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Preventive effect of empagliflozin and ezetimibe on hepatic steatosis in adults and murine models

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ABSTRACT

Background: Even though many oral glucose-lowering or lipid-lowering agents have already been reported to improve hepatic steatosis to some degree, which drug had a more beneficial effect on hepatic steatosis among those drugs has not been precisely explored. We analysed the effect of empagliflozi, a selective sodium-glucose cotransporter 2 inhibitor, and ezetimibe on developing hepatic steatosis.

Methods and results: Using 4005,779 patients with type 2 diabetes mellitus (T2DM) or dyslipidemia provided by the Korean National Health Insurance Service (NHIS) between January 2015 and December 2015, we analyzed the odds ratio (OR) of fatty liver development (fatty liver index [FLI] >60). Additionally, we examined the metabolic effects of ezetimibe and empagliflozin in mice fed with a choline-deficient high-fat diet, mimicking the features of human NAFLD. The experiment for agents was performed for the non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) mouse models independently. In the NHIS data, ORs for the development of fatty liver were significantly lower in all treatment groups than in the reference group, which did not receive ezetimibe or empagliflozin. (Ezetimibe therapy; OR=0.962, empagliflozin therapy; OR=0.527, ezetimibe plus empagliflozin; OR=0.509 compared to reference therapy). Unlike non-alcoholic steatohepatitis mouse model, ezetimibe, empagliflozin, and combination therapy also reduced liver steatosis in the non-alcoholic fatty liver mouse model.

Conclusions: Compared with other agents, empagliflozin and/or ezetimibe treatment reduced the risk of developing hepatic steatosis. Our data suggest that empagliflozin or ezetimibe can be primarily considered in type 2 DM or dyslipidemia patients to prevent hepatic steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an inclusive term for pathological liver conditions in patients without excessive alcohol consumption, ranging from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) [1]. Approximately a quarter of adults have NAFLD in developed countries, including South Korea [2–5]. Considering the economic burden of NAFLD is rising, there is a need to develop a novel drug treatment for NAFLD or consider combining therapies, especially when various molecular pathways and

Abbreviations: ANOVA, one-way analysis of variance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; CD-HFD, choline-deficient high-fat diet; CI, confidence interval; CVD, cardiovascular disease; DNL, de novo lipogenesis; ER, endoplasmic reticulum; FLI, fatty liver index; GGT, γ-glutamyltransferase; ICD-10, International Classification of Disease Tenth version; IPITT, intraperitoneal insulin tolerance test; NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; NHIS, National Health Insurance Service; OGTT, oral glucose tolerance test; OR, odds ratio; PCR, polymerase chain reaction; RT, real time; RER, respiratory exchange ratio; SGLT2i, sodium glucose cotransporter 2 inhibitor; T2DM, type 2 diabetes mellitus; TG, triglyceride; VO_2, oxygen consumption; VCO_2, carbon dioxide production; VLDL, very low-density lipoprotein; WC, weight circumference.

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environmental, genetic, and epigenetic factors contribute to individual NAFLD phenotypes [6]. As NAFLD still had no established treatment options, preventing NAFLD progression is also a therapeutic strategy to decrease NAFLD-related complications. To date, the most common cause of mortality related to NAFLD was the cardiovascular disease (CVD) [7]. Major risk factors for CVD include type 2 diabetes mellitus (T2DM) and dyslipidemia. For this reason, there is increasing attention to the management of T2DM or dyslipidemia patients with NAFLD to reduce the mortality related to NAFLD.

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) are orally administered; they decrease blood glucose and glycosylated hemoglobin levels and improve insulin resistance in patients with T2DM [8]. SGLT2i improves histological hepatic steatosis and inflammation in NAFLD or NASH mouse models [9–14]. Moreover, in recent randomized controlled trials, SGLT2i reduced fatty liver content and improved biological markers in NAFLD patients with T2DM [15].

Ezetimibe, which targets Niemann–Pick C1-Like 1, may improve metabolic, biochemical, and histological abnormalities of NAFLD and may be a promising treatment [16]. Further, ezetimibe ameliorates NAFLD through various mechanisms, such as decreasing liver susceptibility to oxidative injury or modulating autophagy and a hepatocyte-driven exosome pathway, as found in an NAFLD mouse model [17,18].

However, some unresolved issues regarding SGLT2i and ezetimibe treatment for NAFLD exist. First, as insulin resistance plays an essential role in developing NAFLD, many oral glucose-lowering or lipid-lowering agents, as well as SGLT2i and ezetimibe, have already been reported to improve hepatic steatosis to some degree. Nevertheless, which drug had more beneficial effect on hepatic steatosis among those drugs has not been precisely explored. Second, the effects of ezetimibe on hepatic steatosis have not been fully elucidated in clinical studies. Third, it is unclear whether SGLT2i and ezetimibe has therapeutic effects on livers with advanced-stage NAFLD, such as NASH. Since previous studies were cross-sectional, they could not observe whether the therapeutic effect of agents changed with liver disease progression.

Here, we evaluated the therapeutic effect of empagliflozin, a commonly used SGLT2i in clinical practice, and ezetimibe on hepatic steatosis among Korean patients with T2DM or dyslipidemia compared to other agents. Additionally, we sought to identify whether the effects of empagliflozin and ezetimibe treatment differed according to NAFLD stages, NAFL, and NASH in an NAFLD mouse model.

2. Material and methods

2.1. Participants selection criteria

For this study, we initially enrolled all participants with a history of T2DM (ICD-10 codes: E11–E14 along with a prescription for glucose-lowering agents or fasting glucose ≥126 mg/dL or dyslipidemia (ICD-10 code: E78 along with a prescription for lipid-lowering agents or total cholesterol levels ≥240 mg/dL between January 2015 and December 2015 (N = 4005,779). Among them, 749,696 individuals who were re-examined within 1 year of cohort entry and 46,813 participants with missing variables were excluded. Subsequently, 4460 patients aged < 20 years, 16,807 individuals diagnosed with liver cirrhosis (ICD-10 code: K703, K746), 2105 with hepatocellular carcinoma (ICD-10 code: C22), and 561,642 with viral hepatitis, (ICD-10 codes: B15-B19) were also excluded to avoid unexpected effects of pre-existing diseases. To exclude alcohol-induced hepatic steatosis, we also excluded 328,540 heavy alcohol consumers (daily alcohol consumption, ≥30 g for men and ≥20 g for women). To compare the effect of empagliflozin or ezetimibe with that of other oral agents, we also excluded individuals who did not receive any treatment with oral glucose-lowering agents (N = 184,279) or lipid-lowering agents (N = 618,022). In addition, those who were prescribed other SGLT2i agents (dapagliflozin, canagliflozin, and ipragliflozin) were also excluded to avoid the comparative efficacy of empagliflozin (N = 17,872). Additionally, 27,644 participants who were prescribed empagliflozin and/or ezetimibe less than 90 days were excluded. Finally, 274,424 participants with a fatty liver index (FLI) > 60 at enrollment were also excluded to avoid individuals with pre-existing fatty liver disease before the start of treatment. The remaining 1173,475 participants were analyzed and followed up. A summary of the study population selection process is presented in Fig. 1. The study protocol was approved by the Yonsei Severance Hospital Institutional Review Board (institutional review board number: 4–2020–1028) and conducted in accordance with the principles of the Declaration of Helsinki.

2.2. National Health Insurance health examination cohort

The Korean National Health Insurance Service (NHIS) is a national insurer managed by the Korean government, which covers approximately 97% of the total Korean population and conducts mandatory health screening for insured individuals aged > 40 years. Therefore, the Korean NHIS database provides extensive information, including demographic characteristics (i.e., sex and age), prescription records, and diagnostic codes from the International Classification of Disease Tenth version (ICD-10) codes. Additionally, physical parameters, such as height, weight, and laboratory test results, are also available in the NHIS database. Any researcher can use the NHIS database after the official review committee approves the study protocol. Raw data obtained from the NHIS were supplied with anonymized patient identifiers.

2.3. Risk of hepatic steatosis

To assess the risk of hepatic steatosis development in the NHIS cohort, we used the FLI [19–21], which can be calculated using non-invasive parameters based on triglyceride (TG), γ-glutamyl-transferase (GGT), body mass index (BMI), and weight circumference (WC) as follows:

$$FLI = (e^{[0.953 \times ln(TG) + 0.139 \times BMI + 0.718 \times ln(GGT)}$$

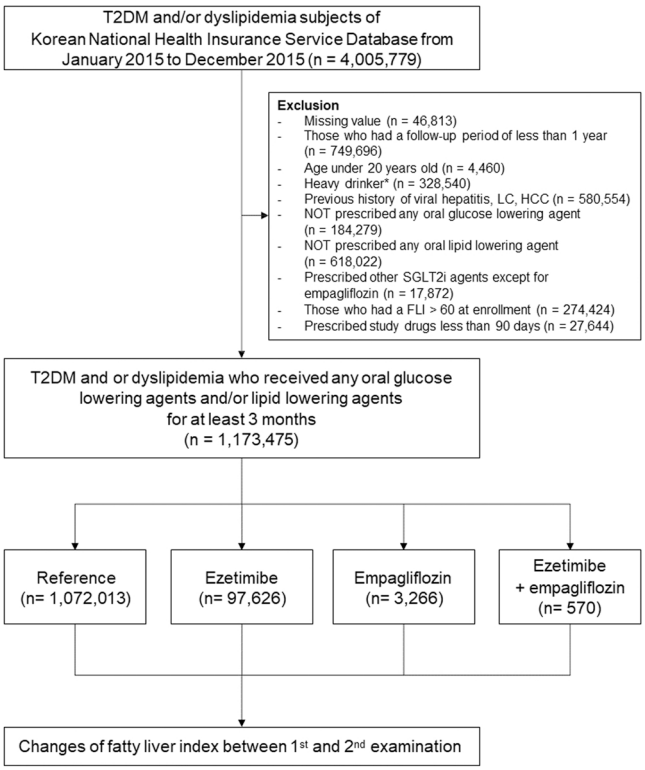

Fig. 1. Flow diagram of the study population.

$+ 0.053 \times WC - 15.745]) / (1 + e\ [0.953 \times ln(TG) + 0.139 \times BMI + 0.718 \times ln(GGT) + 0.053 \times WC - 15.745]) \times 100$.

The Korean Association for the Study of the Liver Guideline for NAFLD reported the cutoff value of FLI for diagnosing hepatic steatosis to be more than 60, with a positive predictive value of 99% [22]. That cutoff value of FLI also showed acceptable accuracy among a Korean population [23,24]. Therefore, we categorized participants as having a fatty liver (FLI ≥60) or without a fatty liver (FLI <60).

2.4. Animal models

In this study, a choline-deficient high-fat diet (CD-HFD; D05010402, Research Diets, New Brunswick, NJ, USA)-induced NAFLD mouse model was used. Six-week-old C57BL/6 N male mice were purchased from Orient Bio (Sungnam, South Korea). The mice had free access to food and water, with a temperature maintained at 23 °C ± 2 °C, humidity of 60% ± 10%, and 12 h light/dark cycles. These mice were randomly assigned to one of the following five groups (five mice in each group): chow diet group, CD-HFD vehicle-treated group, CD-HFD empagliflozin-treated group, CD-HFD ezetimibe-treated group, and CD-HFD ezetimibe plus empagliflozin-treated group. We conducted two experiments; experiment 1, which induced the early stages of NAFLD and NAFL, and experiment 2, which induced the progressive stages of NAFLD and NASH. The experimental design is summarized in Supplementary Fig. 1.

In experiment 1, the mice were fed a CD-HFD for 8 weeks to induce NAFL status. CD-HFD along with 10-mg/kg empagliflozin (empagliflozin group), 10-mg/kg ezetimibe (ezetimibe group), and 10-mg/kg empagliflozin plus 10-mg/kg ezetimibe (combination group) was administered once daily by oral gavage for 8 weeks. In experiment 2, the mice were fed a CD-HFD for 30 weeks to induce NASH. CD-HFD along with 10-mg/kg empagliflozin (empagliflozin group), 10-mg/kg ezetimibe (ezetimibe group), and 10-mg/kg empagliflozin plus 10-mg/kg ezetimibe (combination group) was administered once daily by oral gavage for 12 weeks. The chow diet and CD-HFD with vehicle groups received the same volume of phosphate-buffered saline orally during the experiment. After 8 weeks (experiment 1) and 12 weeks (experiment 2), the mice were anesthetized and sacrificed, and blood was collected via heart puncture. Tissues were harvested, snap-frozen in liquid nitrogen, stored at − 70 °C or fixed in formalin, and embedded in paraffin. All animal studies were approved by the Animal Care and Use Committee of Yonsei University College of Medicine (#2018–0074).

2.5. Drugs and diet

Empagliflozin was purchased from Boehringer Ingelheim Pharma GmbH Co. (KG, Biberach an der Riss, Germany), and ezetimibe was purchased from Cayman Chemical (No. 16331; Ann Arbor, MI, USA). The doses of empagliflozin (10 mg/kg) [11,25] and ezetimibe (10 mg/kg) [18,26,27] were determined based on the previous reports. The chow diet contained 22% protein, 6% fat, and 47% carbohydrate, and the CD-HFD contained 22.6% protein, 23.5% fat (43% energy from fat), and 5.4% fiber.

2.6. Metabolic analysis

To measure the metabolic rate, the mice were individually housed in an indirect calorimetry system (TSE system; PhenoMaster, Bad Homburg, Germany). The mice were acclimatized to the cages for 2 days, and oxygen consumption (VO_2), carbon dioxide production (VCO_2), food intake, and locomotor activity were monitored for 3 days, while food and water were provided ad libitum.

2.7. Histological examination

Histological examination of the liver was performed. Paraffin-embedded sections were stained with hematoxylin and eosin and Sirius red. The severity of histopathology was assessed by a NAFLD scoring system described by Liang et al. [28]. This system consists of two parameters; steatosis and inflammation. First, steatosis was quantified by the microvesicular steatosis, macrovesicular steatosis and hepatocellular hypertrophy. Each parameter was given a score, and each category's severity was assessed as follows: 0 (<5%), 1 (5–33%), 2 (34–66%) and 3 (>66%). Thus, the range of the steatosis scores, which included macrovesicular steatosis, microvesicular steatosis, and hypertrophy, was 0–9. The severity of the inflammation was determined by counting the inflammatory cells. There were four categories into which the inflammatory score was divided: 0 (0.5 foci), 1 (0.5–1.0 foci), 2 (1.0–2.0 foci), and 3 (>2.0 foci). We identified the Sirius-red stained area by utilizing the ImageJ program.

2.8. Biochemical parameters

Immediately before sacrifice, body and liver weights were measured, and the liver-to-body weight ratio was calculated. Blood parameters, including biochemical aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, and triglyceride (TG), were measured.

2.9. Measurement of hepatic triglyceride, cholesterol, and free fatty acid levels

After homogenization, total cholesterol, triglyceride, and free fatty acid contents in liver tissues were measured using a Triglycerides Quantification Kit (BM-TGF-100; Biomax, Rockville, MD, USA), Free Fatty Acid Quantification Kit Colorimetric/Fluorometric (BM FFA-100; Biomax, Rockville, MD, USA), and Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay Kit (BM-CHO–100; Biomax, Rockville, MD, USA), respectively, according to the manufacturer's instructions.

2.10. Oral glucose tolerance test and intraperitoneal insulin tolerance test

To evaluate insulin resistance, the oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (IPITT) were performed. The OGTT protocol was as follows: fasting blood glucose was measured (6-h fast, blood collected from the mouse tail vein) using a glucometer. Glucose was administered by gavage (25% glucose solution, 1 g/kg mice), and blood glucose was measured again at 15, 30, 45, 60, 75, 90, 105, and 120 min after gavage. The IPITT procedure protocol was as follows: fasting blood glucose was measured (4-h fast, blood collected from the mouse tail vein) using a glucometer. Next, insulin was injected intraperitoneally (1 U/kg), and blood glucose was measured again at 15, 30, 45, 60, 75, 90, 105, and 120 min after injection.

2.11. RNA isolation and real-time PCR analyses

Total RNA was extracted from liver tissues using the RiboEx reagent (#305–101; GeneAll, Seoul, South Korea). 1 μg of total RNA was used to generate complementary DNA with the iScript complementary DNA Synthesis Kit (#1708890; Bio-Rad Laboratories, Hercules, CA, USA,), and quantification real-time (RT)-PCR was performed with SYBR green fluorescence on a StepOnePlus instrument RT-PCR system (Applied Biosystems, Waltham, MA, USA). Gene expression was quantified using the ΔΔCt method with the glyceraldehyde-3-phosphate dehydrogenase gene as a reference gene, and the chow diet group was used as the reference group. Primer sequences are listed in Supplementary Table 1.

2.12. Statistical analyses

During analysis of the NHIS data, baseline characteristics were compared using one-way analysis of variance (ANOVA) for continuous variables and chi-square tests for categorical variables; continuous

variables are expressed as mean ± standard error of the mean, and categorical variables are expressed as frequency (percentage). The adjusted odds ratio (OR) with 95% confidence interval (CI) were estimated using multivariable logistic regression analysis. The regression models were sequentially adjusted for age and sex, income, hypertension, smoking, drinking, exercise, and FLI, levels of serum glucose, total cholesterol, HDL, LDL, AST and ALT level, T2DM and dyslipidemia duration, and observation period. In addition, we performed subgroup analyses with participants stratified by age (<65 or ≥65 years) and sex (male or female), duration of T2DM (<5 years or ≥5 years), and duration of dyslipidemia (<5 years or ≥5 years). Multiple comparisons were analyzed using the post-hoc Tukey test after ANOVA. In mouse experiment, the Kruskal-Wallis test followed by the post-hoc Mann-Whitney U test analysis methods were employed to assess the differences among different groups. All statistical analyses were conducted using SAS 9.4 software (SAS Institute, Cary, NC, USA). A P-value $< 0.05$was considered statistically significant. Data visualization and analysis were performed using GraphPad Prism, version 9.13 for Windows (GraphPad Software, La Jolla California, USA).

3. Results

3.1. Baseline characteristics of the study population

Among the 4005,779 participants included in the NHIS cohort, we included 1173,475 patients with T2DM or dyslipidemia newly initiating oral glucose-lowering agents or lipid-lowering agents. Participants were divided into four groups based on treatment—97,626 received ezetimibe treatment, 3266 received empagliflozin treatment, 570 received ezetimibe plus empagliflozin (combination group), and 1072,013 received other oral glucose-lowering agents or lipid-lowering agents other than ezetimibe nor empagliflozin (Fig. 1).

Among the participants enrolled in the Korean National Health Insurance Service cohort, patients with T2DM or dyslipidemia who were prescribed on oral glucose-lowering agents or lipid-lowering agents were included in this study. Heavy drinkers were defined as follows: men consuming > 30 g alcohol per day and women consuming > 20 g alcohol per day. The fatty liver index was used to evaluate the development of fatty liver. Abbreviations: HCC, hepatocellular carcinoma; LC, liver cirrhosis; SGLT2i, sodium-glucose cotransporter 2 inhibitor; T2DM, type 2 diabetes mellitus.

The remaining 1072,013 individuals were included in the reference group. The mean age (SD) of the study population varied across group classifications, ranging from 56.02 (9.44) years in the combination group to 61.08 (10.19) years in the reference group. The proportion of men (number) ranged from 39.76% (38,815) in the ezetimibe group to 50% (285) in the combination group. The duration of T2DM ranged from 1.96 (3.95) years in the ezetimibe group to 7.64 (4.77) years in the empagliflozin group, and the duration of dyslipidemia ranged from 4.54 (4.17) years in the reference group to 5.95 (4.5) years in the combination group (Table 1). Changes in biochemical and metabolic variables are summarized in Supplementary Table 2. There were significant decreases in several variables including BMI, waist circumference, total cholesterol, AST, ALT, rGTP, HDL cholesterol, LDL cholesterol, and triglyceride in all treatment groups after treatment.

3.2. The preventive effect of ezetimibe and empagliflozin on the risk of fatty liver

Using the FLI, we initially analyzed the OR for fatty liver development. Compared to the reference group, the ORs (95% CI) for fatty liver development in the ezetimibe group 0.962 (0.936–0.989). The ORs (95% CIs) for fatty liver development in the empagliflozin group were 0.527 (0.454–0.611). The ORs (95% CIs) for fatty liver development in the combination group were 0.509 (0.362–0.714) (Fig. 2). Ezetimibe and empagliflozin reduced fatty liver development more effectively than other oral glucose-lowering or lipid-lowering agents, and empagliflozin more effectively reduced fatty liver development than ezetimibe. The combination treatment of empagliflozin plus ezetimibe exhibited less fatty liver development than ezetimibe and empagliflozin treatment.

This Forest plot indicates the odds ratio of each agent group over the reference group for the endpoint of FLI ≥ 60. The OR and 95% CI were adjusted for age, sex, income, HTN, smoke, drink, exercise, FLI, glucose, total cholesterol, HDL, LDL, AST, ALT, T2DM duration, DYS duration, Pre exam. ~ Post exam. FLI, fatty liver index; OR, odds ratio; CI, confidence interval; HTN, hypertension; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T2DM, type 2 diabetes mellitus; DYS, dyslipidemia.

Ezetimibe and empagliflozin decreased the risk of fatty liver after data stratification according to age, sex, and T2DM and dyslipidemia duration (Table 2). The protective effect of ezetimibe and empagliflozin against fatty liver development was significant among participants with T2DM (OR, 0.389; 95% CI: 0.22–0.688) and dyslipidemia (OR, 0.334; 95% CI: 0.186–0.6) for < 5 years. These results suggest that combination therapy was effective for those with a shorter disease period than those with a more extended disease period.

3.3. Modeling NAFLD in mice

Based on these results, we further investigated whether ezetimibe and empagliflozin had anti-steatotic effects in an NAFLD mouse model. As we aimed to investigate the therapeutic effect of ezetimibe and empagliflozin on NAFLD according to disease progression, we performed a pilot study before the intervention started. The pilot experiments were divided into experiments 1 and 2, as previously described in Methods. In the NAFL model, liver histology of mice revealed only steatosis, but no significant liver inflammation or fibrosis was identified (Supplementary Fig. 2a). However, in the NASH model, steatosis, inflammatory cell infiltration, and fibrosis were observed in the liver histology of mice fed CD-HFD for 30 weeks (Supplementary Fig. 2a-c). Additionally, C57BL/6 N mice fed CD-HFD developed rapid weight gain and obesity compared to mice fed the chow diet (Supplementary Fig. 2d). As NAFLD is closely linked to the development of metabolic syndrome, insulin resistance was assessed using the IPITT. CD-HFD-fed mice developed significant insulin resistance at 8 weeks, which was sustained for up to 30 weeks (Supplementary Fig. 2e). Overall, these findings suggest that the NAFL model induced in experiment 1 may reflect an early stage of NAFLD, whereas the NASH model derived in experiment 2 may reflect an advanced stage of NAFLD.

3.4. Effect of empagliflozin and ezetimibe on the NAFL model

To confirm whether the preventive effect of agents, which was identified in the human NHIS data, differed according to the disease progression, we conducted two mouse experiments separately. First, to investigate the effect of empagliflozin and ezetimibe on the NAFL model, mice were fed CD-HFD for 8 weeks and received empagliflozin and/or ezetimibe for 8 weeks (Supplementary Fig. 1a). After the intervention started, body weight was comparably reduced in ezetimibe and/or empagliflozin treatment compared to the vehicle group (Fig. 3a, Supplementary Fig. 3). Only significantly less liver weight and liver weight-to-body weight ratio were seen in the combination group compared to the vehicle-treated group (Fig. 3a). Only the ezetimibe plus empagliflozin group ($P = 0.0278$) had a lower histology score for steatosis than the vehicle group (Fig. 3b-c). Furthermore, serum ALT levels and intrahepatic lipid content, which consisted of hepatic cholesterol, hepatic triglyceride, and hepatic free fatty acid, were significantly lower in the empagliflozin, ezetimibe, and combination groups; there was no significant difference among the treatment groups (Fig. 3d, e).

Since impaired glucose metabolism is highly associated with NAFLD, we also performed the IPITT and OGTT to evaluate the effects of

Table 1
Baseline characteristics of the NHIS study population.

	Reference	Ezetimibe	Empagliflozin	Ezetimibe + empagliflozin	P-value	Multiple comparison (Bonferroni)†					
n	1072,013	97,626	3266	570		a	b	c	d	e	f
Sex, male	458,379 (42.76)	38,815 (39.76)	1519 (46.51)	285 (50)	< 0.0001	< 0.0001	< 0.0001	0.0029	< 0.0001	< 0.0001	0.7405
Income, low 25%	206,985 (19.31)	18,456 (18.9)	624 (19.11)	118 (20.7)	0.0173	0.0134	1	1	1	1	1
HTN	616,515 (57.51)	51,915 (53.18)	1899 (58.14)	312 (54.74)	< 0.0001	< 0.0001	1	1	< 0.0001	1	0.7722
Smoke					< 0.0001	0.0419	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.6731
Non	748,805 (69.85)	68,663 (70.33)	2075 (63.53)	336 (58.95)							
Ex	199,106 (18.57)	17,825 (18.26)	649 (19.87)	128 (22.46)							
Current	124,102 (11.58)	11,138 (11.41)	542 (16.6)	106 (18.6)							
Drink					0.003	0.112	0.0308	1	0.008	1	1
Non	753,044 (70.25)	68,929 (70.61)	2221 (68)	394 (69.12)							
Mild	318,969 (29.75)	28,697 (29.39)	1045 (32)	176 (30.88)							
Regular exercise	259,941 (24.25)	23,944 (24.53)	784 (24)	130 (22.81)	0.207	0.3129	1	1	1	1	1
Age	61.08 ± 10.19	59.88 ± 9.66	57.42 ± 9.86	56.02 ± 9.44	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0097
Height (cm)	159.96 ± 8.8	159.96 ± 8.66	161.63 ± 8.49	162.73 ± 8.77	< 0.0001	1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0281
Weight (kg)	61.88 ± 9.35	62.13 ± 9.2	65.58 ± 9.45	66.54 ± 9.46	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1534
BMI (kg/m^2)	24.12 ± 2.6	24.22 ± 2.52	25.05 ± 2.7	25.07 ± 2.53	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1
Waist circum. (cm)	81.89 ± 7.36	81.85 ± 7.22	84.43 ± 7.05	84.43 ± 6.53	< 0.0001	0.5691	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1
SBP	125.77 ± 14.49	124.82 ± 14.3	126.04 ± 14.1	125.25 ± 14.19	< 0.0001	< 0.0001	1	1	< 0.0001	1	1
DBP	76.62 ± 9.41	76.28 ± 9.39	76.54 ± 9.22	76.74 ± 9.39	< 0.0001	< 0.0001	1	1	0.6985	1	1
Glucose (mg/dl)	110.61 ± 33.81	107.9 ± 31.36	150.54 ± 48.76	155.34 ± 54.42	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1991
Total cholesterol (mg/dl)	193.95 ± 48.68	201.18 ± 54.6	172.45 ± 40.1	180.93 ± 47.05	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
HDL (mg/dl)	54.7 ± 14.28	54.98 ± 13.93	50.61 ± 12.34	50.74 ± 12.57	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1
LDL(mg/dl)	114.47 ± 44.56	119.56 ± 50.19	95.89 ± 35.59	102.33 ± 41.03	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0006
TG (mg/dl)	110.94 (110.84–11.04)	119.13 (118.78–119.5)	116.55 (114.67–18.46)	125.09 (120.28–130.09)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0629	0.0962	0.0063
AST (IU/l)	24.31 (24.29–24.32)	24.91 (24.86–24.96)	23.74 (23.43–24.05)	24.56 (23.8–25.33)	< 0.0001	< 0.0001	0.0002	1	< 0.0001	1	0.2909
ALT (IU/l)	22.19 (22.17–22.21)	23.19 (23.13–23.26)	24.56 (24.14–24.98)	26.06 (24.98–27.18)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0632
rGTP (U/l)	24.39 (24.37–24.42)	24.95 (24.86–25.03)	26.7 (26.21–27.2)	27.98 (26.82–29.2)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.3356
GFR	87.77 ± 47.5	88.01 ± 51.24	94.01 ± 69.3	90.77 ± 48.41	< 0.0001	0.842	< 0.0001	0.794	< 0.0001	1	1
FLI	25.95 ± 15.84	27.46 ± 15.98	32.19 ± 16.02	34.02 ± 15.74	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0695
T2DM duration	2.51 ± 4.37	1.96 ± 3.95	7.64 ± 4.77	7.05 ± 4.89	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0423
DYS duration	4.54 ± 4.17	5.47 ± 4.23	5.08 ± 4.5	5.95 ± 4.5	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0367	0.0001
Pre exam. ~ Post exam., days	668.33 ± 151.37	689.25 ± 141.13	723.44 ± 121.67	722.87 ± 121.21	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1

Notes: Data expressed as number, number (%), mean ± standard deviation. †A multiple comparison test following one-way ANOVA was used to compare the differences among groups. a: Reference vs. Ezetimibe; b: Reference vs. Empagliflozin, c: Reference vs. Ezetimibe + empagliflozin, d: Ezetimibe vs. Empagliflozin, e: Ezetimibe vs. Ezetimibe + empagliflozin, f: Empagliflozin vs. Ezetimibe + empagliflozin.
Abbreviations: HTN, hypertension; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; LDL, low density lipoprotein; TG, triglyceride; AST, aspartate aminotransferase; ALT, alanine aminotransferase; rGTP, γ-glutamyl transferase; GFR, glomerular filtration rate; FLI, fatty liver index; T2DM, type 2 diabetes mellitus; DYS, dyslipidemia.

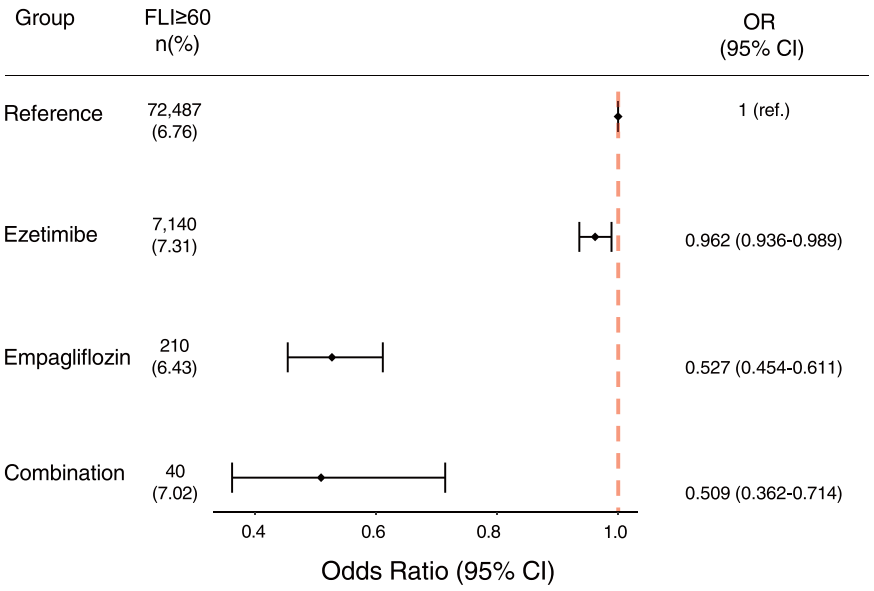

Fig. 2. Comparative risk for fatty liver development between reference, empagliflozin and/or ezetimibe group.

empagliflozin and/or ezetimibe on glucose homeostasis (Supplementary Fig. 4a-b). As expected, treatment with empagliflozin and empagliflozin plus ezetimibe improved glucose tolerance (Supplementary Fig. 4a). Moreover, during the IPITT for measuring insulin resistance, all treatment groups showed reduced insulin resistance compared to the vehicle group in the NAFL model (Supplementary Fig. 4b). To gain insight into the altered metabolic state, the changes in energy expenditure and activity were assessed by using a metabolic cage (Supplementary Fig. 5). The respiratory exchange ratio (VCO2/VO2) of the chow diet group was close to 1, and those of all other CD-HFD-fed groups was close to 0.7 (Supplementary Fig. 5a-c). A comparison of the treatment groups with the vehicle group revealed that only empagliflozin treatment increased the average energy expenditure (Supplementary Fig. 5d); no treatment group showed a statistically significant difference in the activity count or food intake compared to the vehicle group (Supplementary Fig. 5e-f).

(a–e) The 6-week-old C57BL/6 N mice were fed CD-HFD for 8 weeks and then treated with vehicle or ezetimibe and/or empagliflozin for 8 weeks. (a) Body weight change during treatment, body weight at sacrifice, liver weight, and liver weight-to-body weight ratio. (b) Representative hematoxylin and eosin staining images of liver sections from the chow diet group and CD-HFD-fed vehicle-treated and treatment groups. Macrovesicular steatosis (bold circle), microvesicular steatosis (dotted circle). Scale bar, 100 μm. (c) Steatosis histology results in mice fed the chow diet, CD-HFD, a vehicle, or an intervention. (d) Hepatic lipid levels after 8 weeks of treatment. (e) Serum AST, ALT, total cholesterol, and triglyceride levels. * $P < 0.05$, * * $P < 0.01$ compared to the vehicle-treated group, using the post-hoc pairwise Mann-Whitney U tests after Kruskal-Wallis test with Bonferroni correction. CD-HFD, choline-deficient high-fat diet; Eze, ezetimibe; Empa, empagliflozin.

3.5. Effect of empagliflozin and ezetimibe on NASH model

Next, we examined the therapeutic effect of empagliflozin and ezetimibe on NASH, an advanced stage of NAFL. Mice were fed CD-HFD for 30 weeks and administered ezetimibe and empagliflozin for 12 weeks (Supplementary Fig. 1b). Unlike the NAFL model, the body weight, liver weight, serum ALT, and intrahepatic lipid content of the ezetimibe, empagliflozin, and combination groups were not significantly improved compared with those in the vehicle group (Fig. 4a-e). Inflammatory and fibrogenic genes were also not down-regulated in the liver of any intervention group compared to the vehicle group (Supplementary Fig. 6). Regarding the glucose tolerance test, ezetimibe had less effect on glucose tolerance and insulin sensitivity in the NASH mouse model (Supplementary Fig. 4c-d).

(a–e) The 6-week-old C57BL/6 N mice were fed a CD-HFD for 30 weeks and then treated with vehicle or ezetimibe and/or empagliflozin for 12 weeks. (a) Body weight change during treatment, body weight at sacrifice, liver weight, and liver weight-to-body weight ratio. (b) Representative hematoxylin and eosin staining (upper panels) and Sirius red staining (lower panels) images of liver sections from the chow diet group, and CD-HFD-fed vehicle-treated, and treatment groups. Macrovesicular steatosis (bold circle), microvesicular steatosis (dotted circle), and inflammatory foci (arrow). Scale bar, 100 μm. (c) Histology scores of steatosis and inflammation in mice fed chow diet, CD-HFD, a vehicle, or an intervention. (d) Quantification of Sirius-red stained area. (e) Hepatic lipid levels after 12 weeks of treatment. (f) Serum AST, ALT, total cholesterol, and triglyceride levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the vehicle-treated group, using the post-hoc pairwise Mann-Whitney U tests after Kruskal-Wallis test with Bonferroni correction. CD-HFD, choline-deficient high-fat diet; Eze, ezetimibe; Empa, empagliflozin.

3.6. Expression of lipid metabolism-related genes in the liver

To elucidate the relevant metabolic pathways underlying the effects of ezetimibe and empagliflozin on hepatic steatosis, we performed gene expression analyses for an array of lipid metabolic markers. The mRNA levels of de novo lipogenesis (DNL)-related genes, including *Srebf1*, *Fas*, *Acaca*, *Pparg,* and *Gpat1*, were increased more in livers of mice with NAFL and NASH that in livers of chow diet-fed mice (Fig. 5a). In the NAFL model, DNL-related gene upregulation was significantly suppressed in all treatment groups compared to that in the vehicle group. Additionally, the hepatic gene expressions involved in fatty acid uptake were examined. The CD-HFD produced increased gene expression of *Cd36* and *Fabp1* was dramatically down-regulated by the combination therapy (Fig. 5b). *Acox1* and *Pparα* were two of the fatty acid oxidation-related genes whose mRNA expression was considerably increased by the combination group (Fig. 5c). However, no treatment group's expression of VLDL synthesis genes—including *Mttp* and *ApoB*—changed in comparison to the vehicle group's (Fig. 5d). The DNL, fatty acid absorption, fatty acid oxidation, and synthesis of VLDL genes in the NASH model did not alter between the treatment and vehicle groups (Fig. 5a-d).

mRNA expression of genes involved in lipid metabolism in the liver,

Table 2
Subgroup analysis of comparative risk for fatty liver development between reference, empagliflozin and/or ezetimibe.

		FLI ≥ 60, n (%)	Odds ratio†	P for interaction
			Model 3	
Age < 65	Reference	51,483 (7.56)	1 (ref.)	0.5101
	Ezetimibe	5362 (7.91)	0.958 (0.927, 0.989)	
	Empagliflozin	170 (6.81)	0.479 (0.405, 0.567)	
	Ezetimibe + empagliflozin	35 (7.46)	0.494 (0.343, 0.713)	
Age ≥ 65	Reference	21,004 (5.37)	1 (ref.)	
	Ezetimibe	1778 (5.96)	0.981 (0.93, 1.036)	
	Empagliflozin	40 (5.21)	0.578 (0.412, 0.812)	
	Ezetimibe + empagliflozin	5 (4.95)	0.787 (0.308, 2.016)	
Male	Reference	45,365 (9.9)	1 (ref.)	0.9266
	Ezetimibe	4303 (11.09)	0.959 (0.924, 0.994)	
	Empagliflozin	125 (8.23)	0.513 (0.421, 0.625)	
	Ezetimibe + empagliflozin	24 (8.42)	0.493 (0.316, 0.769)	
Female	Reference	27,122 (4.42)	1 (ref.)	
	Ezetimibe	2837 (4.82)	0.967 (0.926, 1.009)	
	Empagliflozin	85 (4.87)	0.475 (0.376, 0.6)	
	Ezetimibe + empagliflozin	16 (5.61)	0.562 (0.329, 0.96)	
T2DM duration < 5 yrs	Reference	57,547 (6.88)	1 (ref.)	0.2726
	Ezetimibe	5835 (7.21)	0.955 (0.926, 0.985)	
	Empagliflozin	94 (8.58)	0.543 (0.431, 0.683)	
	Ezetimibe + empagliflozin	14 (6.25)	0.389 (0.22, 0.688)	
T2DM duration ≥ 5 yrs	Reference	14,940 (6.35)	1 (ref.)	
	Ezetimibe	1305 (7.84)	0.994 (0.931, 1.06)	
	Empagliflozin	116 (5.35)	0.467 (0.382, 0.57)	
	Ezetimibe + empagliflozin	26 (7.51)	0.631 (0.411, 0.968)	
DYS duration < 5 yrs	Reference	39,465 (6.35)	1 (ref.)	0.0508
	Ezetimibe	3232 (6.7)	0.938 (0.9, 0.977)	
	Empagliflozin	112 (6.57)	0.533 (0.433, 0.655)	
	Ezetimibe + empagliflozin	13 (5.08)	0.334 (0.186, 0.6)	
DYS duration ≥ 5 yrs	Reference	33,022 (7.32)	1 (ref.)	
	Ezetimibe	3908 (7.91)	0.984 (0.947, 1.022)	
	Empagliflozin	98 (6.28)	0.462 (0.371, 0.574)	
	Ezetimibe + empagliflozin	27 (8.6)	0.702 (0.459, 1.075)	

Notes: †The odds ratio and 95% confidence interval were adjusted for age, sex, income, HTN, smoke, drink, exercise, FLI, glucose, total cholesterol, HDL, LDL, AST, ALT, T2DM duration, DYS duration, Pre exam. ~ Post exam. Abbreviations: FLI, fatty liver index; T2DM, type 2 diabetes mellitus; DYS; dyslipidemia.

as assessed by real-time (RT)-PCR. (a-b) RT-PCR analysis of mRNA expression of genes related to de novo lipogenesis (a) and fatty acid uptake (b) in the liver. (c-d) RT-PCR analysis of mRNA expression of genes related to fatty acid oxidation (c) and VLDL generation (d) in the liver. N = 5 per group. Data are expressed as mean ± SD. * $P < 0.05$, * * $P < 0.01$ compared to the vehicle-treated group, using the post-hoc pairwise Mann-Whitney U tests after Kruskal-Wallis test with Bonferroni correction. NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; VLDL, very low-density lipoprotein.

4. Discussion

SGLT2is are novel oral glucose-lowering agents that have received attention due to their unique mechanism of inhibiting glucose reabsorption in the proximal renal tubules and increasing urinary glucose excretion [29]. SGLT2i treatment is associated with improvement in hepatic steatosis [15,30–32]. The beneficial effect of SGLT2i on hepatic steatosis was identified even in NAFLD patients without T2DM [33]. However, the sample size of previous studies was < 100 individuals, implying that larger sample sizes are needed to confirm the SGLT2i effect on hepatic steatosis [15,30–32]. Additionally, the effect of ezetimibe on NAFLD is controversial. A previous study demonstrated that ezetimibe treatment reduced the hepatic steatosis and improved insulin resistance via lowering *Cd36* gene expression in the liver [34]. Loomba et al. reported that ezetimibe did not significantly improve hepatic steatosis in a randomized controlled trial [35]. Moreover, in 2019, one randomized controlled trial showed that the use of ezetimibe did not improve hepatic steatosis [36].

Therefore, we investigated empagliflozin and ezetimibe effect on hepatic steatosis with larger samples and longer-term NHIS samples to reach a more comprehensive and reliable conclusion. Ezetimibe and empagliflozin treatments decreased the risk of developing fatty liver disease, as shown by the FLI value, in a large number of Korean patients. To our knowledge, this is the first study to demonstrate that empagliflozin or ezetimibe reduces the FLI compared to other oral glucose-lowering or lipid-lowering agents, supporting our hypothesis. In addition to combination therapy, treatment with ezetimibe or empagliflozin decreased the risk of fatty liver development compared to reference therapy. However, ezetimibe therapy had minimal effect on reducing hepatic steatosis (OR = 0.962, Fig. 2) compared to the reference group. This result was similar with the result of a previous study by Loomba et al., which showed no differential changes in hepatic steatosis between the ezetimibe-treated group and the placebo group [35]. This study revealed that the ezetimibe monotherapy, which targeted the absorption of cholesterol in the intestine, may not be a great therapeutic agent in reducing hepatic steatosis in NAFLD patients.

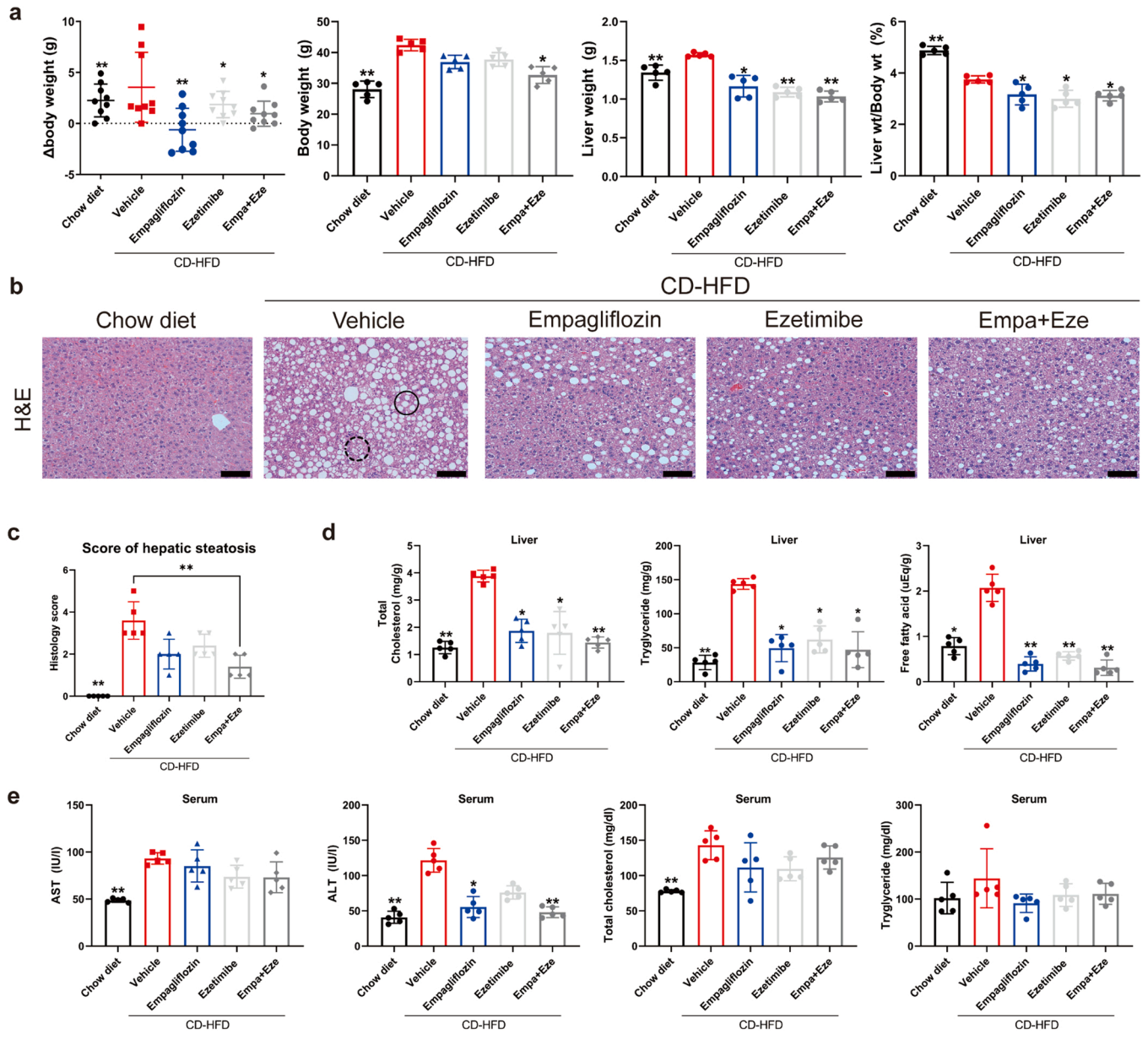

Fig. 3. Empagliflozin and ezetimibe treatment decreases the hepatic lipid contents in the NAFL mouse model.

Furthermore, using a complementary mouse model of NAFLD, we found that ezetimibe and empagliflozin ameliorated hepatic steatosis and decreased intrahepatic lipids. Translational science relies on mouse models of human diseases to improve disease models and elucidate the underlying mechanisms. Therefore, mouse models should be phenocopied as closely as possible to human disease. Regarding NAFLD, models should display all histological, metabolic, and physiological phenotypes of human NAFLD. We used a CD-HFD-induced NAFLD mouse model, which is induced by excessive caloric intake, similar to human NAFLD models. Since a previous study, which analyzed sex differences in the nutritional model of NAFLD, reported that male C57/BL6 mice developed histological features that most closely resemble those seen in human NAFLD [37], we chose male mice for an experiment. Following CD-HFD initiation, mice became obese and insulin resistant and were highly associated with human NAFLD. These phenotypes render it a more relevant model for studying NAFLD than the bile duct ligation model or other diet models, such as methionine choline-deficient high-fat diet, which are not accompanied by body weight gain. Previously, we analyzed the preventive effect of agents based on FLI in the human NHIS data; however, we could not distinguish NAFL status from NASH status. To compensate for this limitation, we performed the mouse experiments according to NAFLD stages, NAFL status, and NASH status. After confirming that the NAFL or NASH phenotypes were well induced by supplementing CD-HFD according to the feeding period, empagliflozin and/or ezetimibe were administered to mouse models. Histological analysis showed that hepatic lipid accumulation was remarkably reduced by the combination of ezetimibe and empagliflozin in the NAFL mouse model. Regarding the mechanisms underlying liver fat improvement, we found that DNL-related genes were significantly decreased in all treatment groups compared to those in the vehicle-treated group. However, only the combination group showed the substantial alterations in the fatty acid uptake- and fatty acid oxidation-related genes. In other words, because combination therapy simultaneously decreased lipogenesis and enhanced fatty acid consumption, it had a greater chance of improving hepatic steatosis than monotherapy. However, we were unable to determine whether combination therapy with empagliflozin and ezetimibe was more effective than monotherapy. Although the amelioration of hepatic steatosis was

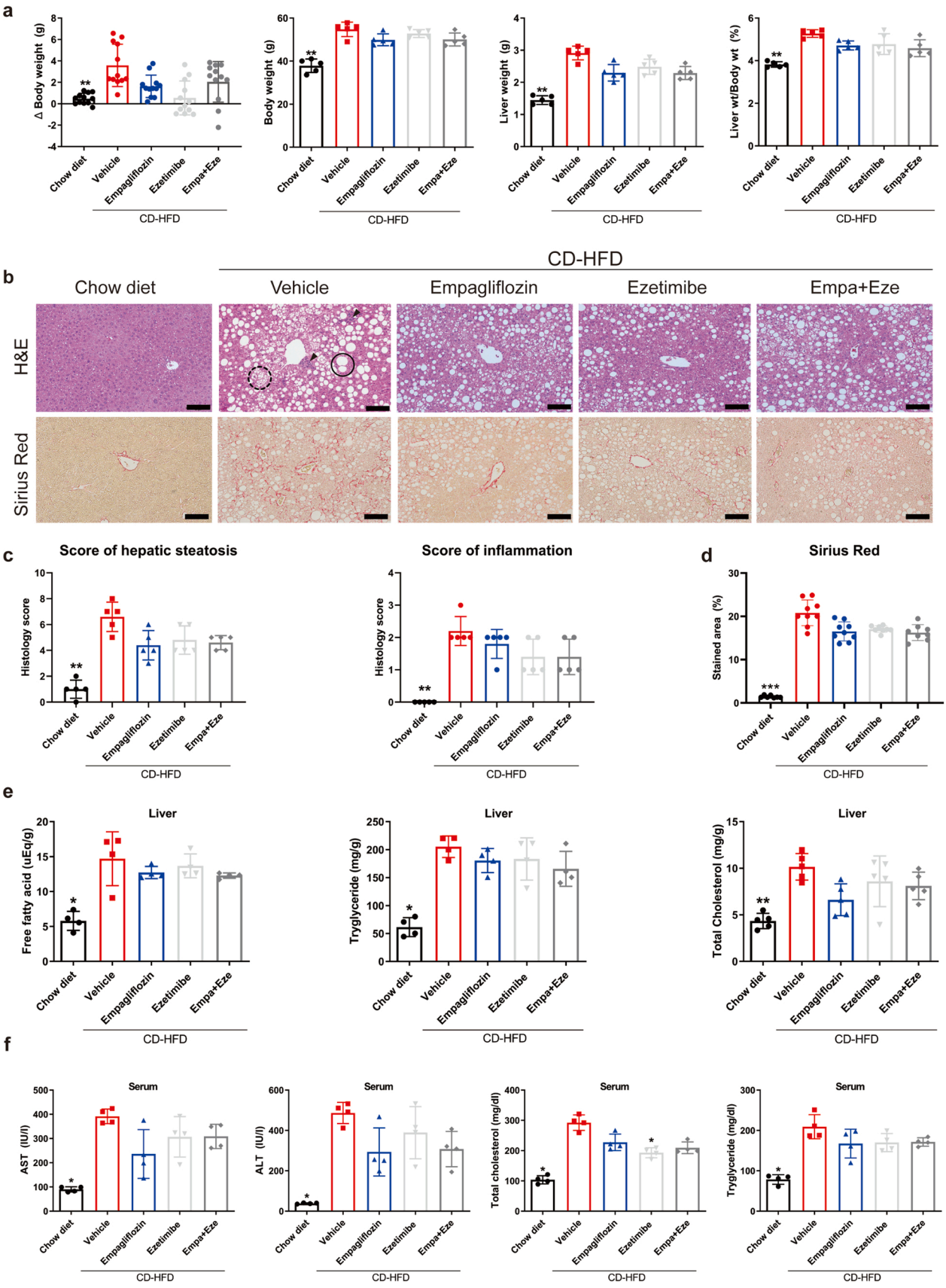

Fig. 4. Empagliflozin and ezetimibe treatment did not exert a therapeutic effect on the NASH mouse model.

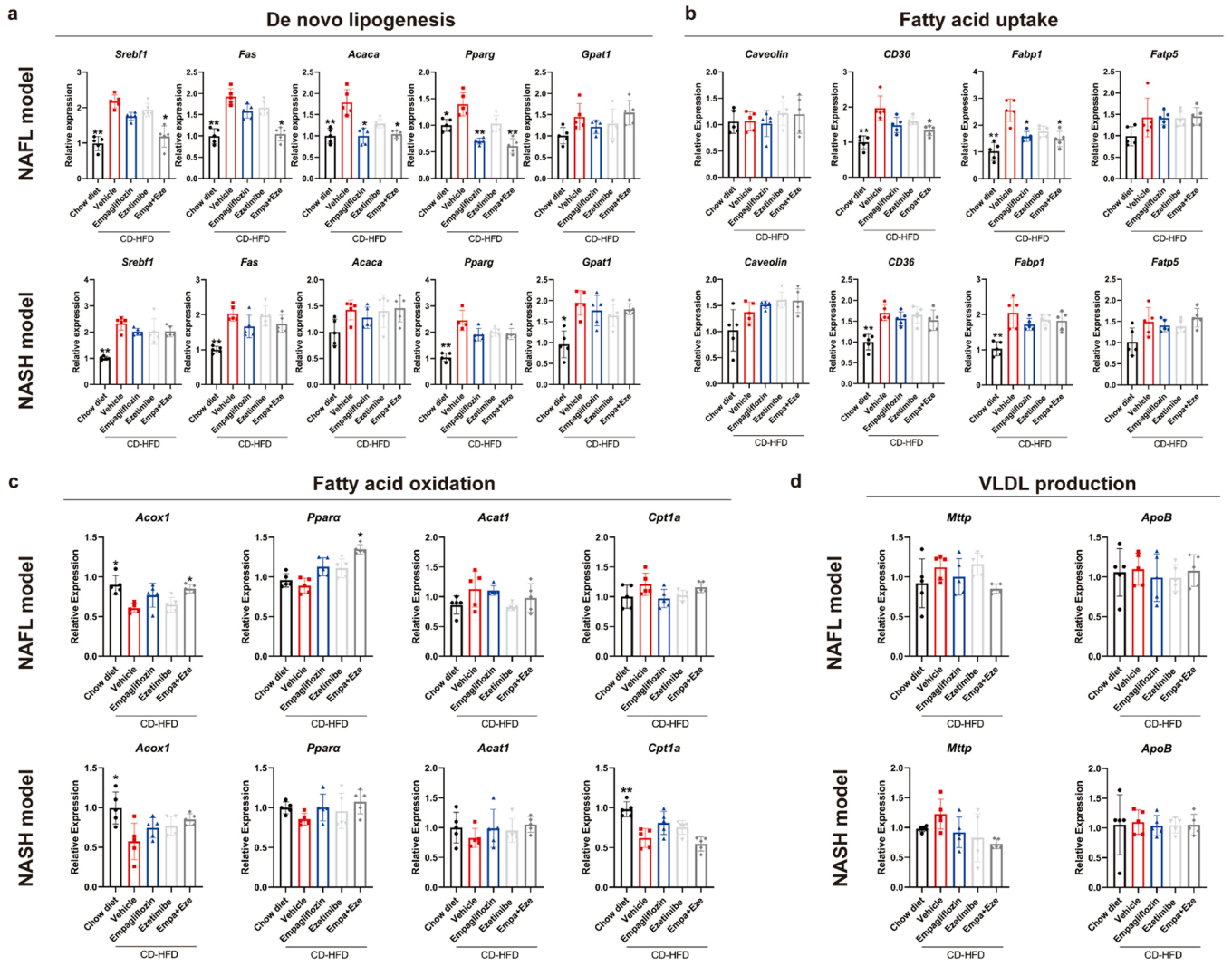

Fig. 5. Empagliflozin and ezetimibe treatment regulates hepatic lipid metabolism in the NAFL mouse model.

most prominent in the combination group, the combination of empagliflozin and ezetimibe did not show a synergistic reduction in hepatic steatosis compared to either ezetimibe or empagliflozin. Nevertheless, the combination therapy may have a several advantages. First, we confirmed that the scores for hepatic steatosis significantly decreased only in the combination group. Second, only combination group had beneficial effects on several lipid metabolism pathways in the RT-PCR, as mentioned above. We believe that by modifying de novo lipogenesis and fatty acid consumption, empagliflozin-based combination therapy has the potential to be used as a therapeutic regimen for treating NAFLD. Further investigation is required to confirm the therapeutic effect of empagliflozin and ezetimibe on NAFLD using a cohort of different ethnicities or different NAFLD mouse models.

We also investigated whether the combination of empagliflozin and ezetimibe could have therapeutic effects in advanced-stage NAFLD in a NASH model. The NASH model derived in experiment 2 reflected advanced-stage NAFLD with inflammation and fibrosis. Unlike in the NAFL model, empagliflozin treatment and empagliflozin plus ezetimibe combination treatment did not significantly improve histological steatosis or inflammation in the liver of the NASH model, implying that the therapeutic effect of empagliflozin and/or ezetimibe could be attenuated in NASH. These findings suggest that empagliflozin-based treatment is more effective in early-stage NAFLD than in advanced-stage NAFLD.

For several years, hepatic steatosis was considered a benign disease as NAFLD progression requires repeated exposure to other factors such as endoplasmic reticulum (ER) stress; however, this notion is now considered outdated [1]. Currently, the most important driver of NAFLD progression is the capacity of the liver to handle the burden of hepatic lipid content. Accumulated hepatic fat content can serve as a substrate for the generation of lipotoxic species that induce ER stress and hepatocellular injury [38]. Given that liver fat accumulation can be a source NAFLD progression, our results have the potential to be applied to therapeutic strategies for patients NAFLD who cannot metabolize hepatic lipid contents. If empagliflozin and ezetimibe treatment can decrease hepatic steatosis, they could subsequently stop progression to NASH, cirrhosis, and hepatocellular carcinoma in patients with NAFLD. Thus, empagliflozin plus ezetimibe may prevent NAFLD progression. Moreover, these results suggested that combination treatment of SGLT2i with other lipid-lowering drugs (e.g., statin) could be beneficial for NAFLD. Further studies on this combination strategy against NAFLD will be needed in the future.

Nevertheless, our study has some limitations. First, there is a limitation in using the FLI to diagnose fatty liver disease, which is due to a lack of data from imaging modalities or liver biopsy to evaluate NAFLD in the NHIS data. To overcome this limitation, we obtained histological evidence from our NAFL mouse model. Second, as empagliflozin and ezetimibe are currently not covered under health insurance for individuals without T2DM or dyslipidemia, the beneficial effect of these agents may not be applicable to the general NAFLD population;

however, in 2021, the Food and Drug Administration approved empagliflozin for the treatment of patients with heart failure with reduced ejection fraction, with or without diabetes, based on the results of the EMPEROR-Reduced trial [39]. Likewise, if the efficiency of empagliflozin in decreasing hepatic fat content is firmly confirmed in the future, SGLT2i treatment can be used for individuals with NAFLD regardless of T2DM status. Third, the interpretation of our mouse experiments needs to be considered. Although ezetimibe and empagliflozin did not exert a therapeutic effect on the NASH mouse model, this result may not apply to all NASH statuses. NASH can be subdivided according to the fibrosis stage (0−4). Liver damage and fibrosis induced by the prolonged administration of CD-HFD in experiment 2 might have been so severe that the medication effect might not have been exerted, suggesting that ezetimibe or empagliflozin can be effective for treating NASH with mild fibrosis. Therefore, the treatment effect needs to be further investigated in depth according to the various NASH statuses. Finally, the detailed therapeutic mechanism of empagliflozin and ezetimibe on hepatic steatosis was not analyzed in the present study. Functional studies of those mechanisms in the future could provide important insights into the clinical practice of NAFLD prevention.

Importantly, our results showed that combination therapy appeared to have more protective effects in patients with T2DM or dyslipidemia of a shorter duration (< 5 years). We recommend combination therapy for the initial treatment of patients with T2DM and/or dyslipidemia to prevent hepatic steatosis development.

5. Conclusions

In conclusion, empagliflozin and ezetimibe, or in combination, reduced the risk of hepatic steatosis development in the human study using NHIS data and NAFL mouse model. Based on these results, empagliflozin or ezetimibe may be considered in individuals with T2DM or dyslipidemia with suspected early-stage NAFLD rather than other oral glucose-lowering or lipid-lowering agents.

Ethics approval and consent to participate

The study protocol was approved by the Yonsei Severance Hospital Institutional Review Board (institutional review board number: 4–2020–1028) and conducted in accordance with the principles of the Declaration of Helsinki. All animal studies were approved by the Animal Care and Use Committee of Yonsei University College of Medicine (#2018–0074).

Consent for publication

All participants granted consent for the publication of the de-identified result.

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CRediT authorship contribution statement

J.Y.P. and H.Y.G. conceived and designed experiments and supervised the research. D.Y.K. and K.S.C. performed experiments and analyzed the data. D.Y.K. analyzed data of the National Health Insurance health examination cohort. D.Y.K. and H.Y.G. wrote the original draft of the manuscript. D.Y.K., K.S.C., JY.P., and H.Y.G. reviewed and edited the manuscript.

Conflict of interest statement

All authors declare no conflicts of interest.

Data Availability

Data will be made available on request. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114445.

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