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ORIGINAL ARTICLE

Incretin combination therapy for the treatment of non-alcoholic steatohepatitis

Aimo Kannt[1,2,3] | Andreas Nygaard Madsen[4] | Claire Kammermeier[1] | Ralf Elvert[1] | Tim Klöckener[1] | Martin Bossart[1] | Torsten Haack[1] | Andreas Evers[1] | Katrin Lorenz[1] | Wolfgang Hennerici[1] | Corinne Rocher[5] | Zsolt Böcskei[5] | Jean-Claude Guillemot[5] | Vincent Mikol[5] | Francois Pattou[6] | Bart Staels[6] | Michael Wagner[1]

[1]Sanofi Research and Development, Frankfurt, Germany

[2]Experimental Pharmacology, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

[3]Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Branch for Translational Medicine and Pharmacology TMP, Frankfurt, Germany

[4]Gubra, Horsholm, Denmark

[5]Sanofi Research and Development, Chilly-Mazarin Cedex, France

[6]Univ Lille, Inserm, CHU Lille, Institut Pasteur de Lille, UMR1011-EGID, Lille, France

Correspondence
Aimo Kannt, Sanofi Research and Development, D-65926 Frankfurt, Germany.
Email: aimo.kannt@web.de

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Abstract

Aims: To test specific mono-agonists to the glucagon-like peptide-1 receptor (GLP-1R), glucagon receptor (GCGR) and glucose-dependent insulinotropic peptide receptor (GIPR), individually and in combination, in a mouse model of diet-induced non-alcoholic steatohepatitis (NASH) and fibrosis in order to decipher the contribution of their activities and potential additive effects to improving systemic and hepatic metabolism.

Materials and methods: We induced NASH by pre-feeding C57BL/6J mice a diet rich in fat, fructose and cholesterol for 36 weeks. This was followed by 8 weeks of treatment with the receptor-specific agonists 1-GCG (20 μg/kg twice daily), 2-GLP1 (3 μg/kg twice daily) or 3-GIP (30 μg/kg twice daily), or the dual (1 + 2) or triple (1 + 2 + 3) combinations thereof. A dual GLP-1R/GCGR agonistic peptide, 4-dual-GLP1/GCGR (30 μg/kg twice daily), and liraglutide (100 μg/kg twice daily) were included as references.

Results: Whereas low-dose 1-GCG or 3-GIP alone did not influence body weight, liver lipids and histology, their combination with 2-GLP1 provided additional weight loss, reduction in liver triglycerides and improvement in histological disease activity score. Notably, 4-dual-GLP-1R/GCGR and the triple combination of selective mono-agonists led to a significantly stronger reduction in the histological non-alcoholic fatty liver disease activity score compared to high-dose liraglutide, at the same extent of body weight loss.

Conclusions: GCGR and GIPR agonism provide additional, body weight-independent improvements on top of GLP-1R agonism in a murine model of manifest NASH with fibrosis.

KEYWORDS

experimental pharmacology, fatty liver disease, GIP, GLP-1, glucagon, incretin therapy

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of hepatic abnormalities, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver fibrosis. Fatty liver is very common, with an estimated global prevalence of approximately 25%, and is strongly associated with other systemic conditions such as obesity, diabetes and dyslipidaemia.[1] Progression to NASH and especially to

NASH with advanced fibrosis is a strong risk factor for the development of cirrhosis and hepatocellular carcinoma, has been linked to increased overall and liver-related mortality, and is becoming the leading cause of liver transplantation.[2–6] From a pathophysiological perspective, fat and triglyceride accumulation in the liver, hepatic and adipose tissue insulin resistance, inflammation, as well as lipotoxicity and oxidative stress, are involved in the development of NAFLD. At present there are no approved pharmacological therapies available to treat NAFLD/NASH. Thus, novel treatment options for NAFLD/NASH are highly warranted.

Lifestyle intervention focusing on weight loss is currently the therapy of choice.[7] Weight loss, independently of whether achieved via diet and exercise,[7] bariatric surgery[8] or pharmacological intervention,[9] has been observed to be associated with reduction in hepatic steatosis and resolution of NASH.

Amongst the various approaches which are being investigated to treat NAFLD/NASH are agonists of the glucagon-like peptide-1 receptor (GLP-1R) or GLP-1-based multi-agonists, as they have been shown to produce significant and sustained weight loss as well as to elicit favourable metabolic effects.[9,10] For example, treatment of patients with biopsy-confirmed NASH with liraglutide, a once-daily GLP-1R agonist, over 48 weeks at a dose of 1.8 mg/d (LEAN study), led to a resolution of NASH in 39% of the treated patients as well as to reduced worsening of fibrosis.[9] In that study, mean placebo-subtracted weight loss under liraglutide was about 4.7 kg but was observed in both responders and non-responders, indicating that weight loss is necessary but not always sufficient for NASH resolution on treatment with a GLP-1R agonist.

Unimolecular dual or triple agonists combining GLP-1R activation with glucagon receptor (GCGR) and/or glucose-dependent insulinotropic peptide receptor (GIPR) agonism are emerging as a promising class of next-generation drug molecules for the treatment of metabolic diseases.[11] Besides their pronounced effects on glycaemic control and body weight, dual GLP-1R/GCGR agonists were shown to improve lipid metabolism and hepatic steatosis in diet-induced obese (DIO) mice.[12] When studied in non-human primates, treatment with an activity-balanced, lipidated dual GLP-1R/GCGR agonist, MEDI0382, led to significant hepatic fat reduction, which was also seen in human clinical studies.[13,14] MEDI0382 is currently under advanced clinical development for treatment of NAFLD/NASH (clinicaltrials.gov: NCT04019561).

Likewise, unimolecular dual GLP-1R/GIPR agonists as well as triple GLP-1R/GIPR/GCGR agonists have been shown to improve glycaemic control and weight loss in DIO mice accompanied by improved liver function and hepatic steatosis.[15,16]

Very little is known, however, about the effects of these multi-incretin approaches in models of obesity and insulin resistance in combination with manifest NASH and advanced fibrosis. Furthermore, the contribution of their individual components – GLP-1R, GCGR and GIPR agonism – to changes in hepatic and metabolic disease in the setting of NASH has not been thoroughly investigated. In the absence of such systematic studies, we have designed acylated, selective GLP-1R, GCGR and GIPR agonists as tool compounds and studied them alone and in combination in a mouse model of diet-induced, biopsy-confirmed advanced NASH and fibrosis to better understand their individual contribution and potential additive effects on improving systemic and hepatic metabolism.

2 | METHODS

2.1 | Animals and experimental design

The peptides were investigated in a mouse model of diet-induced obesity, NASH and fibrosis (DIO-NASH model) as described.[17] All animal experiments were conducted according to the international principles for the care and use of laboratory animals and were covered by personal licences for Jacob Jelsing (2013-15-2934-00784 and 2015-15-0201-00518), issued by the Danish Committee for Animal Research.

Male C57BL/6J mice (5 weeks old) obtained from JanVier (LanVier Labs, Le Genest-Saint-Isle, France) were placed on either a standard rodent chow (2.85 kcal/g, Altromin 1324; Brogaarden, Lynge, Denmark) or an AMLN diet (4.5 kcal/g, D09100301; Research Diets, New Brunswick, New Jersey). An AMLN diet is a NASH-inducing diet rich in fat (40%, including 18% trans-fat), carbohydrates (40%, including 20% fructose) and cholesterol (2%), as previously described.[18] After 33 weeks on the respective diet, a baseline liver biopsy was conducted for histological assessment of individual fibrosis and steatosis staging, as described.[19] Only mice with biopsy-confirmed NASH were included in the study. Mice with fibrosis stage <1 and steatosis score ≤2 were deselected prior to randomization. A total of 96 mice (n = 12 per treatment group) were randomized and stratified according to body weight and liver collagen 1 alpha 1 (COL1A1) quantification as a marker of fibrosis (Figure S1). Ten mice on a chow diet were included as controls. Treatment commenced 36 weeks after starting on the diets and lasted for 8 weeks, with all animals remaining on the same diet as in the pre-treatment phase. Four-hour fasting blood glucose and fasting insulin levels were determined after 6 weeks of treatment. At the end of the intervention, the mice were killed in the non-fasting state and liver tissue and serum samples were collected.

Tested compounds and doses are summarized in Table 1. All compounds were administered twice daily by subcutaneous injection using phosphate-buffered saline as vehicle.

Body weight and body composition analysis, blood sampling, plasma biochemistry and liver tissue biochemistry measurements were performed as previously described.[19]

2.2 | Histology assessment

Baseline liver biopsy and terminal samples were collected from the left lateral lobe (~50–100 mg at baseline and 200 mg at the end) and fixed overnight in 4% paraformaldehyde. Liver tissue was paraffin-embedded and sectioned (3-μm thickness). Sections were stained with haematoxylin and eosin and Sirius Red to quantitatively assess hepatic steatosis and fibrosis, respectively, then analysis with Visiomorph software (Visiopharm, Hørsholm, Denmark) was conducted. COL1A1 and galectin-3 were assessed using immunohistochemical staining. A

TABLE 1 Structure, activities and pharmacokinetics of test compounds. (A) Amino acid sequence and modifications of peptides used in this study. (B) *In vitro* potencies (EC_{50} in pM) in HEK293 cells overexpressing the murine glucagon-like peptide-1 receptor (GLP-1R), the glucagon receptor (GCGR) and the glucose-dependent insulinotropic peptide receptor (GIPR) for peptides used in this study, with potencies determined for human GLP-1, human glucagon and human GIP shown for comparison

(A) Peptide sequences

Compound	Sequence	Twice-daily dose, μg/kg
1-GCG	Tza-s-QGTFTSDYSKQ-K[γGlu-C16]-ESRRAQEFIEWLLAGGPESGAPPPS-NH_2	20
2-GLP1	H-s-EGTFTSDVSKQ-K[γGlu-C16]-EKRAA-Aib-EFIEWLKNTGPSSGAPPPS-NH_2	3
3-GIP	Y-a-EGTFISDYSIA-K[γGlu-C18]-DKIHQQDFVNWLLAQKPSSGAPPPS-NH_2	30
4-dual-GLP1/GCG	H-s-QGTFTSDLSKQ-K[γGlu-C16]-DSRRAQDFIEWLKNGGPSSGAPPPS-NH_2	30
Liraglutide	HAEGTFTSDVSSYLEGQAA-K[γGlu-C16]-EFIAWLVRGRG-OH	100

(B) *In vitro* receptor agonist potencies (cAMP release) in HEK-293 cell lines stably expressing mouse GLP-1, glucagon or GIP receptors

	Mouse EC_{50}, pM		
Compound	GLP-1R	GCGR	GIPR
1-GCG	396	1.3	>10 000
2-GLP1	1	>10 000	>10 000
3-GIP	>10 000	>10 000	3
4-dual-GLP1/GCG	2.3	25	>10 000
Liraglutide	4.4	>10 000	>10 000
hGLP-1	0.9		
Human glucagon	43.5	1.3	>10 000
Human GIP			1.2

Abbreviations: Aib, 2-Aminoisobutyric acid; Tza, Thiazolyl-alanine.

pathologist, blinded to the study, performed the histological assessment and scoring. NAFLD activity score (NAS; steatosis/inflammation/ballooning degeneration) and fibrosis stage were quantified, applying the criteria proposed by Kleiner et al.[20]

2.3 | Hepatic gene expression changes

Liver tissue was harvested from the left lateral lobe, stabilized overnight in RNAlater® solution (Merck KGaA, Darmstadt, Germany) and stored at −80°C. Total RNA isolation was performed with the miRNeasy kit according to the instructions of the manufacturer (QIAGEN GmbH, Hilden, Germany). RNA was quantified with an Agilent RNA 6000 Nano kit using an Agilent 2100 Bioanalyser (Agilent Technologies Inc, Waldbronn, Germany). Gene expression was quantified using digital droplet PCR analysis as described.[21]

2.4 | Statistical analysis

Statistical analysis was performed based on the two major objectives of the study: (1) to test for a treatment effect compared to vehicle (all treatment groups included), and (2) to test for add-on effects of combination treatment or the dual agonist compared to the strongest mono-agonist (2-GLP1, see Results). Statistical significance was evaluated using one-way ANOVA with Dunnett's test. Residuals were optically assessed to show homogeneous variances between groups.

For histological scores, two analyses were performed: (1) response-rate analysis, looking at the percentage of mice in each group showing improvement or worsening using a one-sided Fisher's exact test with Bonferroni correction, with the NASH vehicle group as comparator, and (2) analysis of the magnitude of the response, using a non-parametric two-tailed Wilcoxon test with Bonferroni correction applied to post-treatment scores versus NASH vehicle (all treatment groups), 2-GLP-1 or liraglutide (combination groups and dual agonist group to evaluate add-on effects), as NAS values at baseline were similar between NASH groups.

Data are presented as mean ± SEM. Statistical analysis was conducted using GraphPad Prism v7.03 and Everst@t v6.1.0.

3 | RESULTS

3.1 | Test compounds

For the systematic study of incretin hormone analogues and their combination in the described NASH animal model, specific mono-

agonists of the GLP-1R, GCGR and the GIPR were generated. In addition, an earlier described dual GLP-1R/GCGR agonist[21] was used as well as liraglutide as a GLP-1R agonist standard for comparison. Compounds 1-GCG, 2-GLP1 and 3-GIP were all designed based on the exendin-4 sequence using acylation with either palmitic acid or stearic acid via a γ-glutamate spacer linked to Lys14 to prolong their half-life, similar to liraglutide (Table 1A). In mice, 1-GCG is an equipotent GCGR agonist compared to glucagon itself, with a significantly higher selectivity towards the GLP-1R (>300-fold). 2-GLP1 and 3-GIP were at least 3000-fold more selective for their corresponding receptor in HEK293 cells heterologously expressing the murine receptor (Table 1B)[22,23] or in primary human cells containing the endogenous receptor (Table S1). The dual agonist 4-dual-GLP1/GCG was 10-fold more potent at the murine GLP-1R compared to the murine GCGR *in vitro*. All selected compounds had reasonable pharmacokinetic properties in mice, with half-lives of 2.5 to 4.1 hours after subcutaneous administration (Figure S2). In order to guarantee full daily coverage in our DIO-NASH model the compounds were dosed twice daily, with subcutaneous administration. The doses selected for compounds 1-GCG, 2-GLP1 and 3-GIP were 20 μg/kg, 3 μg/kg and 30 μg/kg twice daily, respectively. These doses were selected based on previous studies in the same model (Figure S3A,B), in DIO mice (Figure S3C,D) and published data[23,24] to elicit a limited body weight response as monotherapy and to allow

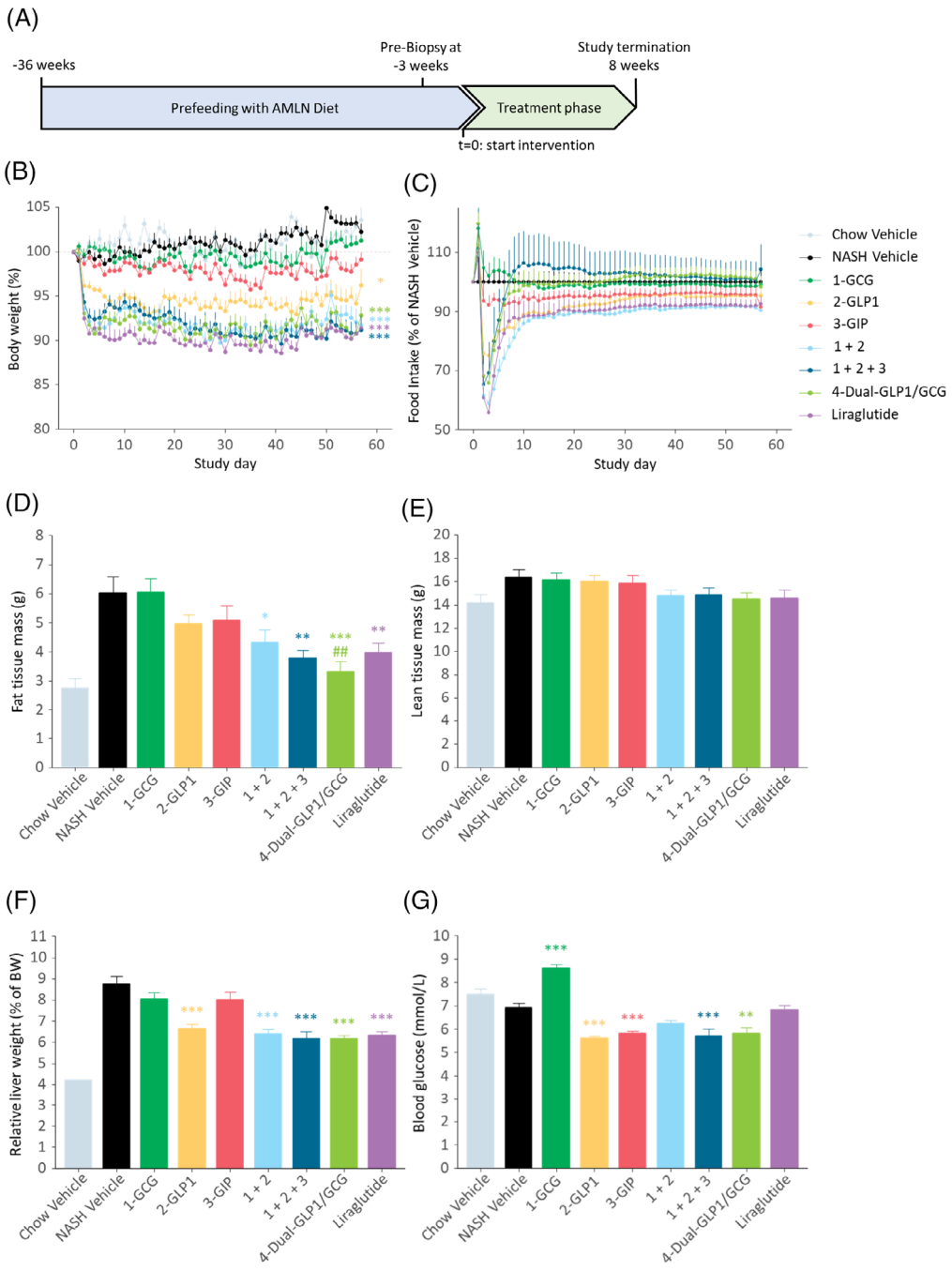

FIGURE 1 **A,** Study layout. **B,** Body weight change (% of day 0) throughout the 8-week treatment period. **C,** Comparative 24-hour food intake relative to non-alcoholic steatohepatitis (NASH) vehicle group. **D,** Lean tissue mass, **E,** fat tissue mass and **F,** relative liver weight at study termination. **G,** Four-hour fasting blood glucose levels after 6 weeks of treatment. Values are mean of n = 10–12 + SEM. **P* <0.05; ***P* <0.01, ****P* <0.001 for treatment groups compared to NASH vehicle, ##*P* <0.01 for combination or dual agonism treatment groups compared to 2-GLP1 group

for the identification of additive or synergistic activity when given in combination at the same individual doses. Because 1-GCG at 100 µg/kg twice daily gave rise to strong weight loss and reduction in hepatic triglycerides as monotherapy (Figure S3A,B), its dose was reduced to 20 µg/kg for the present study. Likewise, 100 µg/kg once daily of 3-GIP had resulted in glucose reduction and, to a small extent, weight loss as monotherapy in DIO mice (Figure S3C,D), and the dose was further reduced to 30 µg/kg twice daily for the study

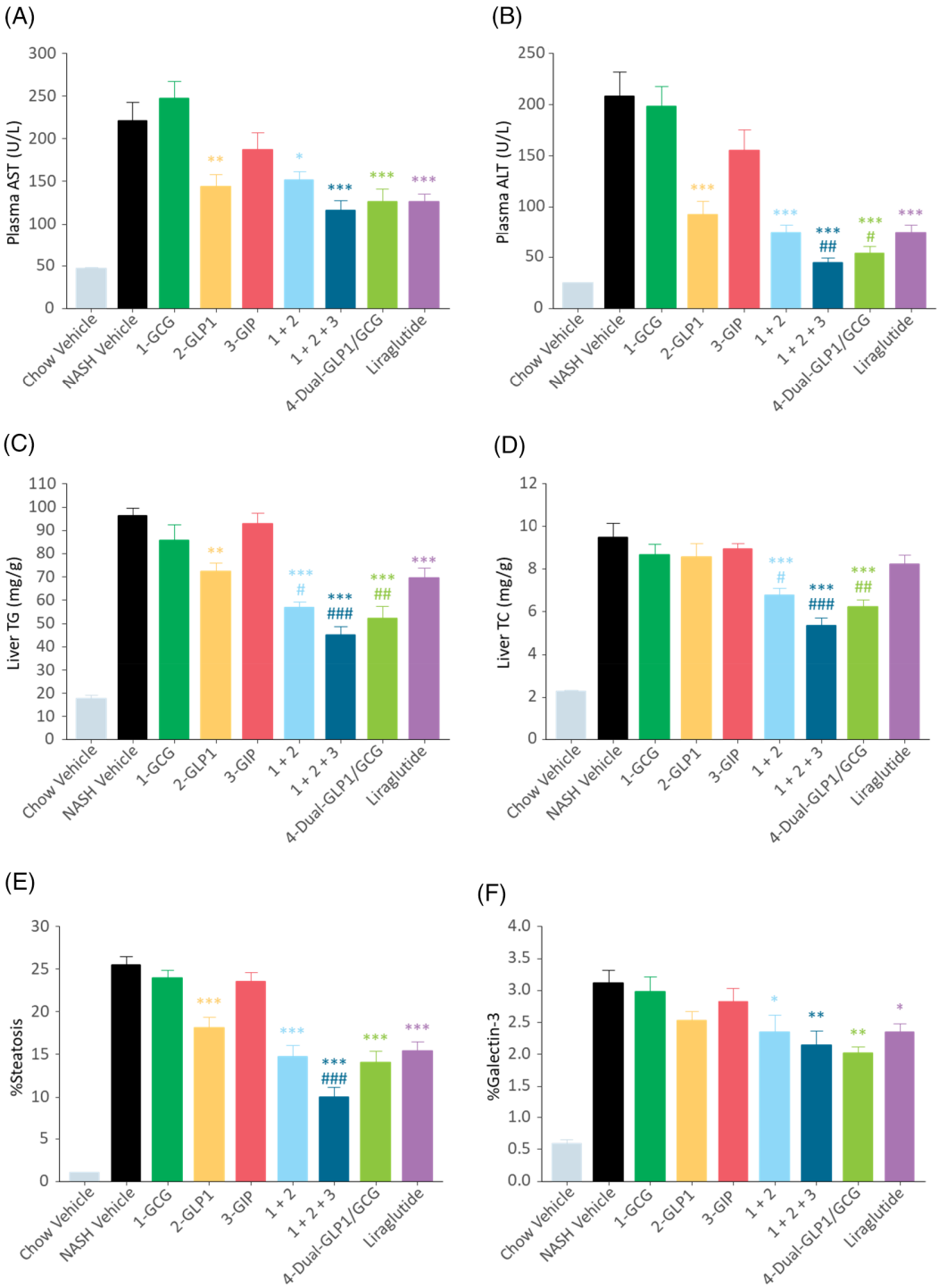

FIGURE 2 **A,** Plasma aspartate aminotransferase (AST) and **B,** plasma alanine aminotransferase (ALT) activities at study termination. **C,** Liver triglycerides and **D,** liver total cholesterol in mg/g wet liver tissue at study termination. **E,** Hepatic fat content and **F,** hepatic galectin-3 content (% fractional area) as determined by histological quantitative assessment (morphometry). Values are mean of n = 10–12 + SEM. **P* <0.05; ***P* <0.01, ****P* <0.001 for treatment groups compared to non-alcoholic steatohepatitis (NASH) vehicle. #*P* <0.05, ##*P* <0.01, ###*P* <0.001 for combination or dual agonism treatment groups compared to 2-GLP1 group

reported here. Liraglutide as a reference GLP-1R agonist was administered at 100 µg/kg twice daily to provide near-maximal effects that can be achieved with a selective GLP-1R agonist. The dose of compound 4-dual-GLP1/GCG was chosen as 30 µg/kg twice daily to achieve an extent of weight loss which is similar to liraglutide at 100 µg/kg twice daily.

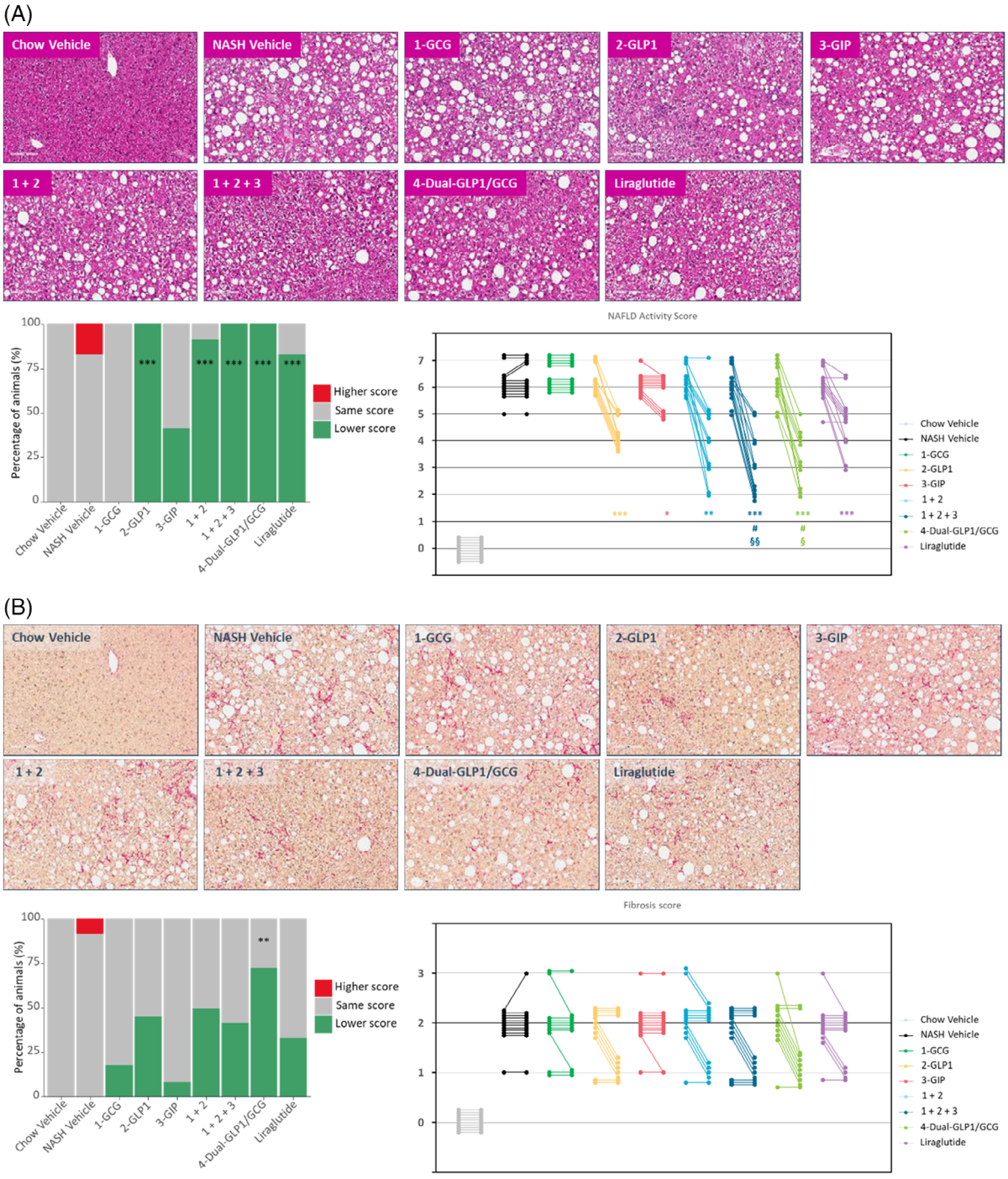

FIGURE 3 **A**, Representative haematoxylin and eosin-stained images (20×) of liver morphology at study termination and individual changes in non-alcoholic fatty liver disease (NAFLD) activity score (post- vs. pre-treatment) for the different treatment groups. **B,** Representative picrosirius red stained images (20×) and individual changes in fibrosis score for the different treatment groups (post- vs. pre-treatment). ***P* <0.01, ****P* <0.001 vs non-alcoholic steatohepatitis (NASH) vehicle (all treatment groups), #*P* <0.05 vs 2-GLP1 (combination treatments and 4-dual-GLP1/GCGR), §*P* <0.05, §§*P* <0.01 vs liraglutide (combination treatments and 4-dual-GLP1/GCGR)

3.2 | Body composition and food intake, blood glucose

The overall study design is summarized in Figure 1A. Mice were placed on the NASH-inducing AMLN diet for 36 weeks and then treated with the different peptides or peptide combinations for 8 weeks while remaining on the AMLN diet. The change in body weight over the treatment period is shown in Figure 1B. The mean (SEM) body weight at the onset of treatment was 36.1 g (± 0.3 g SEM) without significant differences between treatment groups, compared to 31.1 ± 0.4 g for the chow control mice. Whereas vehicle-treated mice gained about 3% over the treatment period, body weight remained nearly constant for mice treated with 1-GCG or 3-GIP alone at the tested doses. Treatment with 2-GLP1 led to body weight loss of 5%, whereas 4-dual-GLP1/GCG, liraglutide or the dual or triple combinations of 1-GCG, 2-GLP1 ± 3-GIP led to 8% to 9% weight loss, all significantly different from vehicle controls and becoming close to the weight of lean control mice. Weight loss was driven primarily by an initial decrease in food intake in the first week of treatment, which then recovered and remained stable for the rest of the treatment period, as observed relative to NASH vehicle (Figure 1C) and for daily and cumulative energy intake (Figure S4A,B). Body weight loss

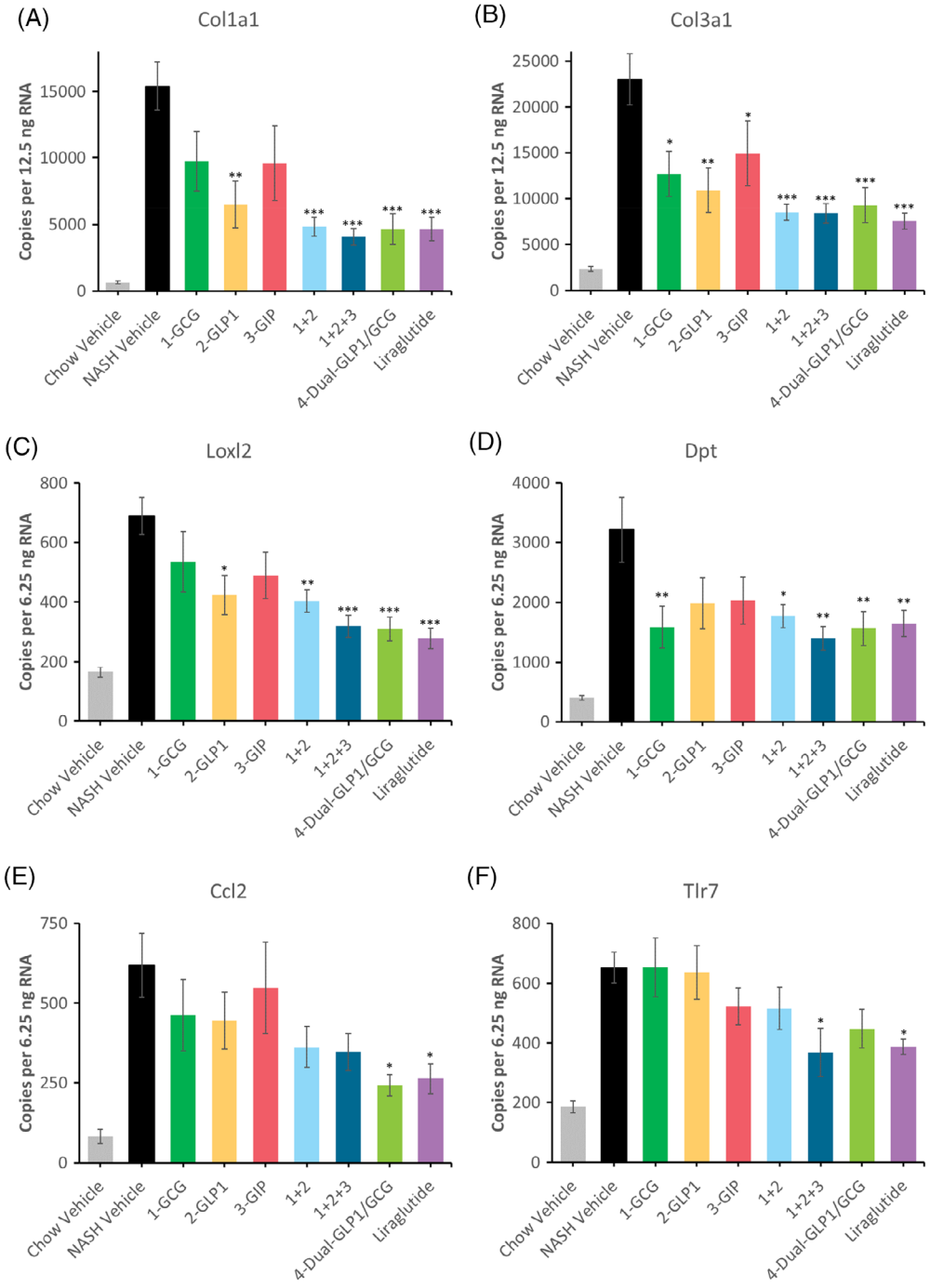

FIGURE 4 Hepatic expression of fibrosis marker genes **A**, Col1a1, **B**, Col3a1, **C**, Loxl2 and **D**, Dpt, inflammation marker genes. **E**, Ccl2 and **F**, Tlr7 at study termination as determined by digital droplet PCR. Values are mean of n = 10–12 + SEM. **P* <0.05; ***P* <0.01, ****P* <0.001 for treatment groups compared to non-alcoholic steatohepatitis (NASH) vehicle

predominantly resulted from loss of fat (Figure 1D), whereas there was no significant change in lean mass (Figure 1E).

Mice with NASH had enlarged livers that had approximately twice the weight of those of the chow control mice (Figure 1F). Whereas treatment with 1-GCG or 3-GIP alone had no effect on liver weight at the selected doses, 2-GLP1 (−24%), the combination of 2-GLP1 with 1-GCG (−27%) or with 1-GCG and 3-GIP (−30%) as well as 4-dual-GLP1/GCG (−30%) and liraglutide (−28%) led to a significant reduction in liver weight.

As expected, treatment with the glucagon receptor agonist 1-GCG led to an increase in blood glucose, measured after 6 weeks of treatment, whereas it remained constant or decreased in all other treatment groups (Figure 1G). Notably, DIO-NASH mice are not diabetic which explains the limited glucose-lowering seen for the GLP-1R agonist- or GIPR agonist-containing treatment groups. There were no differences in 4-hour fasting plasma insulin between the different treatment groups (Figure S4C).

3.3 | Liver enzymes, hepatic steatosis and histopathology

Plasma alanine aminotransferase and aspartate aminotransferase activities were significantly reduced after intervention with 2-GLP1, the 1-GCG + 2-GLP1 dual combination, the 1-GCG + 2-GLP1 + 3-GIP triple combination, 4-dual-GLP1/GCG or liraglutide (Figure 2A and B). In the same groups, total liver triglycerides were lower than in vehicle control mice at the end of the treatment period (Figure 2C). Notably, there was a significant add-on effect of GCGR and GIPR agonism to GLP-1R activation in lowering liver triglycerides, which was also observed for lowering total liver cholesterol (Figure 2D).

Whereas 1-GCG or 3-GIP alone did not lead to a reduction in hepatic steatosis as assessed by quantitative histology, it was significantly lower in all treatment groups containing GLP-1R-agonistic activity (Figure 2E, Figure S4D), with a significantly stronger reduction observed for the 1-GCG + 2-GLP1 + 3-GIP triple combination compared to the GLP-1R agonist 2-GLP1 alone. Hepatic galectin-3 as a macrophage-derived protein involved in hepatic fibrogenesis[24] was decreased in the dual and triple combination group and on treatment with 4-dual-GLP1/GCG or liraglutide (Figure 2F, Figure S4E). While a significant reduction in total COL1A1 was observed for 2-GLP1, the dual and triple combinations and 4-dual-GLP1/GCG (Figure S4F), this difference was lost upon normalization to liver weight (not shown).

Change in the histological NAS after treatment is depicted in Figure 3A. In the pre-treatment biopsy, DIO-NASH mice had, on average, a NAS of 6, primarily driven by a steatosis score of 3 in all DIO-NASH mice, and an inflammation score of 3 in 85% of the DIO-NASH mice, whereas there was little hepatocyte ballooning (35% with score 1, 65% with score 0) at the onset of therapy. Treatment with 1-GCG, 2-GLP1 ± 3-GIP dual and triple combinations or the dual GLP-1R/GCGR-agonist 4-dual-GLP1/GCG led to a stronger decrease in NAS compared to treatment with single GLP-1R-, GCGR- or GIPR-agonistic peptides (Figure 3A), with improvements in all three NAS components steatosis, lobular inflammation and hepatocyte ballooning (not shown). Notably, the dual GLP-1R/GCGR agonist and the dual and triple combination treatments also provided a stronger decrease in NAS compared to liraglutide (Figure 3A), although the amount of body weight loss between these groups was nearly indistinguishable (Figure 1B).

The majority of the DIO-NASH mice had a fibrosis score of 2 in the pre-treatment biopsy. Whereas in the 1-GCG and 3-GIP groups only two mice (18%) and one mouse (8%), respectively, showed an improvement by one point, response rates in mice treated with 2-GLP1 (5/11 or 45%), the dual (6/12 or 50%) or triple peptide combination (5/12 or 42%) or 4-dual-GLP1/GCG (8/11 or 73%) tended to be higher (Figure 3B).

This improvement in histology is also reflected in changes in the expression of marker genes for fibrosis (Col1A1, Col3A1, Loxl2) or inflammation (Ccl2, Tlr4). In addition, dermatopontin (Dpt), previously described to be associated with NASH both in rodents and in people,[25] was found to be regulated in DIO-NASH mice and partially normalized with treatment (Figure 4).

4 | DISCUSSION

Using a mouse model of biopsy-confirmed, diet-induced, advanced NASH with fibrosis, we have demonstrated that combining GLP-1R, GCGR and GIPR agonism provides additive effects in improving hepatic steatosis, liver injury and NAFLD activity.

The GLP-1R agonists are an established therapy for the treatment of diabetes and obesity,[26–32] with positive effects on cardiovascular outcome.[33–37] Recently, dual GLP-1R/GCGR[16,38] and GLP-1R/GIPR[39] agonists have demonstrated clinical proof of concept in lowering body weight and blood glucose in obese patients with type 2 diabetes. Treatment of patients with biopsy-confirmed NASH with the GLP-1R agonist liraglutide led to NASH resolution and inhibition of fibrosis progression,[12] and GLP-1R agonists were shown to improve hepatic and metabolic health in pre-clinical models of NAFLD or NASH.[40–45] In contrast, there is little information on the activity of dual or triple agonists in models of NASH, and the contribution of individual incretin or glucagon effects to the combined activity of these molecules has not been systematically investigated. A dual-active peptide targeting GLP-1R and GCGR, G49, was described to improve hepatic steatosis and ameliorate liver injury in mice on a methionine and choline-deficient diet and partial hepatectomy.[46] The EC_{50} of G49, a pegylated peptide derived from oxyntomodulin, towards the mouse GCGR was about 25 times higher than that for the mouse GLP-1R. However, due to the restrictive diet used in this study, vehicle control mice already lost more than 30% of their initial body weight after 3 weeks on the diet, making it difficult to compare the results with those of the present study in which the mice remained obese. In another study, a unimolecular GLP-1R/GCGR/GIPR triagonist led to an improvement in steatohepatitis in DIO mice.[16] However, these studies did not include specific GLP-1R or GCGR agonists as comparators to delineate the relative contribution of the two components to the observed effects.

Weight loss is a strong predictor of a reduction in hepatic steatosis and resolution of NASH, independently of whether it is induced by diet and exercise,[7] bariatric surgery[8] or pharmacological intervention.[9] Correspondingly, weight loss observed in the present study was tied to improvements in liver metabolism and histology. However, combination of sub-maximal doses of GLP-1R, GCGR and GIPR mono-agonists, as well as administration of a dual GLP-1R/GCGR agonist, provided a more pronounced improvement in NAFLD activity score compared to a high dose of liraglutide eliciting the maximal GLP-1R-mediated response, whereas the body weight loss upon treatment with the triple combination of mono-agonists, the dual GLP-1R/GCGR agonist and liraglutide was very similar. Thus, it is likely that there are additional, weight-independent effects via activation of, for example, liver GCGR leading to inhibition of hepatic *de novo* lipogenesis and stimulation of liver fat utilization.[4748] Recently, it was demonstrated that chronic GCGR activation also stimulates expression of the hepatic leptin receptor (LEPR) although contribution of hepatic LEPR signalling to reduction in liver triglycerides remains unclear.[49] For GIP, the situation is less clear, as the GIPR is not expressed in liver.[50] The effect of GIP on hepatic steatosis is therefore likely to be more indirect, for example, via body weight loss and promotion of adipocyte maturation,[51,52] thereby reducing circulating free fatty acids and triglycerides.

Whether GLP-1R agonism by itself can, independent of body weight changes, directly influence fat deposition in hepatocytes is a matter of debate. While Gupta et al[53] found GLP-1R RNA and protein in primary hepatocytes and linked hepatic GLP-1R signalling via the PDK1/Akt pathway to lead to a decrease in fatty acid synthesis, others could not confirm expression of GLP-1R in hepatocytes.[54,55]

The model of diet-induced NASH used in the present study has been successfully demonstrated to recapitulate clinical findings of improving disease activity and fibrosis, for example, with the FXR agonist obeticholic acid, the PPARα/δ agonist elafibranor, and liraglutide.[44] In the present study, we could confirm previous findings with liraglutide leading to approximately 10% of body weight loss, strong reductions in plasma transaminase activities and hepatic steatosis, and improvement in NAS. The fibrosis response in this study, with an improvement seen in 33% (4/12) of mice treated with liraglutide, was also similar to that reported for the liraglutide-treated NASH patients in the LEAN trial (26%, 6/23),[9] supporting the, at least backward, translatability of the DIO-NASH model.

Although the present study provides the first systematic investigation of individual and combined effects of GLP-1R, GIPR and GCGR agonism in diet-induced NASH, it has certain limitations. Firstly, peptides could only be tested at one dose because including several doses per mechanism alone and in combination would have made the study excessively large and costly. Individual doses were selected according to previous studies in other murine models to produce small effects on weight and metabolic variables. However, the selected dose of 2-GLP1 by itself led to significant weight loss, glucose-lowering and reduction in hepatic steatosis, leaving less room for additive or synergistic effects of 1-GCG and/or 3-GIP on top of 2-GLP1 to be explored. In further studies, lower doses of 2-GLP1 should be included.

Secondly, development of NASH in our model was driven by a diet artificially high in fat, especially trans-fat, fructose and cholesterol. Whether and how results obtained with this murine model translate into clinical efficacy in humans is not clear. In reverse translational studies, several molecules with clinical efficacy in NASH, for example, obeticholic acid, liraglutide and elafibranor, also led to improvements in NASH in our model.[46] However, it remains to be shown that the model also predicts forward translation into humans. Notably, following a US Food and Drug Administration ban on trans-fat as a food component,[56] the NASH-inducing diet has recently been changed to contain palm oil instead of trans-fat.[57]

Finally, it was outside of the scope of this study to further investigate the molecular mechanisms of how GLP-1R-, GCGR- or GIPR-specific agonists and their combinations elicit their beneficial effects on systemic metabolism and steatohepatitis, for example, through comparative expression, proteomics or metabolomics analysis. Such studies are currently under way.

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CONFLICTS OF INTEREST

A.K., C.K., R.E., T.K., M.B., T.H., A.E., K.L., W.H., C.R., Z.B., J.C.G., V.M. and M.W. are employees of Sanofi; some hold Sanofi shares.

AUTHOR CONTRIBUTIONS

A.K., J.C.G., F.P., B.S., V.M. and M.W. designed the concept. A.K., A.N.M., C.K., R.E., T.K., C.R. and Z.B. performed experiments and analysed data. M.B., T.H., K.L., A.E. and M.W. designed and synthetized test compounds. W.H. performed statistical analyses. A.K. and M.W. wrote the paper. All authors edited draft versions and approved the final manuscript.

ORCID

Aimo Kannt https://orcid.org/0000-0002-5197-2286
Ralf Elvert https://orcid.org/0000-0003-1216-307X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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