase (1:500 dilution; Abcam Cat# ab133964) and β -actin (1:2000 dilution; Cell Signaling Technology Cat# 4967, RRID:AB_330288). The fluorescent bands were visualized using a detection system and quantified by densitometry (Amersham ECL Prime; GE Healthcare).

Metabolomics

Metabolite measurements for this study were performed on the liver, skeletal muscle and plasma using CE-MS and LC-MS. CE-MS was performed using an Agilent CE system coupled to an Agilent 6520 Q-TOF (Agilent Technologies). LC-MS was performed using an Agilent 1260 Series coupled to an Agilent 6520 Q-TOF. The HPLC system was equipped with a CAPCELL PAK C18 IF column (2.0 mm I.D. \times 50 mm, 2 μ m) or a Poroshell 120 EC-C18 (2.1 mm I.D. \times 150 mm, 2.7 μ m). For each metabolite measured by metabolome analysis, the relative peak area was calculated and the values were listed.

Clinical study

Fourteen patients with type 2 diabetes admitted in Kyoto University Hospital between March 2018 and March 2020 were included in the clinical study. Entry criteria were patients without a history of SGLT2 use in the previous 3 months, body mass index (BMI) > 20 kg/m², and type 2 diabetes mellitus patients between 20 and 80 years of age. The registration was started after stabilizing the condition among enrolled patients through hospitalization. Patients were assigned to 2 groups: dapagliflozin-treated group (using dapagliflozin 5 mg/day together with other antidiabetic medications) and control group (non-treated group; using antidiabetic medications except SGLT2 inhibitor). Assignments were random, using the minimization method, weighted according to 1) fasting plasma glucose, 2) age, and 3) sex. As differences in pre-intervention blood glucose levels between the two groups, the SGLT2 inhibitor intervention group and the non-intervention group, might affect post-intervention group comparisons, blood glucose levels were included as a factor in stratified randomization. Furthermore, we added age and sex as stratified randomization factors because of the potential influence on metabolite dynamics in the metabolome analysis. The intervention period was a week and metabolic changes were evaluated and metabolites were analyzed in fasting plasma samples using CE-MS and LC-MS, the same analytical methods as in the basic study.

Statistical analysis

All data are expressed as mean \pm SEM. Shapiro–Wilk test and histogram observations were used to analyze

the normality of data distribution. Comparison between two groups was performed using Student's t test as parametric test, Mann–Whitney U test and Wilcoxon signed–rank test as non-parametric test or Fisher's exact test. One-way ANOVA with Tukey post-hoc test was performed when comparing more than two groups. Two-way ANOVA with Bonferroni post-hoc test was performed when comparing groups with repeated measures. Relationships between two groups were assessed using Spearman's rank correlation coefficient. *P* values < 0.05 were considered statistically significant. Statistical analysis was performed using Statview 5.0, JMP Pro 16 and R.

Study approval

All experiments involving animals were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo16584) and complied with the ARRIVE guidelines.¹⁸ Experimental animals were sacrificed by cervical dislocation. The protocol of clinical study (JRCT registration jRCTs051180091) was approved by the Kyoto University Graduate School and Faculty of Medicine ethics committee. The study was carried out at Kyoto University Hospital according to the principles of the Declaration of Helsinki. All participants gave written informed consent.

Role of funding source

No funder had any role in study design, data collection, analysis, interpretation of data, writing of the report or in the decision to submit for publication. The corresponding author had full access to all of the data in the study and has final responsibility for the decision to submit for publication.

Results

Metabolic effects of dapagliflozin in obese diabetic mice under pair feeding

We investigated the effect of dapagliflozin, a SGLT2 inhibitor, using the KK-Ay mouse as a model of obesity and diabetes. We adopted the experimental design of pair feeding to avoid influence of changes in dietary intake, as SGLT2 inhibitors can induce hyperphagia.¹⁴ Dapagliflozin lowered both fed and fasting blood glucose levels for 4 weeks (fed: control, 291.7 \pm 31.7 mg/dL; dapagliflozin, 141.8 \pm 7.8 mg/dL, *P* < 0.05 vs. control; fasting: control, 86.5 \pm 3.2 mg/dL; dapagliflozin, 71.8 \pm 4.7 mg/dL, *P* < 0.05 vs. control; Fig. 1a and b). Intraperitoneal glucose tolerance test (IPGTT) data showed that dapagliflozin ameliorated glucose intolerance (Fig. 1c and d). Insulin tolerance test (ITT) revealed no difference in blood glucose levels by dapagliflozin

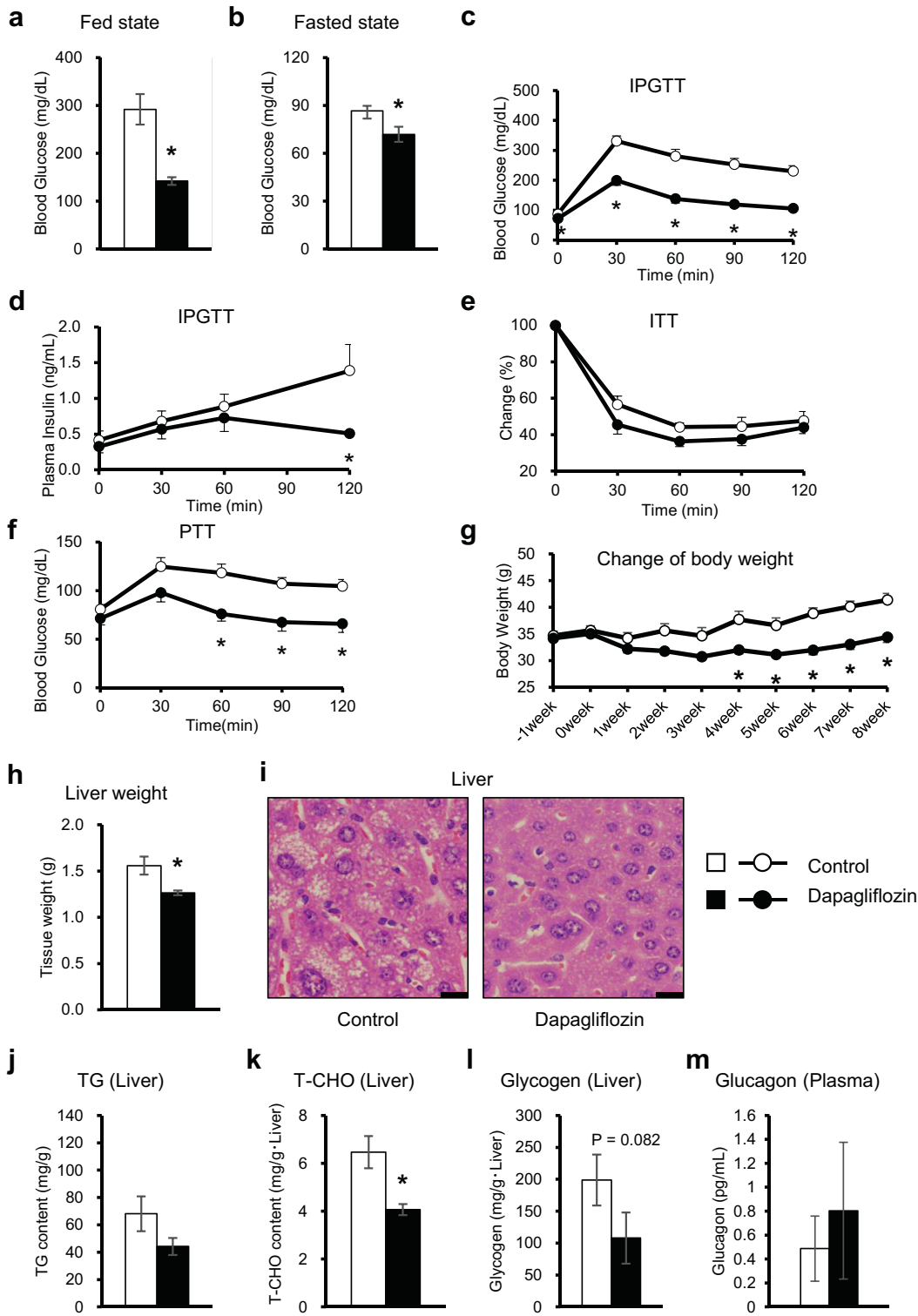


Fig. 1: Effect of Dapagliflozin on blood glucose levels, glucose tolerance, and insulin sensitivity in KK-Ay mice. (a) Fed blood glucose levels. **(b)** Fasting blood glucose levels after 24 h fasting. **(c and d)** Blood glucose **(c)** and insulin **(d)** levels in the intraperitoneal glucose tolerance test (IPGTT). **(e)** Glucose levels in the insulin tolerance test (ITT). **(f)** Glucose levels in the pyruvate tolerance test (PTT). **(g)** Body weight changes. **(h)** Liver weight. **(i)** Hematoxylin-eosin staining of liver. Original magnification, $\times 40$. Scale bars, 16 μm . **(j)** Triglyceride contents (TG), **(k)** total

(Fig. 1e). In pyruvate tolerance test (PTT), dapagliflozin suppressed the elevation of blood glucose levels after pyruvate administration (Fig. 1f). Dapagliflozin decreased LDL-C levels ($P < 0.05$ vs. control) and increased T-KB levels ($P < 0.05$ vs. control) in plasma but did not alter TG or NEFA levels (Supplementary Table 2).

We then examined the influence of dapagliflozin on fat accumulation under pair feeding. Dapagliflozin reduced the increase in body weight (after 4 weeks up to 8 weeks: $P < 0.05$ vs. control; Fig. 1g). Dapagliflozin lowered eWAT weight as well as iWAT weight ($P < 0.05$ vs. control; Supplementary Fig. 1a and b). On the other hand, some of the beneficial effects of dapagliflozin, i.e., the glucose lowering effect in fasted state, the body weight loss effect, and the preventive effect on fat accumulation were attenuated under ad libitum condition compared with those in pair feeding (Supplementary Figure 2). These data indicate that dapagliflozin lowers blood glucose levels and prevents adiposity under pair feeding.

Dapagliflozin decreases lipid accumulation of WAT and liver

We then investigated changes in WAT, liver, skeletal muscle and pancreas by dapagliflozin in KK-Ay mice under pair feeding. Dapagliflozin reduced the adipose cell area of eWAT following the decrease in eWAT weight ($P < 0.05$ vs. control; Supplementary Figure 1c). Dapagliflozin increased the mRNA expression of adipose triglyceride lipase (ATGL) but did not change the expression level of hormone sensitivity lipase (HSL) (ATGL: $P < 0.05$ vs. control; Supplementary Fig. 1d and e), suggesting that SGLT2 inhibitor induced lipolysis of eWAT.

In liver, dapagliflozin lowered weight (control, 1.6 ± 0.1 g; dapagliflozin, 1.3 ± 0.0 g, $P < 0.05$ vs. control; Fig. 1h), lipid accumulation (Fig. 1i) and T-CHO content (control, 6.5 ± 0.7 mg/g liver; dapagliflozin, 4.1 ± 0.2 mg/g liver, $P < 0.05$ vs. control; Fig. 1k) and tended to decrease TG content (control, 68.1 ± 12.7 mg/g liver; dapagliflozin, 44.2 ± 6.1 mg/g liver, $P = 0.12$ vs. control; Fig. 1j). Dapagliflozin also tended to decrease glycogen content in liver (control, 198.8 ± 43.3 mg/g liver; dapagliflozin, 105.5 ± 36.5 mg/g liver, $P = 0.08$ vs. control; Fig. 1l), while plasma glucagon levels were unchanged (control, 0.5 ± 0.3 pg/mL; dapagliflozin, 0.8 ± 0.6 pg/mL, $P = 0.627$ vs. control; Fig. 1m). In addition, dapagliflozin did not alter the weight of skeletal muscle or the size of pancreatic islets (Supplementary Fig. 1f and g). These findings indicate that suppression of fat accumulation in adipose and

liver tissue contributes to amelioration of the systemic metabolic disturbance by SGLT2 inhibitor.

Metabolome analysis of the liver

We then investigated the effects of dapagliflozin on gene expression and metabolite content in the liver. Dapagliflozin increased glucose 6-phosphatase (G6Pase) mRNA and tended to increase mRNA of phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme for gluconeogenesis (*G6pase*: $P < 0.05$ vs. control, *Pepck*: $P = 0.05$ vs. control; Fig. 2a). On the other hand, dapagliflozin did not affect the protein expression of G6Pase and PEPCK (Supplementary Figure 3). Dapagliflozin increased carnitine palmitoyl transferase 1b (*Cpt1b*) mRNA, decreased stearoyl-CoA desaturase 1 (*Scd1*) mRNA and tended to decrease mRNA of sterol regulatory element binding protein 1c (*Srebp-1c*) (*Cpt1b*: $P < 0.05$ vs. control, *Scd1*: $P < 0.05$ vs. control, *Srebp-1c*: $P = 0.07$ vs. control; Fig. 2a). We performed metabolome analysis to assess the dynamics of the metabolites in the liver and to determine which factors participate in the effects of SGLT2 inhibitor by using capillary electrophoresis-mass spectrometer (CE-MS) and liquid chromatograph-mass spectrometer (LC-MS). The orthogonal partial least squares discriminant analysis (OPLS-DA) model enabled us to separate the more distinctly differing ions in the liver in the dapagliflozin group from those in the control group (Fig. 2b and c). Volcano plot revealed that the levels of several metabolites were significantly changed by dapagliflozin, including glucose and pyruvate as the gluconeogenesis substrate (Fig. 2d and e). The differences in the metabolic pathways in the liver between the two groups are shown in Supplementary Figure 4–6.

Autophagy in the liver is induced by SGLT2 inhibitor

Metabolome analysis showed that dapagliflozin increased amino acids overall and, especially, several types of amino acid in the liver (leucine, valine, tryptophan and tyrosine: $P < 0.05$ vs. control; Fig. 3a and Supplementary Figure 7a). As it is thought that increase in amino acid levels may be caused by proteolysis,¹⁹ we evaluated induction of autophagy in the liver. Immunoblot analysis revealed that dapagliflozin increased the LC3-II to LC3-I expression ratio in the liver ($P < 0.05$ vs. control; Fig. 3b and c), strongly suggesting that the autophagy was induced by the SGLT2 inhibitor. p62 protein expression was not altered in the dapagliflozin-treated mice (Fig. 3d). Dapagliflozin treatment tended

cholesterol contents (T-CHO) and (l) glycogen contents in the liver. (m) Plasma glucagon levels. Values are mean \pm SEM (n = 6). Statistical analysis was performed by two-way ANOVA with Bonferroni post-hoc test or Student t-test, * $P < 0.05$ vs. without dapagliflozin.

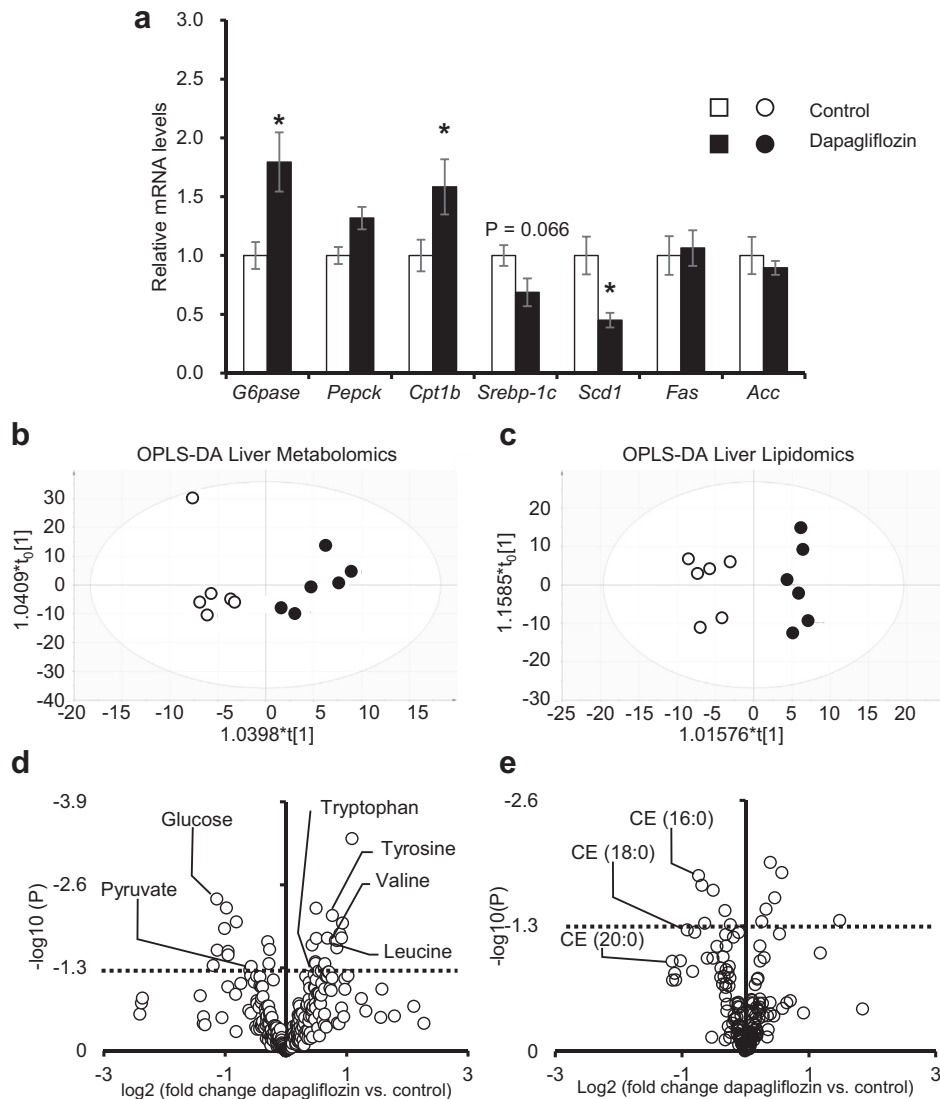


Fig. 2: Metabolome analysis and relative mRNA expression in the liver of control and dapagliflozin treated mice. (a) mRNA expression of gluconeogenesis (*G6Pase* and *PEPCK*), β -oxidation (*Cpt1b*) and fatty acid synthesis- (*Srebp-1c*, *Scd1*, *Fas* and *Acc*) related genes in the liver by qRT-PCR. (b and c) OPLS analysis. Based on the score plots, distinct metabolic profiles in the liver between dapagliflozin group and control group. (b) Metabolomics. (c) Lipidomics. (d and e) Volcano plot of 305 metabolites and 161 lipids, comparing the fold induction after dapagliflozin. The dashed line indicates a P value of 0.05. (d) Metabolomics. (e) Lipidomics. Values are mean \pm SEM ($n = 6$). Statistical analysis was performed by Student t -test, * $P < 0.05$ vs. without dapagliflozin.

to increase the phospho-AMP-activated protein kinase α (AMPK α)-to-AMPK α expression ratio ($P = 0.07$ vs. control; Fig. 3e) and decrease the phospho-p70S6 kinase (S6K)-to-p70S6K expression ratio ($P < 0.05$ vs. control; Fig. 3f), indicating that the autophagy was induced by inactivation of the target of the rapamycin complex 1 (mTORC1), a major negative regulator of autophagy.

We then investigated whether SGLT2 inhibitor induced autophagy in skeletal muscle. Unlike in the liver, amino acids were not increased in the skeletal

muscle of KK-Ay mice under dapagliflozin (Fig. 4a and Supplementary Figure 7b). In addition, dapagliflozin did not increase the LC3-II to LC3-I expression ratio (Fig. 4b and c). p62 protein expression was not altered in the dapagliflozin-treated mice (Fig. 4d). These results indicate that dapagliflozin induces autophagy specifically in the liver. To clarify the mechanism of tissue specificity, we examined glucose content and GLUT4 protein expression in the skeletal muscle, which were not altered by dapagliflozin administration (Fig. 4e and f).

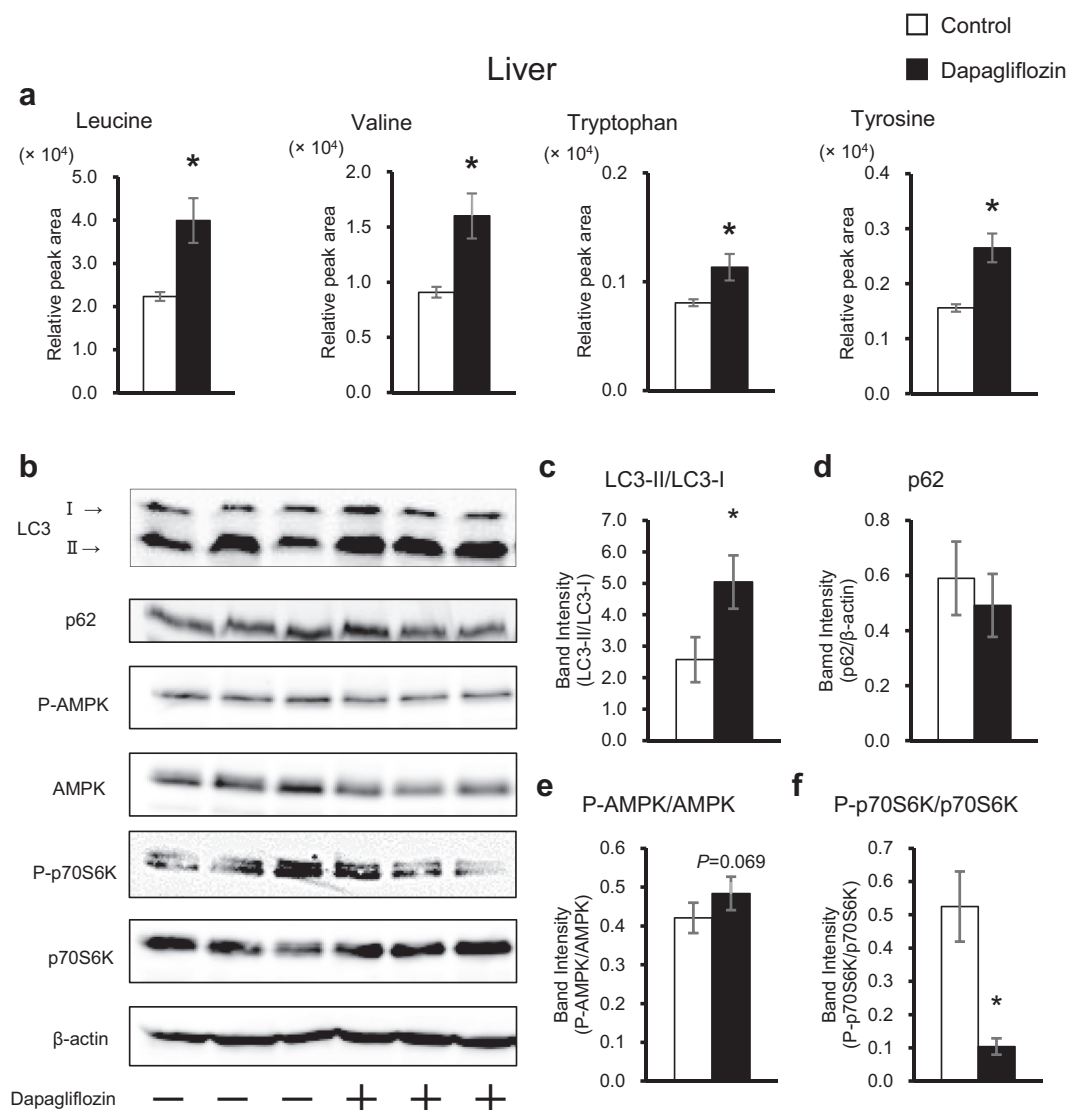


Fig. 3: Autophagy in the liver of KK-Ay mice by dapagliflozin. (a) Amino acid levels by metabolomics in the liver. (b) Immunoblotting of liver lysates. Protein expression levels of LC3-II to LC3-I ratio (c), p62 (d), P-AMPK to AMPK ratio (e) and P-p70S6K to p70S6K ratio (f) in the liver. Values are mean ± SEM (n = 6). Statistical analysis was performed by Student t-test, *P < 0.05 vs. without dapagliflozin.

We also examined induction of autophagy in liver and skeletal muscle under fasting condition. Autophagy tended to be induced in both liver and skeletal muscle after 1 day of fasting, and was significantly induced in skeletal muscle after 3 days of fasting compared to that under fed condition (Supplementary Figure 8). Thus, the organs in which autophagy is readily induced by SGLT2 inhibitor in fed condition may differ from those in fasting condition.

Autophagy in the liver is not induced by insulin

Whether or not the induction of autophagy in liver is specific to dapagliflozin was then investigated. As shown

in Supplementary Fig. 9a–c, insulin glargine administration maintained glucose at levels similar to those in dapagliflozin-treated mice. However, insulin glargine did not affect body weight, liver weight, visceral fat weight or skeletal muscle volume (Supplementary Fig. 9d–g). No reduction in fat accumulation in the liver was observed under insulin glargine administration compared with that of dapagliflozin (Supplementary Figure 9h). Induction of autophagy in the liver was then quantified. Immunoblot analysis revealed that insulin glargine did not increase the LC3-II to LC3-I expression ratio in the liver (Supplementary Fig. 10a and b), indicating that autophagy is not induced by insulin glargine. Furthermore, p62 protein expression was not altered in the

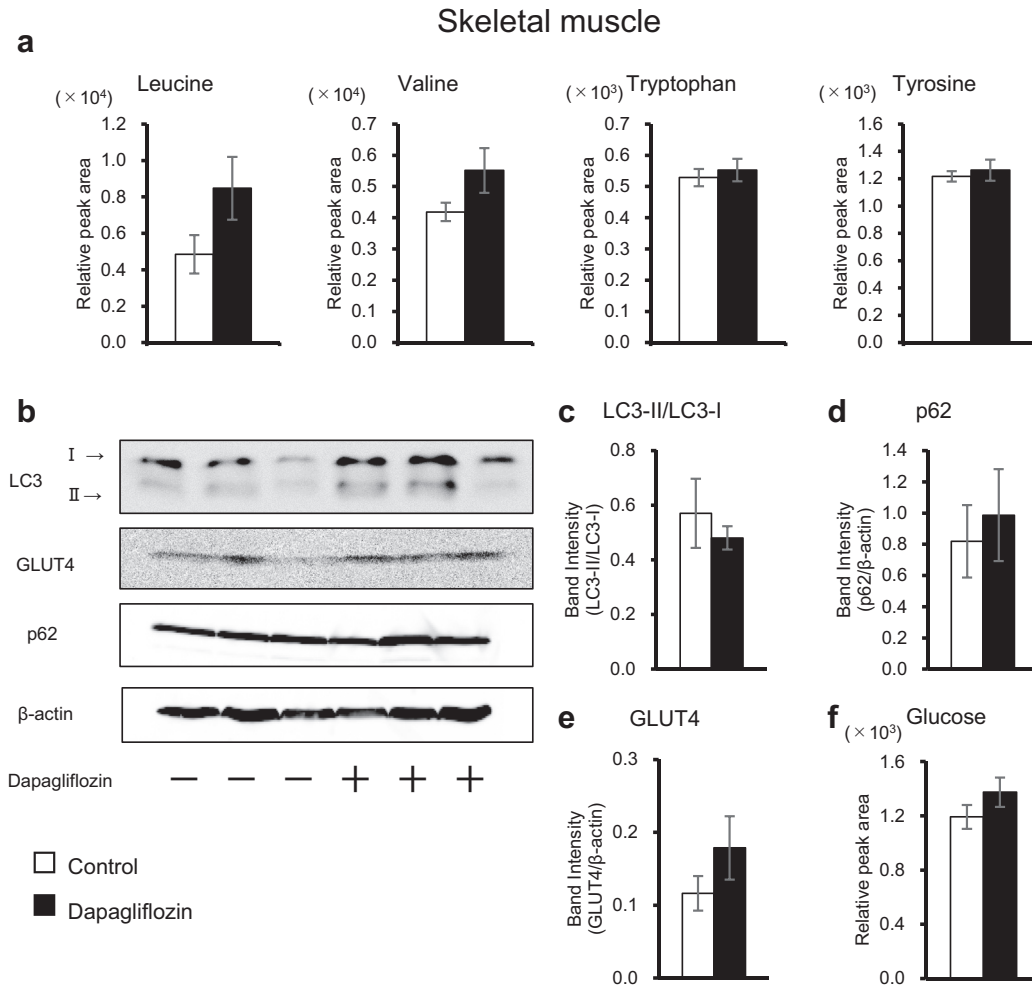


Fig. 4: Autophagy in the skeletal muscle of KK-Ay mice by dapagliflozin. (a) Amino acid levels by metabolomics in skeletal muscle of KK-Ay mice. (b) Immunoblotting of skeletal muscle lysates. Protein expression levels of LC3-II to LC3-I ratio (c), p62 (d) and GLUT4 (e) in the skeletal muscle. (f) Glucose levels in the skeletal muscle. Values are mean \pm SEM (n = 6). Statistical analysis was performed by Student t-test, * $P < 0.05$ vs. without dapagliflozin.

glargine-treated mice (Supplementary Figure 10c). Metabolome analysis revealed that amino acids levels, particularly those of leucine and valine, were not increased in the liver of insulin glargine-treated mice, unlike the case in dapagliflozin-treated mice (Supplementary Figure 11). These findings indicate that induction of liver autophagy by SGLT2 inhibitors does not occur with other diabetes medications such as insulin.

Investigation of biomarkers reflecting the effect of SGLT2 inhibitor

We then analyzed the metabolites in plasma using the same metabolomics approach. Dapagliflozin increased the leucine and valine levels in plasma ($P < 0.01$ vs.

control; Fig. 7a). The dynamics of these plasma amino acid changes was similar to those in the liver under dapagliflozin. In addition, we found by metabolome analysis of the liver that dapagliflozin tended to lower cholesterol esters (CEs) overall, especially CE (16:0) ($P < 0.01$ vs. control; Fig. 5a). CE (16:0) in plasma was significantly lowered by dapagliflozin ($P < 0.01$ vs. control; Fig. 5b). Importantly, we found significant, positive associations between leucine ($r = 0.657$, $P = 0.028$), valine ($r = 0.603$, $P = 0.049$) and CE (16:0) ($r = 0.740$, $P = 0.006$) in the liver and plasma (Fig. 5c–e). These data suggest that leucine, valine and CE (16:0) in plasma are biomarker candidates for assessment of the metabolic effect of dapagliflozin in the liver.

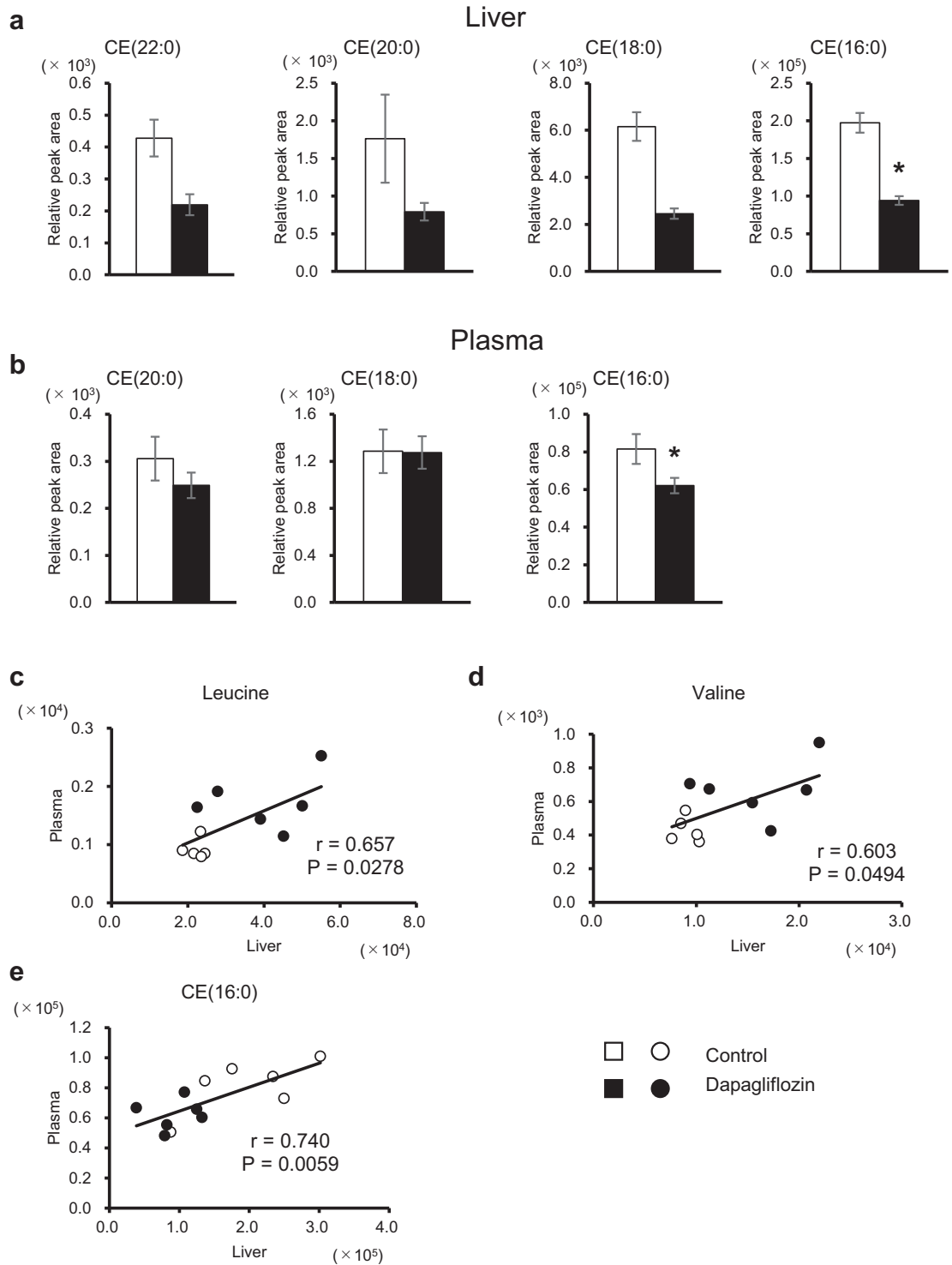


Fig. 5: Investigation of biomarkers reflecting the effect of SGLT2 inhibitor. (a and b) Cholesterol ester (CE) levels by lipidomics in liver (a) and plasma (b). Correlation of valine (c), leucine (d) and CE (16:0) (e) between liver and plasma in KK-Ay mice with dapagliflozin. Throughout, r is the Spearman correlation coefficient between liver and plasma. Values are mean \pm SEM ($n = 6$). Statistical analysis was performed by Student t -test, * $P < 0.05$ vs. without dapagliflozin.

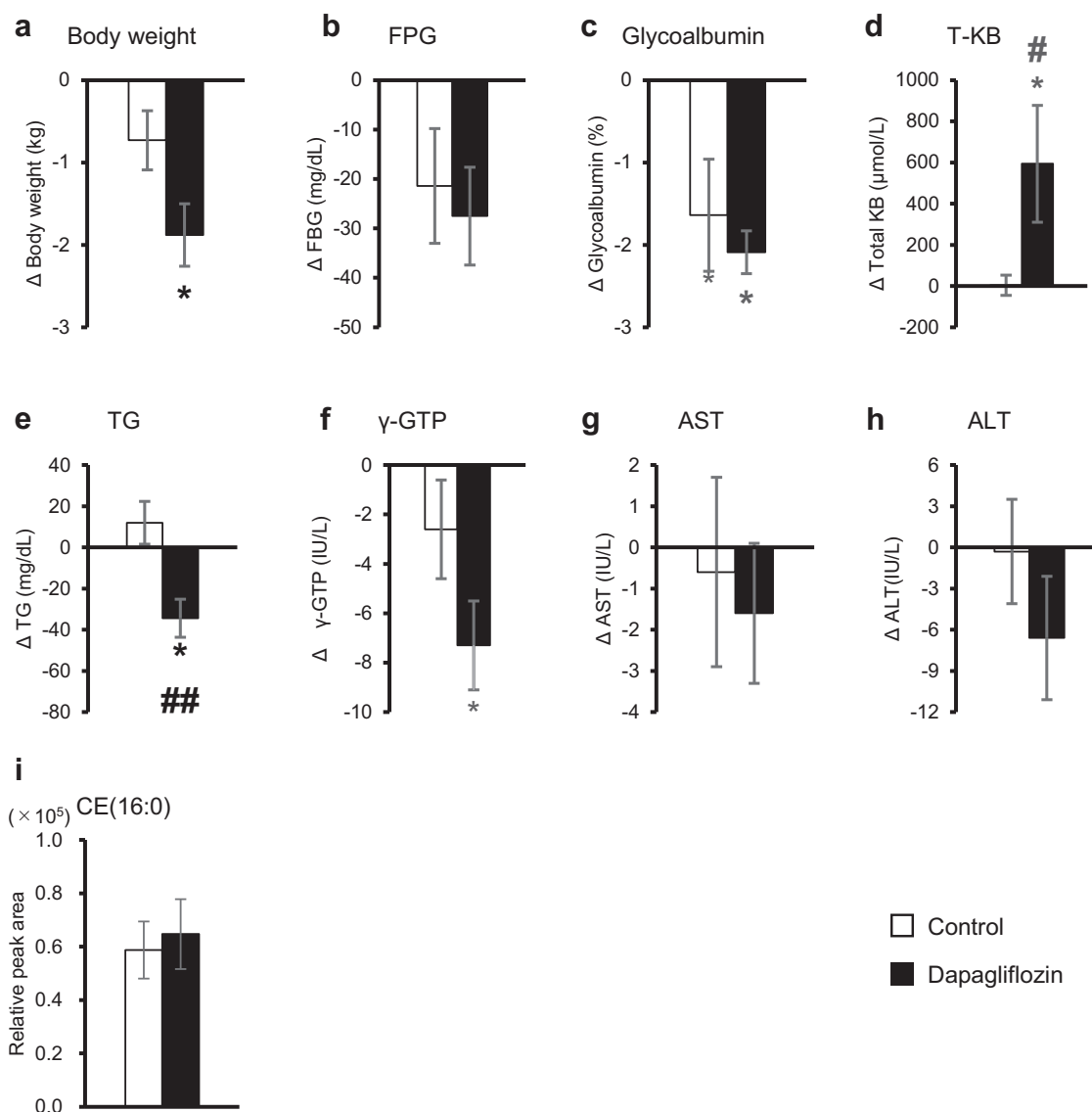


Fig. 6: Changes in clinical parameters and metabolites in patients with type 2 diabetes after 1 week of dapagliflozin treatment. Changes in body weight (a), FPG (b), serum Glycoalbumin (c), serum T-KB (d), serum TG (e), serum γ -GTP (f), serum AST (g) and serum ALT (h) in patients with type2 diabetes during a week of dapagliflozin treatment. Effect of dapagliflozin on plasma levels of CE (16:0) (i) by metabolomics. Values are mean \pm SEM (n = 7). Statistical analysis was performed by Mann-Whitney U test, * $P < 0.05$ vs. baseline, # $P < 0.05$, ## $P < 0.01$ vs. control group.

Plasma valine and leucine levels are higher after dapagliflozin administration in patients with type 2 diabetes

We performed a clinical study as translational research to explore the potential clinical applications of the biomarker candidates found in the present animal study; hospitalized patients with type 2 diabetes were enrolled. The subjects were allocated to a dapagliflozin-treated group or a non-treated group after stabilization through hospitalization. After 1 week intervention under strict dietary conditions, metabolic changes were evaluated and metabolome analysis of fasting plasma

samples using CE-MS and LC-MS was performed. The baseline clinical characteristics of each group are shown in [Supplementary Table 3](#). Body weight was significantly reduced in dapagliflozin group compared with baseline ($P < 0.05$ vs. baseline; [Fig. 6a](#)). On the other hand, glycoalbumin was decreased in both the control and dapagliflozin groups compared with baseline ($P < 0.05$ vs. baseline; [Fig. 6b](#) and c). The increase in serum ketone body (T-KB) levels was significantly greater in dapagliflozin group compared with that in control group ($P < 0.05$ vs. control; [Fig. 6d](#)). T-KB levels was significantly increased in dapagliflozin group compared with

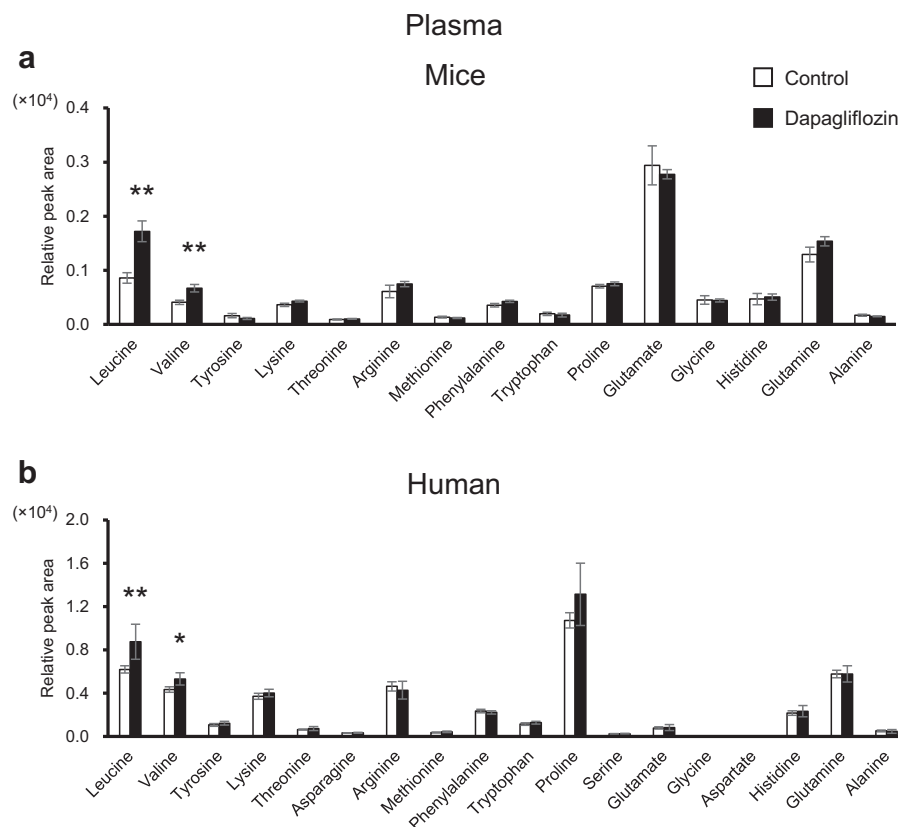


Fig. 7: Plasma valine and leucine can serve as useful biomarker candidates reflecting the effect of SGLT2 inhibitor in clinical practice. (a and b) Amino acid levels by metabolomics in plasma of mice (a) and human (b). Values are mean ± SEM (n = 6–7). Statistical analysis was performed by Student t-test, *P < 0.05, **P < 0.01 vs. without dapagliflozin.

baseline ($P < 0.05$ vs. baseline; Fig. 6d). Serum TG levels and γ -GTP levels were significantly reduced in dapagliflozin group compared with baseline ($P < 0.05$ vs. baseline; Fig. 6e–f). The decrease of serum TG levels was significantly greater in dapagliflozin group compared with that in control group ($P < 0.05$ vs. control; Fig. 6e). Serum AST, ALT and γ -GTP levels were not different in dapagliflozin group (Fig. 6f–h).

After 1 week intervention, plasma valine and leucine levels were higher in dapagliflozin-treated group than those in non-treated group (valine: $P < 0.05$ vs. control, leucine: $P < 0.01$ vs. control; Fig. 7b). On the other hand, a difference in plasma CE (16:0) levels between the two groups was not observed in the current study design (Fig. 6i). These data suggest that plasma valine and leucine, which we found in animal study, can serve as useful biomarkers reflecting the effects of SGLT2 inhibitor in clinical practice (Fig. 7).

Discussion

We investigated the factors participating in the metabolic action of the SGLT2 inhibitor dapagliflozin in liver. Dapagliflozin inhibits the reabsorption of glucose

filtered by glomeruli in kidney and increases urinary glucose excretion and lowers blood glucose levels.²⁰ In the present study, we investigated the effect of an SGLT2 inhibitor using KK-Ay mice, an obesity and diabetes mouse model. It is known that SGLT2 inhibitor exacerbates hyperphagia in a rodent model.¹⁴ The effects of SGLT2 inhibitor found in clinical practice such as hypoglycemic action, suppressant effect on fat accumulation and metabolic effects on respective tissues were not obtained using rodent models under ad libitum diet.¹⁴ We performed experiments using dapagliflozin under pair feeding condition, which insured that food intake was identical between groups with and without dapagliflozin. We thereby obtained findings closer to clinical effects, and exploration of factors contributing to the metabolic action of the SGLT2 inhibitor dapagliflozin was facilitated.

Autophagy is a self-devouring system for its role in supplying amino acids in response to nutrient starvation.^{21,22} Liver autophagy has been known to be induced by fasting.²³ Several recent reports have suggested that SGLT2 inhibitors ameliorate fatty liver by induction of autophagy.^{24–26} Li L et al. reported that dapagliflozin improved fatty liver by inducing autophagy via the

AMPK-mTOR pathway in ZDF rats.²⁵ Nasiri-Ansari N et al. reported that the SGLT2 inhibitor empagliflozin induces autophagy to mitigate progression to fatty liver in ApoE-deficient mice.²⁶ We also report that the SGLT2 inhibitor dapagliflozin induces liver autophagy. Previous study reported that several amino acids including valine and leucine in the liver were elevated, reflecting proteolysis by autophagy.²⁷ Our data demonstrates that dapagliflozin increases amino acids overall, especially valine, leucine, tryptophan and tyrosine. Furthermore, no increase in autophagy or valine and leucine levels in the liver was observed with insulin, suggesting that the effects are specific to SGLT2 inhibitors. Recent studies report that autophagy participates in the mechanism of glucose and lipid metabolism.^{28,29} Rubicon, a protein that inhibits autophagy, exacerbates fatty liver and promotes fat accumulation and cell death.³⁰ Singh et al. reported that lipid droplets in hepatocytes are degraded by autophagy.²⁹ This supports the possibilities that enhancement of autophagy by dapagliflozin reduces fatty liver. In addition, we showed CPT1b mRNA expression is increased by dapagliflozin, suggesting that lipid oxidation is enhanced resulting in decreased lipid accumulation in liver.³¹⁻³³

As the action of SGLT2 inhibitor is unique, it has been receiving attention regarding its safety and adverse events from well before its launch.⁶ Sarcopenia, a decrease in muscle volume, has been considered to be a risk of SGLT2 inhibitor, as excessive autophagy in muscle can cause muscle atrophy.³⁴⁻³⁶ However, a recent clinical study reported that ipragliflozin, a SGLT2 inhibitor, did not affect skeletal muscle volume.³⁷ Also, in our study using a rodent model, dapagliflozin did not decrease skeletal muscle volume. Since previous study reported that autophagy in skeletal muscle was induced under fasting conditions, we expected autophagy to be activated in skeletal muscle as it was in liver by dapagliflozin.^{23,27} This was not the case, intriguingly. Furthermore, the increase in valine and leucine, which are supposed to reflect the activation of autophagy, was not observed in skeletal muscle. The cause of the difference in autophagy induction by SGLT2 inhibitor among organs is unknown, but distinct intra-cellular starvation responses, which induce autophagy, may well be involved.²² Indeed, intra-hepatocellular glucose is deprived by dapagliflozin, while the intra-muscular glucose level is not altered (Figs. 2d and 4f). Thus, tissue-specific autophagy, such as liver-specific autophagy, may underlie some of the beneficial effects of dapagliflozin in diabetes therapy on liver metabolism and decreased risk of sarcopenia.

It is also difficult to predict responders and non-responder to SGLT2 inhibitor in clinical settings.³⁸ SGLT2 inhibitor may cause hyperphagia in compensation for increased urine glucose excretion clinically, which makes it difficult to evaluate the effect of the drug in individual cases.^{39,40} It is therefore desirable to

identify biomarkers that similarly reflect the metabolic effects in liver and whole body under SGLT2 inhibitor administration. We find valine, leucine and CE (16:0) to be such candidate plasma biomarkers in the present animal study. These metabolites have the same dynamics in liver and blood under dapagliflozin administration using rodent models, and can serve as plasma markers for evaluation of the metabolic status of the liver under dapagliflozin treatment. Valine and leucine, which are branched chain amino acids (BCAAs), are not metabolized in the liver. Increased BCAAs in liver are known to be secreted into blood and partially taken into skeletal muscle.⁴¹ In our study, valine and leucine levels in liver as well as in plasma were increased by SGLT2 inhibitor administration, which may indicate that increased valine and leucine in the liver are secreted into the blood and taken up by skeletal muscle. A recent study suggested that SGLT2 inhibitor improves grip strength and is an anti-sarcopenia reagent.⁴² This supports the possibility that increased BCAA induced by dapagliflozin can be taken into skeletal muscle to maintain skeletal muscle volume. On the other hand, other amino acids that are not altered under dapagliflozin may be consumed within the liver.⁴³ Our study also shows that CE (16:0), which is decreased in the liver, is also decreased in plasma. CE is composed of cholesterol and fatty acid, and accumulation of CE stored as lipid droplets leads to macrophage foam cells, which can induce the development of atherosclerotic plaques.⁴⁴ Thus, CE (16:0) in plasma may reflect not only the metabolic status of the liver under dapagliflozin more sensitively than other lipids but also early signs of arteriosclerosis.

We also performed a translational clinical study to ascertain whether these biomarker candidates may be applicable to clinical use. Plasma valine and leucine levels were markedly higher in dapagliflozin group compared to those in control group. The relationship between higher plasma concentrations of BCAA, i.e., leucine, isoleucine, and valine and insulin resistance has been acknowledged since Newgard et al. reported a correlation between BCAAs and insulin resistance in obese individuals in 2009.⁴⁵ However, a recent study reported that glucose metabolism was improved by administration of BCAA (20 g/day) for 4 weeks in a clinical study.⁴⁶ In addition, an animal study showed that BCAA intake for 8 weeks decreased fat accumulation in the liver.⁴⁷ These recent findings suggest that BCAA does not worsen insulin resistance but rather improves it. In our study, higher plasma levels of valine and leucine, which are BCAAs, were found by 1-week intervention of SGLT2 inhibitor, suggesting that these amino acids may be contributing factors to the metabolic ameliorating effects of SGLT2 inhibitor.

Limitations and future issues to be considered based on this study include the following: although dapagliflozin was used as the SGLT2 inhibitor in this study,

many SGLT2 inhibitors are used in clinical practice, and it is necessary to verify whether this effect is a class effect. This study also used KK-Ay mice as an animal model of diabetes and obesity. There have been reports on the hypoglycemic and weight-loss effects as well as the effects on fatty liver of SGLT2 inhibitors using wild-type mice on a high-fat diet and db/db mice.^{24,26,48} Further studies on the effects on autophagy and liver metabolism using other model animals under different loading conditions such as a high-fat diet are needed. Although, the search for candidate biomarkers reflecting the effect of the SGLT2 inhibitor dapagliflozin led to the amino acids valine and leucine, in a study by Mulder S et al. no change in plasma levels of valine or leucine was found.⁴⁹ Differences in experimental design may be involved, such as the 1-week intervention period in our study and the 12-week intervention period in the other.⁴⁸ As the main study limitations, we must mention the small sample size. The 14 patients with type 2 diabetes included in our clinical trial may not be a representative sample of the wider population of interest within the scope of this research. Further validation studies are required to establish plasma valine and leucine as an accepted biomarkers of treatment response, such as by confirming an increase only in patients with metabolic and/or hepatic histology improvement versus those patients who do not.

In conclusion, our data suggest that hepatic autophagy contributes to the metabolic effect of dapagliflozin in liver and that regulation of autophagy in liver may be a therapeutic target for NAFLD/NASH.^{50,51} In addition, plasma valine and leucine levels may reflect its effect. Further studies are required to elucidate the mechanism of its effect in liver and to utilize leucine and valine in plasma as biomarkers to evaluate the clinical efficacy of SGLT2 inhibitors.

Contributors

F.F. researched data, performed data analysis, contributed to discussion, and wrote, reviewed, and edited the manuscript. Y.F. researched data, performed data analysis, contributed to discussion, and wrote, reviewed, and edited the manuscript. N.M. researched data, performed data analysis, and contributed to discussion. H.M., Y.O., N.I., K.I., K.T., Y.L., A.K., and F.M. performed data analysis. N.I. contributed to discussion and wrote, reviewed, and edited the manuscript. Dr. Yoshihito Fujita and Nobuya Inagaki are the guarantors of this work, had full access to all the data, and take full responsibility for the integrity of the data and the accuracy of data analysis.

Data sharing statement

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Yoshihito Fujita (yfujita9@kuhp.kyoto-u.ac.jp).

Declaration of interests

Y.F. received clinical commissioned/joint research grants from AstraZeneca K.K. and Ono Pharmaceutical Co., Ltd. N.I. received clinical commissioned/joint research grants from Daiichi Sankyo, Terumo, and

Drawbridge Inc.; speaker honoraria from Kowa, MSD, Astellas Pharma, Novo Nordisk Pharma, Ono Pharmaceutical, Nippon Boehringer Ingelheim, Takeda, Sumitomo Dainippon Pharma and Mitsubishi Tanabe Pharma; and scholarship grants from Kissei Pharmaceutical, Sanofi, Daiichi Sankyo, Mitsubishi Tanabe Pharma, Takeda, Japan Tobacco, Kyowa Kirin, Sumitomo Dainippon Pharma, Astellas Pharma, MSD, Eli Lilly Japan, Ono Pharmaceutical, Sanwa Kagaku Kenkyusho, Nippon Boehringer Ingelheim, Novo Nordisk Pharma, Novartis Pharma, Teijin Pharma and Life Scan Japan. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jebiom.2022.104342>.

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