Comparison of Tofogliflozin and Glimepiride Effects on Nonalcoholic Fatty Liver Disease in Participants With Type 2 Diabetes: A Randomized, 48-Week, Open-Label, Active-Controlled Trial

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OBJECTIVE

Nonalcoholic fatty liver disease (NAFLD) is a liver phenotype of type 2 diabetes and obesity. Currently, the efficacy of sodium–glucose cotransporter 2 (SGLT2) inhibitors and sulfonylureas in liver pathology and hepatic gene expression profiles for type 2 diabetes with NAFLD are unknown.

RESEARCH DESIGN AND METHODS

We conducted a 48 week, randomized, open-label, parallel-group trial involving participants with biopsy-confirmed NAFLD. A total of 40 participants were randomly assigned to receive once daily 20 mg tofogliflozin or 0.5 mg glimepiride. The primary outcome was the percentage of participants with at least an improvement in all individual scores for histological categories of steatosis, hepatocellular ballooning, lobular inflammation, and fibrosis by at least 1 point. The secondary end points were the changes in liver enzymes, metabolic markers, and hepatic gene expression profiles.

RESULTS

Fibrosis scores improved in the tofogliflozin group (60%, $P = 0.001$), whereas the change from baseline did not differ significantly between the groups ($P = 0.172$). The histological variables of steatosis (65%, $P = 0.001$), hepatocellular ballooning (55%, $P = 0.002$), and lobular inflammation (50%, $P = 0.003$) were improved in the tofogliflozin group, whereas only hepatocellular ballooning was improved in the glimepiride group (25%, $P = 0.025$). Hepatic gene expression profiling revealed histology-associated signatures in energy metabolism, inflammation, and fibrosis that were reversed with tofogliflozin.

CONCLUSIONS

Tofogliflozin and, to a lesser degree, glimepiride led to liver histological and metabolic improvement in participants with type 2 diabetes and NAFLD, with no significant difference between the agents. The hepatic expression of the genes involved in energy metabolism, inflammation, and fibrosis was well correlated with liver histological changes and rescued by tofogliflozin. We need further confirmation through long-term larger-scale clinical trials of SGLT2 inhibitors.

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Nonalcoholic fatty liver disease (NAFLD), ranging from simple fatty liver to nonalcoholic steatohepatitis (NASH), is a liver phenotype of metabolic disorders, such as diabetes, obesity, and dyslipidemia (1). NAFLD and type 2 diabetes share epidemiological and pathophysiological features. Specifically, hyperglycemia is closely associated with liver fibrosis (2), which is associated with liver cirrhosis, hepatocellular carcinoma, and prognosis in participants with NASH (3–5).

To date, some antidiabetes agents have been tested in participants with NAFLD (6–9). The guidelines in the Asian Pacific, European, and American associations recommended the administration of a peroxisome proliferator–activated receptor-γ (PPAR γ) agonist (pioglitazone) and glucagon-like peptide receptor agonists (GLP-1 RA) for the treatment of diabetes with NAFLD/NASH (10–12). However, there are concerns about adverse effects, such as weight gain, edema, fractures, and carcinogenesis, with pioglitazone or gastrointestinal adverse effects and medication burden as an injection with GLP-1 RA. Because all of these antidiabetes agents significantly reduced glycemic levels compared with placebo, liver histological improvement may be theoretically attributable to glucose reduction itself.

Both sodium–glucose cotransporter 2 (SGLT2) inhibitors and sulfonylureas are chosen as second-line therapy when glycemic control cannot be achieved with metformin or as first-line therapy when metformin is contraindicated or not tolerated (13).

In animal models of NAFLD/NASH, SGLT2 inhibitors protect against fibrosis (14,15), steatosis (15), and inflammation (15). Ipragliflozin improved liver histology due to reduction of the hepatic triglycerides and lipotoxicity in NASH-model mice with type 2 diabetes (15). To our knowledge, among past studies investigating effects of SGLT2 inhibitors on NAFLD in participants with type 2 diabetes, most of them have demonstrated that SGLT2 inhibitors exert protective effects on liver enzymes (16–20) and liver steatosis evaluated with MRS (21–26). To date, three studies have evaluated liver histology. Two were single-arm observation studies lacking a control group (27,28). However, these studies lacked a control group or histological examination, which precludes meaningful conclusions since the natural course of the disease or tight glycemic control may ameliorate liver histology in some

participants with NAFLD (2). Only one study evaluated the effects of ipragliflozin versus conventional treatments on liver histology in a 72 week randomized controlled trial (29). In this study, ipragliflozin reduced ballooning and fibrosis, but, unexpectedly, not steatosis scores.

Sulfonylureas are still reliable and potent antidiabetes agents in insulinopenic participants with type 2 diabetes and therefore are used as second-line therapy, especially when the cost is a significant issue. Past studies suggest that sulfonylureas are associated with NAFLD progression or adverse outcomes such as hepatocellular carcinoma (30–32), possibly via exaggerating insulin secretion and thereby enhancing weight gain and SREBP-1c–driven de novo lipogenesis. On the other hand, sulfonylureas reduce glucose and thereby may reduce carbohydrate response element binding protein (ChREBP)-1–driven de novo lipogenesis. Therefore, sulfonylureas may render positive and negative effects, respectively, on liver pathology in NAFLD/NASH. In the phase 3 trial, canagliflozin was noninferior to glimepiride for the reduction of hemoglobin A_{1c} (HbA_{1c}) at 52 weeks (33). However, the differences between SGLT2 inhibitors and sulfonylureas on NAFLD participants with type 2 diabetes under similar glucose level reduction remain uncertain.

The clinicopathological analyses revealed that the reduction in HbA_{1c} and the use of insulin independently contribute to the reduction in liver fibrosis scores during the histological course of NAFLD development (2). These findings led us to hypothesize that glycemic control and insulin ameliorate or protect against the histological progression of liver fibrosis in participants with NAFLD.

In the current study, we investigated the efficacy of SGLT2 inhibitor tofogliflozin and sulfonylurea glimepiride, which lower glucose levels similarly with reduction and elevation in circulating insulin levels, respectively, in NAFLD participants with type 2 diabetes for 48 weeks by examining liver histology as well as hepatic enzymes, metabolic markers, and hepatic gene expression profiles.

RESEARCH DESIGN AND METHODS
Participants
This randomized, open-label, active-controlled trial was conducted at a single center

(Kanazawa University Hospital) in Japan. The trial consisted of a 48 week treatment period. The protocol was approved by the Kanazawa University Certified Review Board, Ishikawa, Japan (CRB4180005). This trial was registered with ClinicalTrials.gov number NCT02649465 and the Japan Registry of Clinical Trials (jRCTs041180132) from UMIN 000020544.

Eligibility for the trial was determined at screening using standard blood tests, clinical history (including written confirmation of drug history, where necessary), and physical examination/observations to identify other illnesses or contraindications.

Fatty liver is clinically diagnosed when a bright liver or hepatorenal echo contrast is observed on the abdominal ultrasonography. We excluded all other liver disorders in each patient. All participants reported drinking <20 g/day of ethanol. All liver biopsies were performed during hospitalizations. All biopsies were obtained after a thorough clinical evaluation and obtaining a receipt of signed informed consent from each patient. The trial entry criteria are based on a diagnosis of "definite" NAFLD with type 2 diabetes on liver biopsy specimen obtained within 12 weeks of screening. All the participants had to be ≥20 years of age at the time of the initial screening.

Key exclusion criteria included hepatic virus infections, autoimmune hepatitis, primary biliary cirrhosis, and the use of agents known to induce steatosis or excessive alcohol consumption. Full eligibility criteria are in the protocol study (34).

Study Design
The schedules for the study visits and data collection are summarized in Supplementary Table 1. All of the participants were asked to attend each visit under a minimum 8 h fasting state before each visit. A follow-up liver biopsy was obtained under ultrasound guidance after completion of the 48 week study treatment.

Participants were randomly assigned on a 1:1 ratio to receive once-daily tofogliflozin at a dose of 20 mg or glimepiride at an initial dose of 0.5 mg. Randomization was performed with the use of computer-generated randomization. The participants in the SGLT2 inhibitor group received tofogliflozin (fixed dose of 20 mg/day; brand name: Deberza, Kowa Company Ltd., Nagoya, Japan), and the participants in the sulfonylurea group received glimepiride

(starting from 0.5 mg/day and titrated up to 6.0 mg/day; Sanofi-Aventis, Quebec, Canada) for 48 weeks (Supplementary Fig. 1). Previous treatment with oral antidiabetes drugs and metabolic-related medications was continued at the same dose in participants from 12 weeks before enrollment. The participants were not allowed any new prescriptions or dose changes.

In addition to study medications, the participants continued to undergo lifestyle modifications (i.e., exercise, weight loss, and dietary adjustment) and management of various coexisting illnesses throughout the trial. The participants were asked to limit alcohol consumption to <20 g/day for women and 30 g/day for men. All of the participants received an hour of nutritional counseling by an experienced dietitian before the 48 week treatment period. The experienced dietitians were unaware of the study assignments. In addition, all of the participants were given a standard calorie diet (30 kcal/kg/day; carbohydrates, 50–60%; fat, 20–30%; and protein, 15–20%) and exercise (5–6 MET estimations for 30 min daily) counseling before the study.

Screening biopsy results were used as the baseline for histological variables, and a second biopsy was performed at week 48. A single pathologist (K.H.), who was blind to both clinical information (e.g., treatment assignments, participants' characteristics, and the order in which the biopsy specimens were obtained), histologically evaluated all biopsy specimens. The biopsied tissues were scored for steatosis (from 0 to 3), stage (from 0 to 4), and grade (from 0 to 3), as previously described (35), according to the standard criteria of Brunt et al. (36). The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2) (37).

Outcomes

The primary outcome measure was assessed using an intention-to-treat analysis of the percentage of participants with at least 1 point improvement in each histological score of steatosis, hepatocellular ballooning, lobular inflammation, and fibrosis between liver biopsies at baseline and after 48 weeks of treatment.

Confirmatory secondary histological end points included the changes in the overall NAS, individual components of NAS, and fibrosis stages. Other secondary end points included changes in serum liver-related markers, glucose metabolism, body compositions, lipid profiles, oxidative stress markers, and cytokines levels; details are in the protocol study (34).

The body composition was predicted using a segmental bioelectrical impedance analysis (InBody 720). This device provides information about body fat mass, percentage of body fat, and skeletal muscle mass, among others. The measurement procedure required the subject to stand with bare feet on the analyzer and to hold a pair of handgrips, one in each hand. These conditions refer to the manufacturer's recommended standard conditions for the InBody 720 device, which works by the segmental multifrequency-bioelectrical impedance analysis method.

The Fibrosis-4 (FIB-4) index is a noninvasive tool (i.e., FIB-4 index = age × AST/[platelet count × $(ALT)^{1/2}$]) for assessing liver fibrosis. The FIB-4 index is easy to use in clinical practice, and its diagnostic capability for advanced fibrosis is comparable to that of magnetic resonance elastography (38).

Serial gene expression analyses were performed using liver biopsy samples obtained from participants before and after administration of tofogliflozin or glimepiride. The liver biopsy specimens stored in liquid nitrogen were once immersed in RNAlater (Ambion, Austin, TX) overnight and homogenized in lysis buffer by TissueLyser (Qiagen, Hilden, Germany). Total RNA was isolated using the RNAqueous kit (Ambion), as previously reported (39). The quality of the isolated RNA was estimated after electrophoresis using an Agilent 2100 Bioanalyzer (Palo Alto, CA). RNA sequencing (RNA-seq) was performed by using the SMART-Seq Stranded Kit (Takara Bio, Kusatsu, Shiga, Japan) and NovaSeq 6000 Sequencing System (Illumina, San Diego, CA). Expression data were processed by BRB-ArrayTools (https://brb.nci.nih.gov/BRB-ArrayTools). In brief, the library preparation was performed by using SMART-Seq Stranded Kit (Takara Bio) following the manufacturer's recommendations. PCR was performed for 5 cycles before rRNA depletion and 15 cycles during the last library amplification. The libraries were sequenced on the Illumina Nova-Seq 6000. The paired-end reads of each sample were aligned to the human genome (hg38) using Subread (40), and transcript abundance was shown by the count data using high-throughput sequence analysis (41). Count data were filtered and normalized by BRB-ArrayTools (https://brb.nci.nih.gov/BRB-ArrayTools). Differentially expressed genes of paired samples were obtained by edgeR with the generalized linear model likelihood ratio test approach. Functional ontology enrichment analysis was conducted to compare the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the gene ontology of the biological processes distribution of the differentially expressed genes. Least squares/Kolmogorov-Smirnov permutation tests were performed for pathway comparison ($P < 0.005$) (BRB-ArrayTools). Gene set enrichment analysis was done using the single-cell RNA-seq gene signature (42). To characterize which cell components contributed to the gene expression, representative gene sets of hepatocytes (1–6), cholangiocytes, central liver sinusoidal endothelial cells (LSECs), periportal LSECs, portal endothelial cells, stellate cells, inflammatory macrophages, noninflammatory macrophages, $\alpha\beta T$ cells, $\gamma\delta T$ cells-1, $\gamma\delta T$ cells-2, natural killer cells, mature B cells, plasma cells, and erythroid cells were retrieved from a previously reported study (42). Functional ontology enrichment analysis was conducted to compare the distribution of the differentially expressed genes in each cell component.

Statistical Analysis

At the time of the study design, we had no available data to estimate the histological response with a 48 week treatment using tofogliflozin and glimepiride. Therefore, we estimated the sample size based on findings of other studies on non-SGLT2 inhibitors as follows: Based on clinical trials of non-SGLT2 inhibitors for NAFLD that had improvements in liver histology as a primary end point, we assumed that 52.6–69.0% of participants undergoing treatment would demonstrate an improvement in NAFLD (7,43). We estimated that there would be an improvement in liver histology in 17.6–19.0% of the placebo-control arm participants, based on the literature (7,43). We calculated a sample size of 14 in each group, for a significance level of 0.05 (type I error) and a power of 0.90 (type II error). This design required 40 evaluable participants in the treatment group. The published literature

in NAFLD trials reported, an average participant withdrawal rate of 10–20% (43,44).

The statistical analyses were performed with an intention-to-treat principle to ascertain safety and adverse effects. Baseline characteristics of the two study groups are summarized with frequencies for categorical variables and with means (SD) in the normally distributed variables or median (interquartile range) in the nonnormally distributed continuous variables. We performed the Shapiro-Wilk test to evaluate the assumption of a normal distribution. The between-group comparison at baseline was performed with the χ^2 test or the Fisher test for categorical variables and with the Mann-Whitney U test in the nonnormal distribution or the two-sample t test in the normal distribution for continuous parameters.

We statistically evaluated changes in each histological score before and after the intervention with the Wilcoxon signed rank test and between the groups for the treatment effect (change from baseline) with the χ^2 test, according to analyses of previous hepatic histological scores (6). Primary outcomes measures are eight because each of the four histological features was analyzed in the two treatments arms. The significance of differences was defined as $P < 0.00625$ (0.05/8), adjusting for the Bonferroni multiple testing.

The intergroup comparison of continuous parameters was performed with the Mann-Whitney U test in the nonnormal distribution or the two-sample t test in the normal distribution. The internal group comparison between baseline and 48 weeks was performed with the Wilcoxon signed rank test. Data pertaining to the major clinical events of interest are presented as frequencies and percentages for categorical variables. We examined the association between the baseline characteristics, the change in the laboratory data, and the change in histology in both groups by using the Spearman analysis. A P value of <0.05 was regarded as statistically significant. All analyses were performed with SPSS Statistics 25.0 software (IBM Corp., Armonk, NY).

RESULTS

From March 2016 through December 2019, a total of 40 participants were randomly assigned to receive once daily tofogliflozin at a dose of 20 mg (20 participants) or to receive once daily glimepiride at a final mean dose of 0.8 mg (20 participants). All 40 participants (100%) completed the trial. Information for the primary and confirmatory secondary outcomes related to a biopsy at week 48 was available for 39 participants (97.5%). For only one patient with a serious adverse event (pancreatic cancer) (Supplementary Fig. 1), the liver histology scores were imputed as nonresponse using an intention-to-treat analysis. All of the participants analyzed were >80% in compliance of study medication. A total of 15 of 20 participants assigned to tofogliflozin and 16 of 20 assigned to glimepiride achieved 100% compliance with the study medication in the current study (Supplementary Table 2).

Demographic and baseline clinical characteristics, except for sex, were similar in both groups (Tables 1 and 2, and Supplementary Tables 3–6). All participants were Japanese and had type 2 diabetes. The mean age was 53.9 years, mean NAS was 4.45, mean HbA$_{1c}$ was 8.2%, and mean weight was 82.0 kg (Table 1). A total of 18 participants (45.0%) had stage F1 fibrosis, 11 (27.5%) had stage F2, and 5 (12.5%) had stage F3 (Table 2).

Fibrosis scores improved in the tofogliflozin group (60%, $P = 0.001$ for the comparison of scores before and after treatment), but the change from baseline did not differ significantly between the tofogliflozin group and the glimepiride group ($P = 0.172$) (Table 2). In addition, subjects who received tofogliflozin had significant histological improvements from baseline to 48 weeks in all variables (ratios of the participants with improvement in steatosis, hepatocellular ballooning, and lobular inflammation were 65, 55, and 50%, respectively). In the glimepiride group, the histological improvement from baseline to 48 weeks was the tendency to be a reduction in only hepatocellular ballooning, adjusting for the Bonferroni multiple testing.

NAS improved significantly compared with baseline values in both groups, and the beneficial effects were greater in the tofogliflozin group ($P = 0.002$) (Supplementary Table 4). There was an early and highly significant decrease in ALT and AST levels in the tofogliflozin group (Fig. 1A and B). The changes from baseline did not differ significantly between the tofogliflozin group and the glimepiride group (Supplementary Table 4).

The changes of γ-glutamyl transferase were significantly reduced in the tofogliflozin group ($P < 0.001$ for the comparison with glimepiride) (Fig. 1C and Supplementary Table 4). Moreover, the FIB-4 index was significantly reduced in the tofogliflozin group, and the effects were greater in the tofogliflozin group ($P = 0.015$) (Fig. 1D and Supplementary Table 4).

The decrease in glycemic parameters, such as fasting plasma glucose and HbA$_{1c}$, were similar (Fig. 1E and Supplementary Table 5). Weight, BMI, and percentage of body fat were significantly reduced in the tofogliflozin group (a mean weight decrease of 4.2 kg at week 48, $P < 0.001$ compared with glimepiride) (Fig. 1F and Supplementary Table 5). These changes occurred in the first 12 weeks and were sustained throughout the period. In contrast, the changes in C-peptide response, lipid profile, oxidative stress markers, and cytokines were similar in both groups (Supplementary Tables 5 and 6).

We examined the association between the change in various clinical parameters and the change in liver histology in both treatment arms by the Spearman analysis (Supplementary Table 7). The reduction in body weight was significantly associated with the reduction in steatosis scores in the tofogliflozin group. The reduction in HbA$_{1c}$ was significantly associated with the reduction in steatosis scores in both groups. Higher baseline HbA$_{1c}$ (ρ = 0.582, $P = 0.007$) and the reduction in HbA$_{1c}$ (ρ = 0.524, $P = 0.018$) were significantly associated with the reduction in fibrosis scores only in the tofogliflozin group.

To understand molecular signatures of tofogliflozin and glimepiride in the liver, we examined global hepatic gene expression profiles using RNA-seq before and after the interventions. There was a significant difference ($P < 0.005$) in the number of genes expressed in the liver of the tofogliflozin group compared with the glimepiride group (663 genes in the tofogliflozin group vs. 51 genes in the glimepiride group). The pathway analyses of differentially expressed genes according to the KEGG pathways (Table 3) and the gene ontology of the biological processes (Supplementary Table 8) in serial hepatic gene expression profiles showed unique metabolic signatures in the tofogliflozin group compared with the glimepiride group. Genes involved in gluconeogenesis, fatty acid catabolism/

Table 1—Baseline characteristics of the participants (n = 40)

Characteristics	Normal range	All (N = 40)	Tofogliflozin (n = 20)	Glimepiride (n = 20)	P value†
Male, n (%)		21 (53)	7 (35)	14 (70)	0.027
Diabetes, n (%)		40 (100)	20 (100)	20 (100)	NA
Hypertension, n (%)		24 (60)	12 (60)	12 (60)	1.000
Dyslipidemia, n (%)		32 (80)	16 (80)	16 (80)	0.653
Age, years		56.5 (40.5–65.0)	59.0 (43.0–64.8)	50.5 (38.3–65.0)	0.445
AST, IU/L	13–33	28.0 (22.0–51.0)	28.0 (24.3–54.5)	30.0 (21.3–49.0)	0.602
ALT, IU/L	6–27	40.0 (28.0–73.0)	36.0 (28.0–77.5)	48.0 (35.5–60.0)	0.398
γ-Glutamyl transferase, IU/L	10–47	46.0 (36.0–63.0)	50.0 (36.5–77.8)	42.5 (30.0–59.8)	0.221
Alkaline phosphatase, IU/L	115–359	224.0 (200.0–283.0)	238.0 (173.5–286.0)	218.5 (191.8–258.0)	0.947
Total bilirubin, mg/dL	0.3–1.2	0.70 (0.70–1.00)	0.70 (0.63–0.90)	0.80 (0.60–1.00)	0.602
FIB-4 index		1.12 (0.76–1.50)	1.10 (0.83–1.48)	0.95 (0.50–1.49)	0.277
Liver steatosis, as assessed by FibroScan, dB/m	100–220	291.4 (38.3)	288.6 (37.9)	300.4 (28.9)	0.223
Liver stiffness, as assessed by FibroScan, kPa	1.5–5.0	6.3 (4.8–9.1)	5.7 (4.3–7.3)	6.4 (4.7–11.3)	0.581
Total activity score for NALFD		4.45 (1.48)	4.40 (1.76)	4.50 (1.19)	0.904
Fasting plasma glucose, mg/dL	69–109	143.0 (123.0–158.0)	144.0 (120.0–157.8)	141.0 (128.3–158.0)	0.947
HbA$_{1c}$, %	4.6–6.2	8.1 (7.3–8.8)	7.9 (7.4–8.4)	8.2 (7.3–9.2)	0.565
HbA$_{1c}$, mmol/mol	27.0–44.0	64.0 (56.0–73.0)	63.0 (57.0–67.8)	65.5 (55.3–76.8)	0.565
C-peptide immunoreactivity, ng/mL	0.80–2.50	2.84 (0.91)	2.81 (0.92)	2.86 (0.92)	0.852
Body weight, kg		82.0 (21.9)	79.3 (18.2)	84.7 (25.4)	0.449
BMI, kg/m^2		31.5 (7.7)	31.0 (6.7)	32.0 (8.8)	0.705
Systolic blood pressure, mmHg		129.6 (13.6)	129.3 (12.7)	130.0 (14.8)	0.864
Pulse rate, bpm		82.7 (12.7)	81.2 (13.1)	84.2 (12.6)	0.471
Total cholesterol, mg/dL	128–219	173.3 (34.7)	170.1 (28.1)	176.5 (40.7)	0.567
Triglycerides, mg/dL	30–149	137.0 (120.0–218.0)	140.0 (115.0–204.0)	140.5 (123.0–228.0)	0.602
HDL cholesterol, mg/dL	40–99	42.7 (10.1)	44.8 (11.6)	40.7 (8.1)	0.204

Categorical variables are presented as n (%). Continuous variables are presented as mean (SD) or median (interquartile range). NA, not analyzed. †The between-group comparison at baseline was performed with the χ^2 test or Fisher test for categorical variables and the Mann-Whitney U test in nonparametric parameters or the two-sample t test in normal distribution for continuous parameters.

oxidation, and amino acids catabolism in peroxisome were coordinately upregulated in the tofogliflozin group. On the other hand, genes involved in cell death, stress response, inflammation, T-cell response, and fibrosis were substantially downregulated in the tofogliflozin group but not in the glimepiride group (Table 3 and Supplementary Table 8).

Next, we performed gene set enrichment analyses using gene sets associated with resident cells in the liver defined by single-cell RNA-seq analyses and corresponding liver histological scores before and after the tofogliflozin treatment (Fig. 2 and Supplementary Table 9) to further address which components of resident cells participate in the tofogliflozin-mediated alleviation of NAFLD pathology.

Figure 2 shows one-way hierarchical clustering of 51 representative genes involved in central LSECs and zone 2 and 3 hepatocytes (left) and 59 genes involved in γδT cells, inflammatory macrophages, stellate cells, and plasma cells (right). Histological scores of fibrosis, lobular inflammation, NAS, and steatosis (%) are shown in individual patients before and after treatment, respectively. Gene expression patterns were well correlated with histological changes. The 51 genes involved in LSECs and zone 2 and 3 hepatocytes were coordinately downregulated in the liver with severe steatosis before treatment (left side of left panel). Tofogliflozin upregulated the expression of these genes (right side of left panel). The 59 genes, representative of γδT cells, inflammatory macrophages,

stellate cells, and plasma cells, showed a similar gene expression pattern and clustered in each cell component. These genes were coordinately upregulated in the liver with severe steatosis before treatment (left side of right panel). Tofogliflozin downregulated these genes expression (right side of left panel). In contrast, glimepiride rather upregulated the pathway for γδT cells (Supplementary Table 9).

All adverse events occurred during the on-treatment observation period. Data are reported for all of the participants. The incidences of adverse events were significantly higher in the tofogliflozin group. This difference was attributable to the increased incidence of genital and urinary tract symptoms

Table 2—Hepatic histological scores

Histologic features	Tofogliflozin (n = 20)			Glimepiride (n = 20)			P value (tofogliflozin vs. glimepiride)‡
	Before	After	P value†	Before	After	P value†	
Steatosis							
Score, n subjects							
0 (<5%)	0	5		0	0		
1 (5–33%)	8	11		6	11		
2 (33–66%)	8	3		9	5		
3 (>66%)	4	1		5	4		
Improvement, %		65	0.001		30	0.058	0.141
Hepatocellular ballooning							
Score, n subjects							
0 (None)	3	10		1	5		
1 (few balloon cells)	10	9		14	11		
2 (many balloon cells)	7	1		5	4		
Improvement, %		55	0.002		25	0.025	0.098
Lobular inflammation							
Score, n subjects							
0 (0 focus)	1	4		0	0		
1 (<2 foci per 200*field)	11	16		13	14		
2 (2–4 foci per 200*field)	7	0		7	6		
3 (>4 foci per 200*field)	1	0		0	0		
Improvement, %		50	0.003		15	0.655	0.064
Fibrosis							
Score, n subjects							
0 (none)	3	10		2	6		
1 (perisinusoidal or periportal)	7	7		11	7		
2 (perisinusoidal and portal or periportal)	8	1		3	3		
3 (bridging fibrosis)	2	2		3	4		
4 (cirrhosis)	0	0		1	0		
Improvement, %		60	0.001		35	0.096	0.172

†The P values were calculated with the Wilcoxon signed rank test. ‡The between-group comparison for the effect of treatment (change from baseline) was performed with the χ^2 test.

(itching in the genital area, n = 7; urinating pain, n = 1; cystitis, n = 1; nocturia, n = 1; vaginal candida, n = 1) in the tofogliflozin group. Most adverse events were mild or moderate, and no participants discontinued the treatment. Two participants in the glimepiride group experienced mild or moderate hypoglycemia: one patient in reduced prandial dosing of rapid-acting insulin (total 36 to 30 units) due to hypoglycemia. One serious adverse event, pancreatic cancer, occurred in the glimepiride group but was considered not related to the study (Supplementary Table 10).

CONCLUSIONS

In this open-label, randomized, parallel trial, tofogliflozin and, to a lesser degree, glimepiride significantly reduced the score of liver histology for 48 weeks in participants with liver biopsy specimen-confirmed NAFLD and type 2 diabetes, with no significant difference between the agents under a similar reduction in glucose levels.
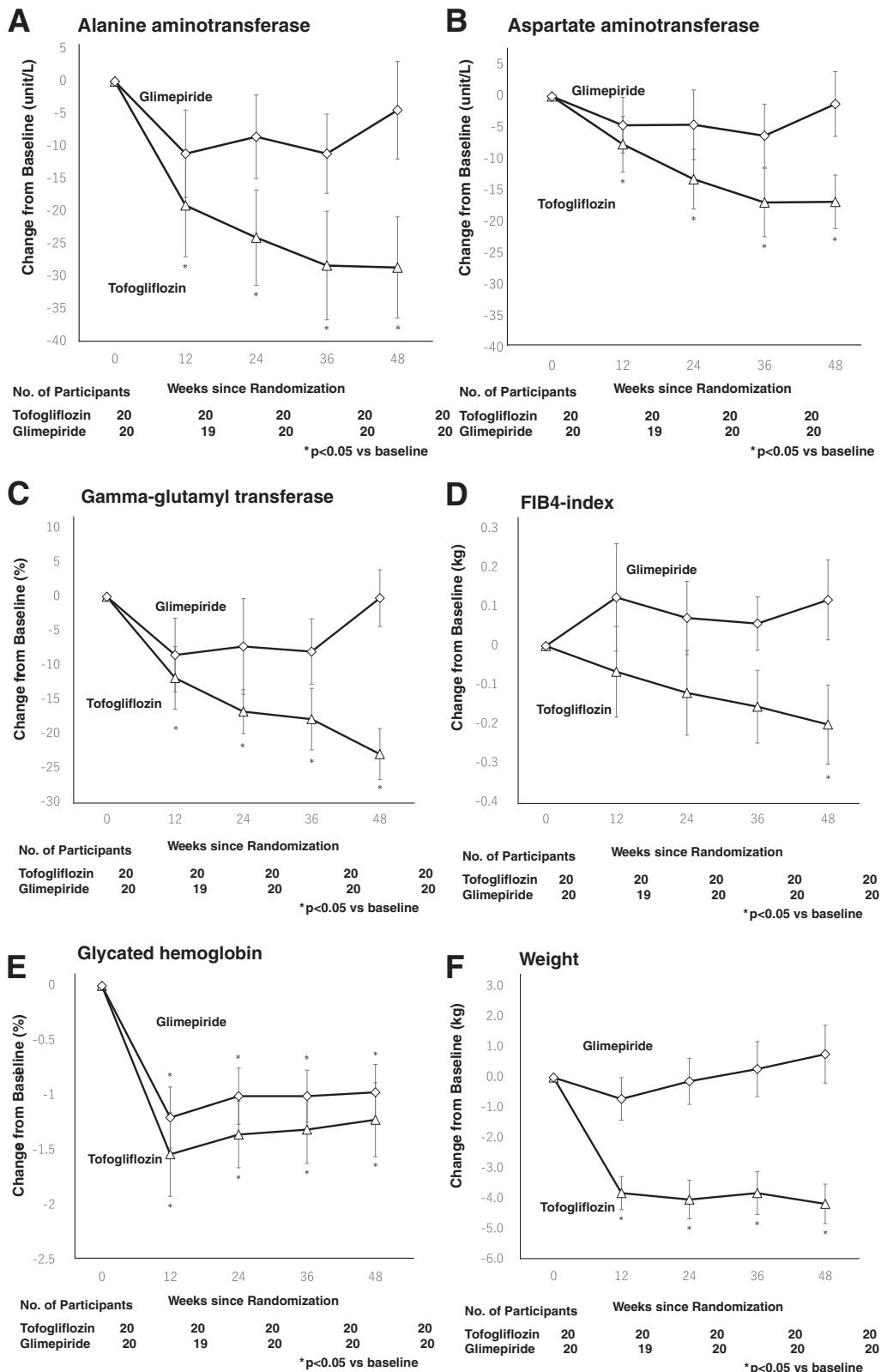
The percentage of participants in the tofogliflozin group who had an improvement in the fibrosis stage after 48 weeks in this trial was 60%. As shown in Supplementary Table 11, the tofogliflozin-mediated improvement in the fibrosis stage in the current study is greater than that reported with liraglutide treatment (8), obeticholic acid treatment (45), semaglutide treatment (9), pioglitazone treatment (6,7), and vitamin E treatment (7). The Spearman analysis (Supplementary Table 7) showed that the baseline fibrosis scores did not affect the changes in histology scores after the treatments. Unexpectedly, the percentage of participants in the tofogliflozin group who had an improvement in steatosis, hepatocellular ballooning, and lobular inflammation in this trial (65, 55, and 50%, respectively) was not greater than that reported with liraglutide treatment (8), obeticholic acid treatment (45), and

pioglitazone treatment (6,7). These findings suggest that tofogliflozin may preferentially ameliorate liver fibrosis compared with liraglutide, semaglutide, obeticholic acid, liraglutide, pioglitazone, and vitamin E.

The systematic review (46) reported that SGLT2 inhibitors improved liver histology, such as liver steatosis and fibrosis, in participants with NAFLD according to findings from the single-arm clinical trials (27,28). However, a recent systematic review demonstrated SGLT2 inhibitors may not reduce liver fibrosis (47). Our randomized controlled trial study is the first to characterize the greater improvement in NAFLD histology, especially fibrosis, with tofogliflozin.

The percentage of participants with improvement in the fibrosis stage in the glimepiride group in this trial was 35%, similar to the percentage of participants with obeticholic acid treatment (45) and greater than that reported in the placebo participants (6–9). On the other

Figure 1—Changes from baseline in liver-related parameters, HbA₁c, and weight, according to the study group. Mean values are shown for changes from baseline (the value at follow-up minus the baseline value) for ALT levels (*A*), AST levels (*B*), γ-glutamyl transferase (*C*), FIB-4 index (*D*), HbA₁c (*E*), and weight (*F*) among the 20 subjects in the tofogliflozin group and the 20 subjects in the glimepiride group.

hand, the percentage of participants in the glimepiride group who had an improvement in steatosis, hepatocellular

ballooning, and lobular inflammation in this trial was not greater than with other agents and placebo. Past studies reported

that sulfonylureas may exacerbate liver histology, NAFLD progression, and adverse outcomes such as hepatocellular

Table 3—Differential signaling pathways in the liver of NAFLD participants altered by treatment with tofogliflozin or glimepiride

No.	KEGG pathway	Pathway description	Genes, n	LS permutation P value	KS permutation P value	Up or down	Representative genes
Tofogliflozin							
	Metabolism						
1	hsa00071	Fatty acid degradation	43	0.00001	0.00001	Up	ACSL5, GCDH, ACADSB
2	hsa00250, hsa00260, hsa00280, hsa00340, hsa00380	Amino acids metabolism (Ala, Asn, Gln, Gly, Ser, Thr, Val, Leu, Ile, His, Trp)	172	0.00001	0.00001	Up	AGXT, GNMT, BCKDHB
3	hsa00980	Metabolism of xenobiotics by cytochrome P450	64	0.00001	0.00009	Up	GSTA2, ALDH3A1, UGT1A7
4	hsa03320	PPAR signaling pathway	64	0.00001	0.00001	Up	SLC27A5, APOA5, ACOX2
5	hsa04146	Peroxisome	79	0.00001	0.00001	Up	PXMP2, PHYH, MLYCD
6	hsa00051	Fructose and mannose metabolism	35	0.00005	0.00079	Up	PMM1, ALDOB, FBP1
7	hsa00830	Retinol metabolism	59	0.00020	0.00022	Up	CYP2A6, CYP2A7, CYP1A1
8	hsa00010	Glycolysis/gluconeogenesis	58	0.00047	0.00363	Up	PCK1, ENO3, G6PC
9	hsa00020	Citrate cycle (TCA cycle)	29	0.00374	0.06582	Up	PCK2, ACO1, SDHB
10	hsa00190	Oxidative phosphorylation	97	0.00891	0.00175	Up	COX6C, COX17, NDUFB7
	Cell cycle						
1	hsa03030	DNA replication	36	0.00001	0.0026	Down	MCM2, MCM6, PRIM2
2	hsa04110	Cell cycle	124	0.00001	0.00001	Down	CDC7, CHEK1, CCNB1
	Apoptosis/ inflammation						
1	hsa04612	Antigen processing and presentation	62	0.00015	0.00165	Down	TAP1, HLA-DQB1, CIITA
2	hsa05340	Primary immunodeficiency	32	0.00042	0.00183	Down	CD3E, JAK3, IL2RG
3	hsa04210	Apoptosis	84	0.00256	0.00223	Down	BIRC3, TNFRSF10D, FAS
4	hsa04010	MAPK signaling pathway	229	0.00349	0.00942	Down	PDGFRB, CD14, FGF2
5	hsa04670	Leukocyte transendothelial migration	105	0.01046	0.00038	Down	CXCR4, CLDN7, ICAM1
	Fibrosis						
1	hsa04510	Focal adhesion	190	0.00001	0.00001	Down	COL4A1, COL1A1, LAMA3
2	hsa04512	ECM-receptor interaction	80	0.00001	0.00057	Down	HMMR, ITGA9, PDGFRA
3	hsa04514	Cell adhesion molecules	122	0.00002	0.00009	Down	CNTNAP2, SDC2, CLDN7
Glimepiride							
	Metabolism						
1	hsa04146	Peroxisome	79	0.0017	0.00001	Up	PEX7, HACL1, GNPAT
2	hsa00071	Fatty acid degradation	43	0.0085	0.00048	Up	ADH7, ALDH3A2, ADH1A
3	hsa00980	Metabolism of xenobiotics by cytochrome P450	64	0.0271	0.00445	Up	UGT2B15, UGT1A1, UGT2B10

ACADSB, acyl-CoA dehydrogenase short/branched chain; ACO1, aconitase 1; ACOX2, acyl-CoA oxidase 2; ACSL5, acyl-CoA synthetase long chain family member 5; ADH1A, alcohol dehydrogenase 1A (class I), α polypeptide; ADH7, alcohol dehydrogenase 7 (class IV), μ or sigma polypeptide; AGXT, alanine–glyoxylate and serine–pyruvate aminotransferase; ALDH3A1, aldehyde dehydrogenase 3 family member A1; ALDH3A2, aldehyde dehydrogenase 3 family member A2; ALDOB, aldolase, fructose-bisphosphate B; APOA5, apolipoprotein A5; BCKDHB, branched chain keto acid dehydrogenase E1 subunit beta; BIRC3, baculoviral IAP repeat containing 3; CCNB1, cyclin B1; CD14, CD14 molecule; CD3E, CD3e molecule; CDC7, cell division cycle 7; CHEK1, checkpoint kinase 1; CIITA, class II major histocompatibility complex transactivator; CLDN7, claudin 7; CNTNAP2, contactin associated protein 2; COL1A1, collagen type I α 1 chain; COX17, cytochrome C oxidase copper chaperone; COX6C, cytochrome C oxidase subunit 6C; CXCR4, C-X-C motif chemokine receptor 4; CYP1A1, cytochrome P450 family 1 subfamily A member 1; CYP2A6, cytochrome P450 family 2 subfamily A member 6; CYP2A7, cytochrome P450 family 2 subfamily A member 7; ENO3, enolase 3; FAS, Fas cell surface death receptor; FBP1, fructose-bisphosphatase 1; FGF2, fibroblast growth factor 2; G6PC, glucose-6-phosphatase catalytic

Table 3—Continued
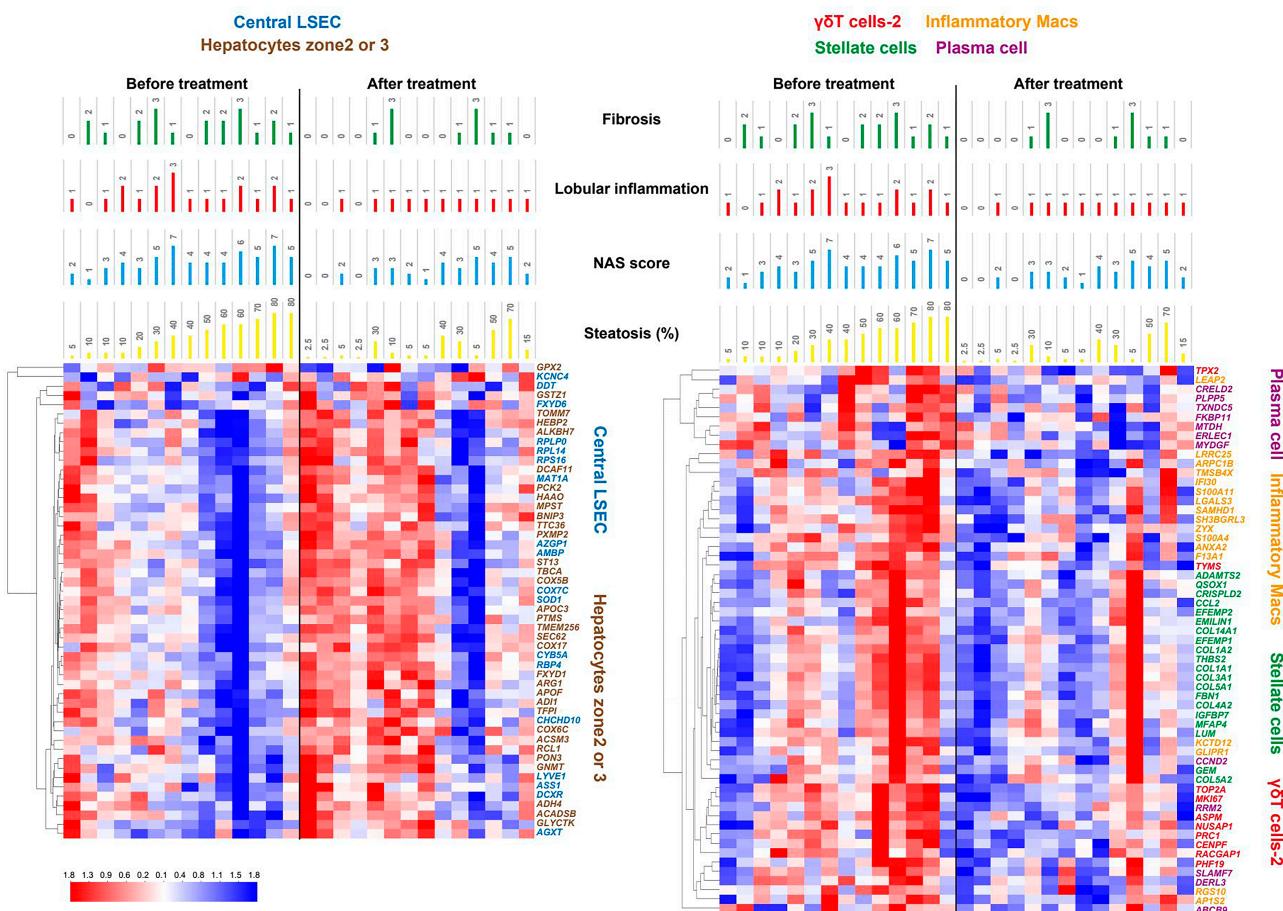
subunit 1; *GCDH*, glutaryl-CoA dehydrogenase; *GNMT*, glycine *N*-methyltransferase; *GNPAT*, glyceronephosphate *O*-acyltransferase; *GSTA2*, glutathione *S*-transferase α 2; *HACL1*, 2hHydroxyacyl-CoA lyase 1; *HLA-DQB1*, major histocompatibility complex, class II, DQ beta 1; *HMMR*, hyaluronan mediated motility receptor; *ICAM1*, intercellular adhesion molecule 1; *IL2RG*, interleukin 2 receptor subunit γ; *ITGA9*, integrin subunit α 9; *JAK3*, Janus kinase 3; KS, Kolmogorov-Smirnov; *LAMA3*, laminin subunit α 3; LS, least squares; MAPK, mitogen-activated protein kinase; *MCM2*, minichromosome maintenance complex component 2; *MCM6*, minichromosome maintenance complex component 6; *MLYCD*, malonyl-CoA decarboxylase; *NDUFB7*, NADH:ubiquinone oxidoreductase subunit B7; *PCK1*, phosphoenolpyruvate carboxykinase 1; *PCK2*, phosphoenolpyruvate carboxykinase 2, mitochondrial; *PDGFRA*, platelet derived growth factor receptor α; *PDGFRB*, platelet derived growth factor receptor beta; *PEX7*, peroxisomal biogenesis factor 7; *PHYH*, phytanoyl-CoA 2-hydroxylase; *PMM1*, phosphomannomutase 1; PPAR, peroxisome proliferator-activated receptor; *PRIM2*, DNA primase subunit 2; *PXMP2*, peroxisomal membrane protein 2; *SDC2*, syndecan 2; *SDHB*, succinate dehydrogenase complex iron sulfur subunit B; *SLC27A5*, solute carrier family 27 member 5; *TAP1*, transporter 1, ATP binding cassette subfamily B member, ATP binding cassette subfamily B member; TCA, tricarboxylic acid; *TNFRSF10D*, TNF receptor superfamily member 10d; *UGT1A1*, UDP glucuronosyltransferase family 1 member A1; *UGT1A7*, UDP glucuronosyltransferase family 1 member A7; *UGT2B10*, UDP glucuronosyltransferase family 2 member B10; *UGT2B15*, UDP glucuronosyltransferase family 2 member B15.

carcinoma (30–32). However, these findings in our study suggest that glimepiride also preferentially ameliorates liver fibrosis. Collectively from the characteristic improvement of liver histology by tofogliflozin and glimepiride, glucose reduction may reduce liver fibrosis, at least partly, independently of steatosis and inflammation, which is consistent with our initial hypothesis.

As we have expected, the reduction of fasting plasma glucose and glycated

Figure 2—Heat maps of gene set enrichment analyses using gene sets of resident cells in the liver defined by single-cell RNA-seq analyses and corresponding liver histological scores before and after the tofogliflozin treatment. The heat maps show one-way hierarchical clustering of 51 representative genes involved in central LSECs and zone 2 and 3 hepatocytes (left) and 59 genes involved in γδT cells, inflammatory macrophages (macs), stellate cells, and plasma cells (right). Histological scores of fibrosis, lobular inflammation, NAS, and steatosis (%) are shown in individual patients before and after treatment, respectively. Gene expression patterns were well correlated with histological changes. The 51 genes involved in LSECs and zone 2 and 3 hepatocytes were coordinately downregulated in the liver with severe steatosis before treatment (left side of left panel). Tofogliflozin upregulated these genes expression (right side of left panel). The 59 genes, representative of γδT cells, inflammatory macrophages, stellate cells, and plasma cells, showed a similar gene expression pattern and clustered in each cell component. These genes were coordinately upregulated in the liver with severe steatosis before treatment (left side of right panel). Tofogliflozin downregulated these genes expression (right side of left panel).

hemoglobin was similar in both groups, consistent with a phase 3 noninferiority trial (33). Weight, BMI, and fat mass were reduced only in the tofogliflozin group. In the subanalysis (Supplementary Table 7), reduction in steatosis scores was significantly associated with the tofogliflozin-mediated reduction in HbA$_{1c}$ and weight. On the other hand, reduction in fibrosis scores was significantly associated with HbA$_{1c}$ at baseline and the tofogliflozin-mediated glycemic control but not the reduction in weight. These findings suggest that glycemic control, rather than weight reduction, contributes to liver fibrosis alleviation.

Pioglitazone has several safety concerns, such as weight gain, heart failure, fluid retention, bone fracture, and bladder cancer. The GLP-1 RAs often cause gastrointestinal symptoms (8,9). The reduction in body fat with tofogliflozin may have a potentially helpful therapeutic effect on the future risk of cardiovascular events and premature death in participants with NAFLD, although longer-term outcome studies are needed to confirm this point.

The mechanisms by which SGLT2 inhibitors ameliorate NAFLD pathology remain underinvestigated in humans. In the current study, pathway analyses of differentially expressed genes according to the KEGG pathways and gene ontology of the biological processes in serial hepatic gene expression profiles showed unique metabolic signatures in the tofogliflozin group compared with the glimepiride group. In the tofogliflozin group, genes involved in gluconeogenesis were coordinately upregulated, consistent with clinical observations in which the SGLT2 inhibitor is associated with an elevated endogenous glucose production (48,49). Genes involved in fatty acid catabolism/oxidation and amino acids catabolism in peroxisome were coordinately upregulated in the tofogliflozin group, indicating enhanced lipolysis and protein catabolism for gluconeogenesis (50). The findings were compatible with the upregulated pathways of cytochrome P450 and retinol metabolisms, both activated by peroxisome proliferation in mouse models (51).

Genes involved in cell death, stress response, inflammation, T-cell response, and fibrosis were substantially downregulated in the tofogliflozin group but not in the glimepiride group, which are compatible with the tofogliflozin-mediated alleviation of liver inflammation, hepatocellular damage, and liver fibrosis.

Since glimepiride-mediated glucose lowering did not alter the inflammation- and fibrosis-related pathways, tofogliflozin may exert unique pleiotropic effects beyond glucose lowering.

To further address possible responsive resident cells in the tofogliflozin-mediated alleviation of NAFLD pathology, we performed gene set enrichment analyses using single-cell RNA-seq gene signatures. Genes involved in zone 3 hepatocytes, which are rich in peroxisomes, and LSECs were coordinately upregulated in the liver with severe steatosis. On the other hand, genes involved in γδT cells, inflammatory macrophages, stellate cells, and plasma cells, which play essential roles in the pathogenesis of NAFLD, were coordinately upregulated in the liver with severe steatosis. Tofogliflozin rescued these gene expression patterns; it upregulated the genes involved in zone 3 hepatocytes and LSECs and, in contrast, downregulated the genes involved in γδT cells, inflammatory macrophages, stellate cells, and plasma cells. Rescuing zone 3 hepatocytes could enhance various redox signalings, such as glutathione pathways, that remove reactive oxygen species induced by oxidative lipid (50). Therefore, it might be possible that tofogliflozin suppresses inflammation and fibrosis via the recovery of zone 3 hepatocytes. Considering similar gene expression patterns between zone 3 hepatocytes and LSECs, LSECs may be novel therapeutic targets for NAFLD, the hypothesis of which should be confirmed by further investigations.

Our study has some limitations. First, the study is an open-label design, including two open-label active treatment arms without a placebo group. Therefore, we cannot exclude the possibility that the improvement in liver histology in both groups could be attributed to lifestyle modification through diet and exercise counseling at baseline. Nevertheless, the strength of the current study is to evaluate the effects of the SGLT2 inhibitor on NALFD pathology by comparing with those of the sulfonylurea as an active control, both of which similarly lower glucose levels with reduction and elevation in circulating insulin levels, respectively, to clarify the role of glucose and insulin separately.

Second, the percentage of sex was unbalanced in both groups after randomization.

However, there was no association between sex and the changes in liver histology (Supplementary Table 7). Furthermore, we analyzed the effects of the agents on liver histology separately by sex, as summarized in Supplementary Tables 12 and 13. Almost similar results were obtained, except that hepatocellular ballooning and lobular inflammation in men of the tofogliflozin group and hepatocellular ballooning in both sexes of the glimepiride group remain in the tendency or insufficient statistical significance, possibly due to the small number of subjects. Based on these findings, we concluded that sex differences unlikely affected the conclusion of the current study.

Third, although tofogliflozin improved all of the liver histological scores, there was no statistical difference in the effects with glimepiride. Past NAFLD clinical trials (6–8) also observed that active arms significantly improve fibrosis scores but with no statistically significant differences between the active arms and comparative agents/placebo. Such discrepancies may be attributed to the short study duration, the small number of subjects, and the variations in the assessment of liver histology by means of percutaneous liver biopsy.

In conclusion, among participants with biopsy specimen-confirmed NAFLD and type 2 diabetes, tofogliflozin administration was associated with a significant liver histology improvement compared with glimepiride under similar glucose level reduction. Tofogliflozin coordinately altered hepatic expression of the genes involved in energy metabolism, inflammation, and fibrosis, which may underlie liver pathology. SGLT2 inhibitors may have a hepatoprotective effect, in addition to the previously recognized cardiorenal protective effects, and could be promising agents in the treatment of type 2 diabetes with NAFLD. Long-term larger-scale placebo-controlled clinical trials of SGLT2 inhibitors for participants with type 2 diabetes and NAFLD are needed to confirm our findings and to establish evidence in hepatocarcinogenesis, incident major adverse cardiovascular events, overall survival, and medical economics to be adopted as the therapeutic guidelines for type 2 diabetes and NAFLD.

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