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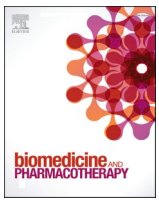

Chronic treatment with glucagon-like peptide-1 and glucagon receptor co-agonist causes weight loss-independent improvements in hepatic steatosis in mice with diet-induced obesity

Emma Rose McGlone [a,b], David C.D. Hope [b], Iona Davies [b], Marian Dore [c], Rob Goldin [b], Ben Jones [b], Zhigang Liu [b], Jia V. Li [b], Panagiotis A. Vorkas [d,e], Bernard Khoo [f], David Carling [g], James Minnion [b], Stephen R. Bloom [b], Tricia M-M. Tan [b,*]

[a] Department of Surgery and Cancer, Imperial College London, London, UK
[b] Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK
[c] Genomics facility, MRC Laboratory of Medical Sciences (LMS), Imperial College London, London, UK
[d] Institute of Applied Biosciences, Centre for Research and Technology Hellas (INAB|CERTH), Thessaloniki 57001, Greece
[e] School of Cardiovascular and Metabolic Medicine & Sciences, King's College London, London, UK
[f] Endocrinology, Division of Medicine, University College London, London, UK
[g] Cellular Stress group, MRC LMS, Imperial College London, London, UK

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ABSTRACT

Objectives: Co-agonists at the glucagon-like peptide-1 and glucagon receptors (GLP1R/GCGR) show promise as treatments for metabolic dysfunction-associated steatotic liver disease (MASLD). Although most co-agonists to date have been heavily GLP1R-biased, glucagon directly acts on the liver to reduce fat content. The aims of this study were to investigate a GCGR-biased co-agonist as treatment for hepatic steatosis in mice.
Methods: Mice with diet-induced obesity (DIO) were treated with Dicretin, a GLP1/GCGR co-agonist with high potency at the GCGR, Semaglutide (GLP1R monoagonist) or food restriction over 24 days, such that their weight loss was matched. Hepatic steatosis, glucose tolerance, hepatic transcriptomics, metabolomics and lipidomics at the end of the study were compared with Vehicle-treated mice.
Results: Dicretin lead to superior reduction of hepatic lipid content when compared to Semaglutide or equivalent weight loss by calorie restriction. Markers of glucose tolerance and insulin resistance improved in all treatment groups. Hepatic transcriptomic and metabolomic profiling demonstrated many changes that were unique to Dicretin-treated mice. These include some known targets of glucagon signaling and others with as yet unclear physiological significance.
Conclusions: Our study supports the development of GCGR-biased GLP1/GCGR co-agonists for treatment of MASLD and related conditions.

1. Introduction

Excessive fat deposition in the liver secondary to obesity and metabolic dysfunction now affects 25 % of the global population[1]. Metabolic dysfunction-associated steatotic liver disease (MASLD, also known as non-alcoholic fatty liver disease) is a progressive condition that can lead to steatohepatitis, cirrhosis, liver failure and hepatocellular cancer [2]. Although it responds well to weight loss[3–5], at present there is a paucity of pharmaceutical treatments for MASLD[6], although the FDA has recently approved resmetirom, an oral thyroid hormone receptor beta selective agonist, for the treatment of metabolic dysfunction-associated steatohepatitis (MASH) [7].

Abbreviations: MASLD, Metabolic dysfunction-associated steatotic liver disease; DIO, diet-induced obesity; GCGR, glucagon receptor; GLP1R, glucagon-like peptide-1 receptor; Sema, Semaglutide; WM, calorie restricted to match the weight of the other treatment groups "weight-matched"; HOMA-IR, homeostatic model assessment of insulin resistance; HFD, high fat diet.

* Correspondence to: Dept of Metabolism, Digestion and Reproduction, Imperial College London, 6th Floor Commonwealth Building, Du Cane Road, London W12 0NN, UK.
E-mail address: t.tan@imperial.ac.uk (T.M.-M. Tan).

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Glucagon-like peptide-1 (GLP1) is an incretin hormone secreted in response to eating, which activates its cognate receptor GLP1R leading to increased insulin secretion, satiety, delayed gastric emptying and reduced food intake. GLP1R agonists such as liraglutide and semaglutide are now accepted treatments for diabetes and obesity[8]. In clinical trials, chronic treatment with liraglutide and semaglutide improves hepatic steatosis, steatohepatitis and insulin sensitivity, but the effects are limited by gastrointestinal side effects which include nausea, vomiting and constipation[9,10]. The development of unimolecular co-agonists of GLP1R and glucagon receptor (GCGR) has shown promising beneficial effects on body weight and metabolic health over those obtainable with GLP1R agonists alone[11,12]. Oxyntomodulin, the endogenous GLP1/GCGR co-agonist, additionally promotes satiety and increases energy expenditure via its interaction with GCGR[13,14]. GLP1/GCGR co-agonists may lead to greater improvement in MASLD than GLP1R agonism alone[15–17]. This could be due to direct effects of GCGR agonism in increasing hepatic fatty acid oxidation[18] and decreasing de novo lipogenesis[19]. Indeed, GCGR agonism is a treatment for liver steatosis in cows[20], while chronic treatment with the glucagon antagonist LY2409021 leads to liver fat accumulation in patients with type 2 diabetes mellitus[21]. Despite the potential direct effects of additional glucagon receptor agonism to reduce liver fat, co-agonists also lead to substantial weight loss, and it is unclear whether improvements seen after treatment with these agents are weight loss-dependent or if there is a weight loss-independent component. Previous studies claiming weight loss-independent changes, e.g. with oxyntomodulin, have examined acute changes in gene expression after a few hours' treatment[15]. Furthermore, due to concerns that glucagon could promote hyperglycemia, most dual agonists trialed have exhibited a strong GLP1R bias[12,22,23], which could reduce the potential benefits of GCGR action on hepatic fat content.

The aims of this study were to investigate the effects of a GCGR-preferring GLP1/GCGR co-agonist (Dicretin) on hepatic steatosis and metabolic health in mice with diet-induced obesity; to compare these outcomes to weight-matched mice undergoing treatment with diet alone or a GLP1R agonist; and to investigate the hepatic transcriptome and metabolome of mice undergoing these treatments to gain mechanistic insight into pathways that are activated with glucagon agonism.

2. Materials and methods

2.1. Animal studies

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and carried out in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Experiments were also approved by and conducted in accordance with the Animal Welfare Ethical Review Board (AWERB) of Imperial College London.

2.1.1. Housing and diets

C57BL/6 J male mice (Charles River, UK) were housed in cages at controlled temperature (22°C) with a 12 h light dark cycle with free access to water. Food deprivation and subsequent interventions were performed during the light cycle. Mice were weaned on standard chow (Special Diet Services RM3), then from 5 weeks of age were given free access to a high fat diet (HFD) containing 60 % kcal from fat (Research Diets D12492). Mice were group housed from birth until age 17 weeks, from which point they were individually housed to allow monitoring of food intake. At age 19 weeks mice that had failed to gain weight on the HFD or whose weight had failed to stabilize during the period of single-housing (approximately 10 %) were excluded, and remaining mice were allocated to study groups using stratified (by weight) random sampling.

2.1.2. Pharmacological intervention

Mice were allocated to the following groups, with nine mice in each: (1) Free access to HFD + vehicle (Vehicle); (2) Calorie-restricted HFD + vehicle (WM); (3) Free access to HFD + semaglutide (Sema); (4) Free access to HFD + Dicretin (Dicretin). All mice were maintained on HFD throughout the study period with free access to water. All mice had subcutaneous injections three times weekly, on Monday, Wednesday and Friday. Based on the results of a dose-finding exercise in a separate cohort of mice, starting doses were 15 nmol/kg semaglutide and 150 nmol/kg Dicretin, with 2 g of HFD for mice in group 2. All peptides were supplied by WuXi AppTec Biologics (China). Dicretin was formulated with zinc as a chelation agent (1:1.2 molar ratio). Vehicle was saline only, and doses were made up such that an equivalent volume was injected. Daily body weight and food intake was recorded, and doses were adjusted to ensure that groups 2, 3, and 4 remained weight-matched, with a target weight loss of between 20 % and 25 % of total body weight over the 24-day treatment period.

2.1.3. Cull and tissue harvest

After the study period, mice were culled via decapitation following a 5-hour period of food deprivation to allow collection of tissues for further assay. Plasma was obtained from blood collected in tubes on ice that had previously been flushed with heparin 1000 IU/ml and contained 1 μl protease inhibitor (Sigma) and immediately spun at 4°C. Serum was obtained from blood allowed to clot at room temperature for 10 minutes before spinning at 4°C. Samples were separated and aliquots of plasma and serum were stored at −80°C. Organs were harvested rapidly, weighed, and either snap-frozen in liquid nitrogen or fixed in 2 % paraformaldehyde for subsequent histopathological examination.

2.1.4. Glucose tolerance testing

Glucose tolerance testing was performed in mice following 5-hour food restriction, 24 hours after the last injection of peptide or vehicle. Blood was sampled from the tail vein before and at time intervals following intraperitoneal injection of 20 % glucose (2 mg/g lean body weight, estimated to be 31 g based on the mean weight of a cohort of mice from the same batch fed standard chow throughout the study period). Glucose was measured using a calibrated glucometer (GlucoRx, Nexus, UK). Incremental area-under-curve (iAUC) was calculated from baseline glucose reading to account for differences in fasting glucose. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the formula (fasting glucose mmol/l × fasting insulin mU/l)/22.5 [24].

2.1.5. Hepatic histology and biochemistry

Lipids were extracted from tissue by homogenization in ethanol (volume of ethanol [ml] = liver weight [mg] x 0.03) [25]. Triglyceride content of samples was measured using a GPO-PAP Triglyceride assay (Randox Laboratories Ltd, UK) and cholesterol using Amplex Red Cholesterol Assay Kit (ThermoFisher Scientific, UK). Liver histology was assessed using the Non-alcoholic fatty liver disease Activity Score (NAS) [26] by a pathologist with a special interest in liver disease and blinded to treatment assignment, using hematoxylin and eosin-stained paraffin sections.

2.1.6. Clinical chemistry

Plasma from mice after 5-hour food restriction was assayed using ultra-sensitive mouse insulin, leptin and adiponectin enzyme-linked immunosorbent assay kits (Crystal Chem, Netherlands). Glucagon with Cisbio HRTF and total GLP1 with the Meso Scale Discovery system (MSD, USA). L-amino acids, alanine and branched-chain amino acids were measured with colorimetric assays from Sigma-Aldrich. All assays had inter and intra-assay CVs of ≤10 %. Serum triglyceride was assayed using a GPO-PAP Triglyceride assay (Randox Laboratories Ltd, UK).

2.2. Cell studies

2.2.1. Cell lines

AD293 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin and cultured at 37°C in a 5 % CO_2 atmosphere.

2.2.2. cAMP assays

Cells were transfected for 24 hours with plasmids encoding full length wild-type human or mouse GLP1R or GCGR (Azenta Life Sciences) using Lipofectamine 2000 (Invitrogen). Cells were then stimulated with agonists for 30 min in serum-free DMEM with 0.1 % BSA. cAMP was assayed using the Cisbio cAMP Dynamic 2 HTRF kit. 3-parameter logistic fitting was performed with fitting to the global maximum for the assay. Ratio of the cAMP response for Dicretin relative to the native ligand at each receptor, was calculated by taking the antilog of the difference between delta pEC_{50} at each receptor and using GLP1R as a reference (arbitrarily assigned 1).

2.2.3. Receptor binding assays

GLP1R and GCGR binding assays were performed using lung and liver tissue harvested from C57BL/6 J male mice, respectively. Peptides were iodinated using I^{125} from Hartmann Analytic (Braunschweig, Germany). The method has been described in full previously[27].

2.3. Transcriptomics, metabolomics and lipidomics

RNA, metabolite and lipid extraction, preparation and analysis are described in detail in Supplementary Methods. RNA data generated from this study is openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/, reference number GSE243681.

2.4. Quantification and statistical analyses

Glucagon-alanine index was calculated as the product of fasting plasma glucagon (pmol/l) and fasting plasma alanine (mmol/l). Unless stated otherwise, data are presented as mean and standard error of mean. GraphPad Prism 9.0.0 (GraphPad Software, USA) was used for graphing and statistical analysis. Datasets were compared using one-way ANOVA, followed by Dunnett's or Sidak's multiple comparison post-hoc tests. For comparison of non-normally distributed data, a Kruskal Wallis test with Dunn's multiple comparison post-hoc test was used. Comparisons were made with reference to the vehicle group (Vehicle) and the weight-matched group (WM) for each intervention.

3. Results

3.1. Dicretin is a dual GCGR and GLP1R agonist which is GCGR-preferring

Dicretin is a peptide analogue of oxyntomodulin, which has been formulated with 2-aminoisobutyric acid (AIB) at position 2. This protects the molecule from degradation by dipeptidyl peptidase-4. It is also co-administered with zinc, which allows slow release from subcutaneous tissues. Dicretin has similar potency for cAMP production to native glucagon, as measured in AD293 cells transfected with a full length wild-type human or mouse GCGR (Table 1; Fig. 1A-C). It is less potent than GLP1 at both human and mouse GLP1R: the ratio of the cAMP response for Dicretin relative to the native ligand at each receptor was around 15:1 at the human receptors and 10:1 at the mouse receptors (GCGR: GLP1R). Similarly, the binding affinity ratio to the endogenous mouse GCGR and GLP1R, measured using lung and liver tissue (respectively) harvested from C57BL/6 J male mice, was 14:1 (GCGR: GLP1R).

Table 1
Dicretin is a GCGR-preferring GCGR/GLP1R co-agonist in mice and humans with similar potency to native glucagon. Mean ± SEM presented, n.d. not determined; pIC_{50}: negative logarithm of half maximal inhibitory concentration; pEC_{50}: negative logarithm of half maximal effective concentration; Delta pEC_{50} and pIC_{50} represent the pEC_{50} or pIC_{50} value relative to the native ligand for each receptor; SEM: standard error of the mean.

cAMP potency (pEC_{50})	**Mouse GLP1R**	**Mouse GCGR**	**Human GLP1R**	**Human GCGR**
GLP1	8.95 ± 0.08	n.d.	9.13 ± 0.07	n.d.
GCG	6.41 ± 0.08	9.30 ± 0.15	6.70 ± 0.06	9.47 ± 0.13
Dicretin	7.94 ± 0.04	9.32 ± 0.11	8.33 ± 0.07	9.85 ± 0.09
ΔpEC_{50} (Dicretin – native ligand)	-1.01 ± 0.04	0.02 ± 0.05	-0.80 ± 0.06	0.38 ± 0.08
Binding affinity (pIC_{50})	**Mouse GLP1R**	**Mouse GCGR**		
GLP1	9.24 ± 0.02	n.d.		
GCG	n.d.	8.36 ± 0.03		
Dicretin	8.17 ± 0.07	8.45 ± 0.02		
ΔpIC_{50} (Dicretin – native ligand)	-1.07 ± 0.05	0.09 ± 0.01		

3.2. Dicretin is associated with weight loss-independent improvements in hepatic steatosis

We treated obese mice with Dicretin, the GLP1R monoagonist semaglutide (Sema), vehicle or with food restriction ("weight-matched"; WM) such that the weights of all treatment groups were matched. Over a 24-day period, all treatment groups lost around 25 % of their original body weight, stabilizing for the final few days at a similar weight to a lean mouse maintained on standard chow[28] (Fig. 1D and E; Table 2). For equivalent weight loss, cumulative food intake was higher in the Sema and Dicretin-treated cohorts than the calorie-restricted weight--matched cohort (Fig. 1F). Weight loss was associated with an improvement in glucose tolerance in all treated groups; to a greater extent in WM and Dicretin-treated mice than Sema-treated mice (Fig. 1G and H). Fasting insulin decreased following both Sema and Dicretin treatment (Fig. 1I); and homeostatic model assessment of insulin resistance (HOMA-IR) was improved in mice undergoing all weight-loss treatments (Fig. 1J). All treated mice lost substantial amounts of white adipose tissue from both subcutaneous and gonadal regions, as well as brown fat (Fig. 1K-M).

At the end of the study, liver triglyceride was significantly reduced in all treated cohorts, however the degree of improvement was greater in Dicretin-treated mice than in WM mice, indicating a weight loss-independent effect (Fig. 2A). Hepatic cholesterol content was comparable between the groups whilst NAS score was significantly improved in all treated cohorts (Fig. 2C and D; Table 2).

Circulating leptin fell in all treated groups as expected from reductions in fat mass, although adiponectin was not significantly altered (Fig. 3A-C). The adiponectin:leptin ratio increased in all treated groups (vehicle vs WM $p=0.06$; vehicle vs Sema $p=0.004$; vehicle vs Dicretin $p=0.08$). Total plasma amino acids including the glucogenic amino acid alanine decreased in mice treated with Dicretin when compared to vehicle control, but not in WM nor Sema groups; branched-chain amino acids were lower in the groups treated with Sema and Dicretin (Fig. 3D-F). The GLP1 assay detected semaglutide in the Sema group and there was a numerical reduction in fasting glucagon in Sema and Dicretin groups (Table 2). Glucagon-alanine index, a surrogate marker of glucagon resistance [29], was numerically lower in all treatment groups (Table 2).

A

GCG	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe	Val	Gln	Trp	Leu	Met	Asn	Thr					
GLP-1	His	Ala	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr	Leu	Glu	Gly	Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg	NH2				
Dicretin	His	AIB	His	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ala	Lys	Arg	Ala	Gln	Glu	Phe	Ile	Glu	Trp	Leu	Leu	Gln	Ser	His	His	His	His	Pro	Ser	Trp

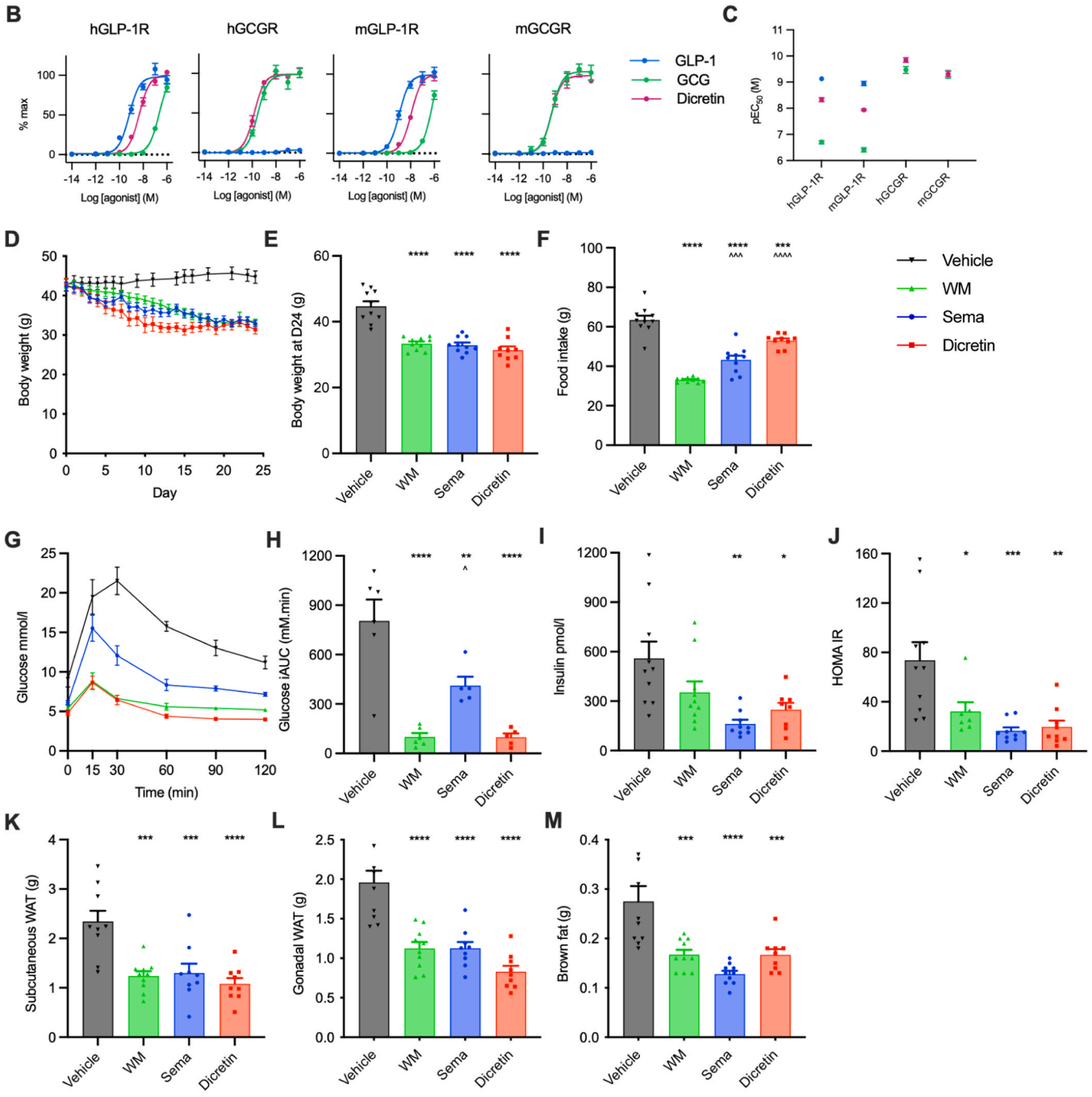


Fig. 1. Treatment with Dicretin decreases body weight and improves glucose tolerance. A: amino acid sequence of GLP1, GCG and Dicretin. Red letters indicate conserved amino acids. AIB = aminoisobutyric acid. B: stimulation of cAMP accumulation with human (h) and mouse (m) receptors as indicated, in AD293 cells. 3-parameter logistic fitting of n=4, with fitting to the global maximum for the assay. C: pEC_{50} of the three peptides at each receptor. Following 14 weeks on a high fat diet, obese mice were treated with either vehicle (Vehicle; black), semaglutide (Sema; blue); Dicretin (red) or calorie restriction to match the weight of the other treatment groups (WM; green). A: weight trajectory over time; B: final weight at day 24; C: cumulative food intake over study period; D: blood glucose in response to intra-peritoneal glucose tolerance test; E: $iAUC_{0-120}$ for glucose during tolerance test; F: fasted (5 hours) serum insulin levels of mice at the end of the study period; G: homeostatic model assessment of insulin resistance (HOMA-IR) calculated from 5-hour fasted glucose and insulin levels at end of study period; H-J tissue weights at end of study period. n=9 in each group, mean and SEM plotted. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to vehicle; ^p <0.05, ^^p<0.01, ^^^p<0.001, ^^^^p<0.0001 when compared to WM (one-way ANOVA followed by Sidak's multiple comparison test).

Table 2
Body weight and biomarkers of MASLD at the end of the study period. Mice culled at the end of the treatment period after 5-hour food deprivation. Mean and SEM unless otherwise stated; $^{\$\$}$p<0.01; $^{\$\$\$}$p<0.001 for comparison with vehicle (Kruskal-Wallis test with Dunn's post-hoc test);*p<0.05, **p<0.01, ***p<0.001 for comparison with vehicle (one-way ANOVA and Dunnett's post-hoc test).

	Vehicle	WM	Sema	Dicretin
Body weight at end of study period (g)	44.7 ± 1.6	33.4 ± 0.6	32.9 ± 0.8	31.4 ± 1.1
Median NAS (IQR)	4 (3–4)	1 (0–1)$^{\$}$ $^{\$\$}$	1 (0.75–2)$^{\$\$}$	1 (0–1.5)$^{\$}$ $^{\$\$}$
Serum triglyceride (mg/dl)	63.8 ± 5.0	85.4 ± 8.8	49.4 ± 5.2	50.3 ± 6.6
Plasma glucagon (pmol/l)	26.4 ± 8.1	27.0 ± 13.2	14.7 ± 3.4	16.7 ± 7.1
Glucagon-alanine index	18.6 ± 2.2	13.8 ± 3.4	15.3 ± 8.4	10.4 ± 3.9
Plasma GLP1 (pmol/l)	14.7 ± 0.04	16.5 ± 1.9	124.0 ± 4.0***	16.7 ± 1.2

3.3. Dicretin drives weight loss-independent changes in the hepatic transcriptome and metabolome

To investigate how hepatic metabolic pathways were affected by the different treatments, we performed RNA-sequencing, NMR-based metabolomics and LC-MS-based lipidomics of liver tissue at the end of the study period. Unsupervised principal component analysis demonstrated clear separation between gene expression profiles of mice treated with Dicretin and the other groups (Fig. 4A). When compared to vehicle, we found many differentially expressed genes (p<0.05 in both groups). Semaglutide affected the expression of 466 genes, 190 of which (41 %) overlapped with those treated by WM (Fig. 4B). In contrast, Dicretin affected a larger number of genes (4148), of which just 519 (12.5 %) overlapped with WM.

For small molecules, while Vehicle and WM groups were clustered, mice treated with Dicretin were separated from the other groups along the 2nd principal component (Fig. 4C). Supervised analysis of OPLS-DA showed a significant separation between Dicretin and vehicle ($p=1.57\times10^{-5}$, Q^2Y (predictivity of the model) =0.86, R^2X=63.4 %; Fig. 4D). OPLS-DA analysis was not significant for vehicle versus WM nor vehicle versus Sema, indicating no significant differences between the pairs (Supplementary Figure 1A and B). Relative hepatic levels of glucose, glutamate, lactate and tyrosine were found to be higher in the vehicle group compared to the Dicretin group, whereas levels of acetate, phosphocholine and trimethylamine were significantly higher in Dicretin-treated mice (Fig. 4E).

Furthermore, a clear separation was observed in lipid profiles between Dicretin and vehicle groups in both positive and negative ionization modes (Fig. 4F and G); significant changes contributing to the group separation comprised 80 metabolites (Supplementary Table 1). Only 13 lipid changes were observed with vehicle versus WM (Supplementary Figure 1C and D; Supplementary Table 2), and for Sema versus vehicle three changes were observed in negative ionization mode only (Supplementary Figure 1E; Supplementary Table 3). Although some low abundance triglycerides (TG) were higher in Dicretin-treated mice, the TGs with the highest abundances (which accounted for the majority of TGs) were reduced in Dicretin-treated mice (Supplementary Table 4). This is consistent with an overall reduction in hepatic triglyceride in Dicretin-treated mice (Fig. 2A). The high abundance TGs which were reduced in the Dicretin-treated group incorporated fatty acyl chains in the range of 50–52 C and low unsaturation levels (2–4 double bonds).

Five genes were significantly (q<0.01) downregulated in all three treatment groups when compared to vehicle (Supplementary Table 5). All of these are known to be implicated in development or progression of MASLD. Cell death inducing DFFA like effector A and C genes (CIDEA and CIDEC) control hepatic lipid droplet growth and are upregulated in mouse models of diet-induced hepatic steatosis[30]. Along with peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PPARGC1α), CIDE genes are also known to regulate cell death. Eight sphingomyelins, members of the sphingolipid pathway which is considered a key regulatory pathway of apoptosis[31], manifested

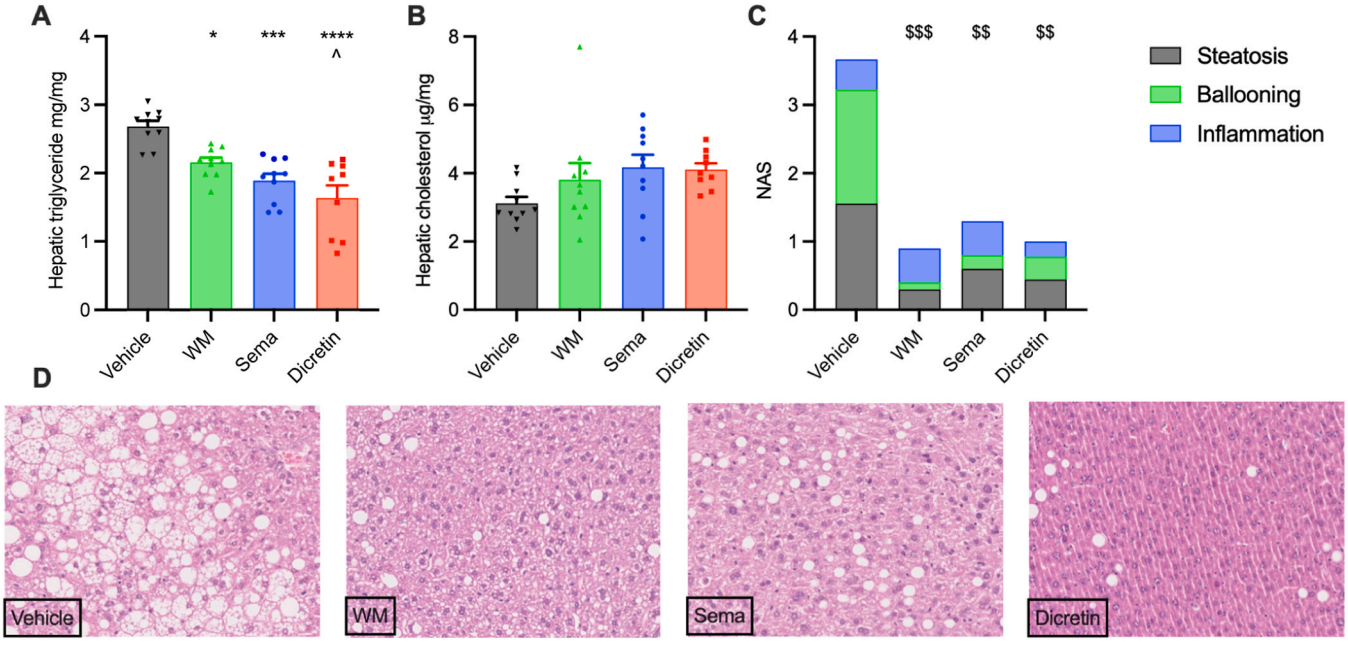


Fig. 2. Dicretin leads to partial weight loss-independent improvements in hepatic steatosis. Following 14 weeks on a high fat diet, obese mice were treated with either vehicle (Vehicle; black), semaglutide (Sema; blue); Dicretin (red) or calorie restriction to match the weight of the other treatment groups (WM; green). A: hepatic triglyceride; B: hepatic cholesterol. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to Vehicle; ^p <0.05 when compared to WM (one-way ANOVA followed by Sidak's multiple comparison test). C: Non-alcoholic fatty liver disease Activity Score (NAS) with three components illustrated. Total median NAS compared using Kruskal-Wallis test with Dunn's post-hoc test; $^{\$\$}$p<0.01; $^{\$\$\$}$p<0.001 for comparison with vehicle; n=9 in each group, mean and SEM plotted. D: illustrative examples of H&E stains of liver for a mouse in each group as labelled, 10X magnification.

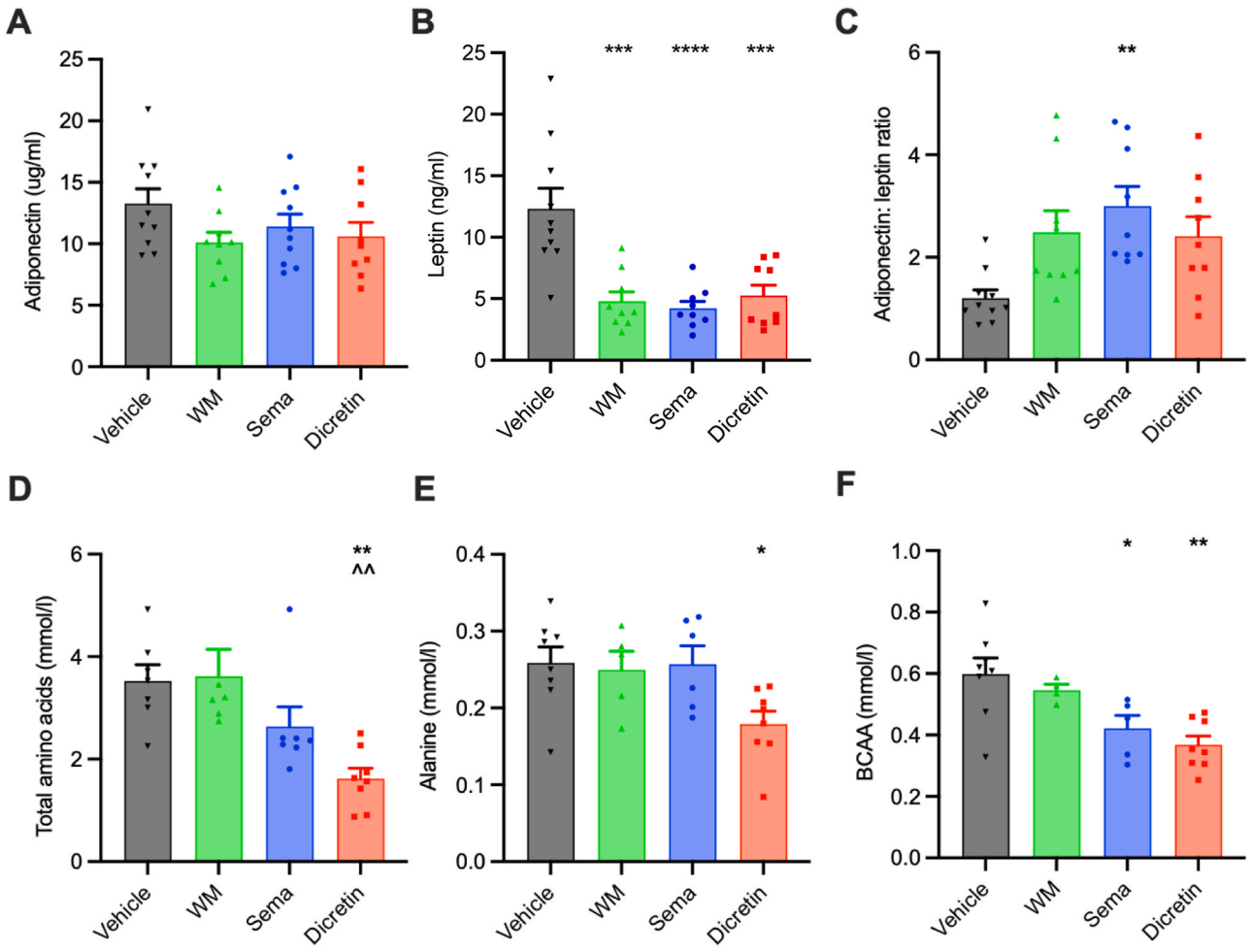


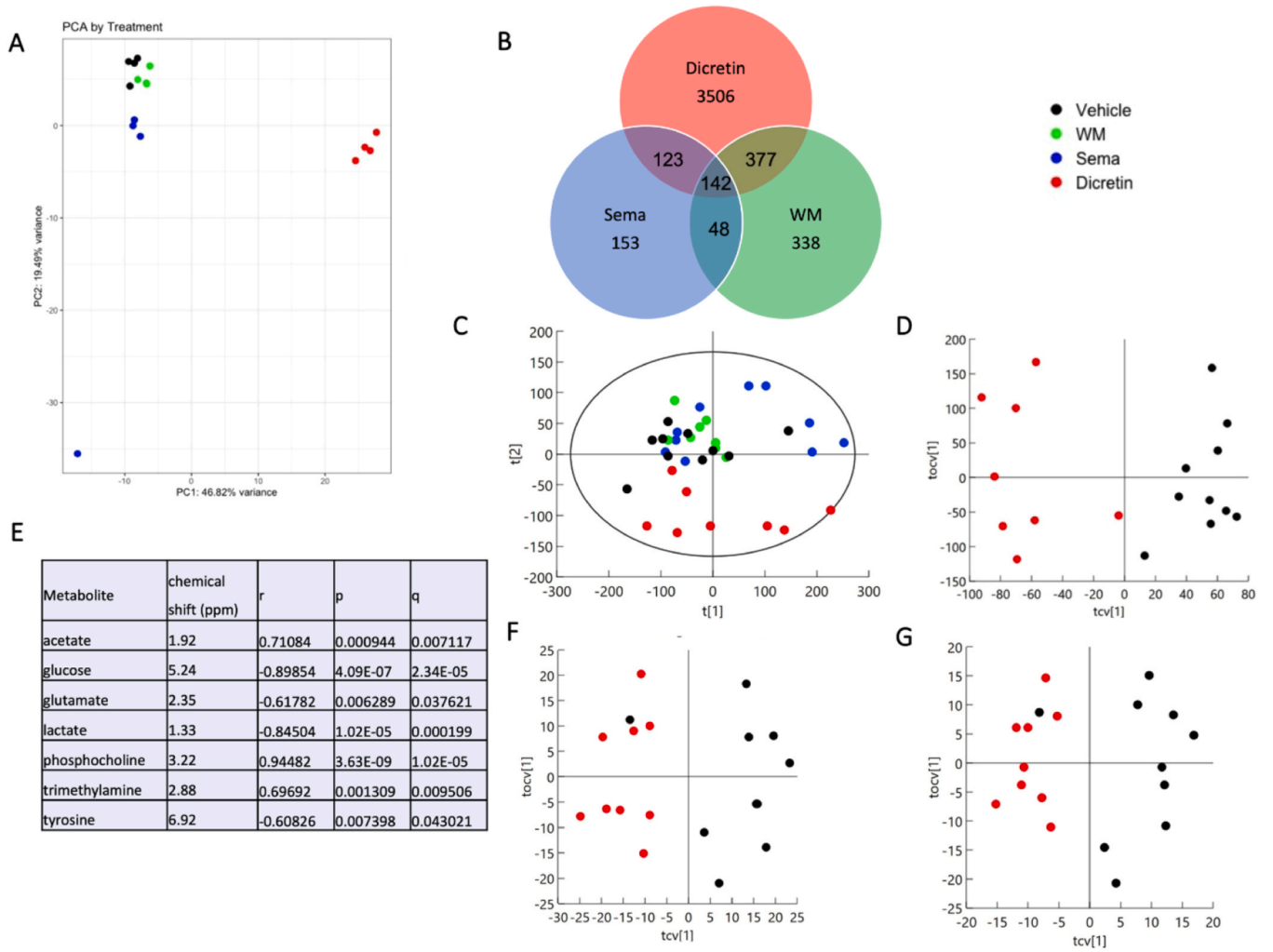
Fig. 3. Dicretin treatment is associated with a reduction in plasma amino acid levels A,B: fasted (5 hours) serum adiponectin (A) and leptin (B) levels of mice at the end of the study period; C adiponectin: leptin ratio; D, E, F: fasted (5 hours) total plasma amino acids, alanine and branched-chain amino acids. n=9 in each group, mean and SEM plotted. *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to Vehicle, ^^p<0.01 when compared to WM (one-way ANOVA and Sidak's post-hoc test).

higher abundances in the Dicretin group up to a log ratio of 1.9 (Supplementary Table 1). This could indicate a shift of the equilibrium between ceramides and sphingomyelins, in favor of the less biologically active sphingomyelins. Reduction of apoptosis has been identified as a mechanism to halt the progression of MASLD[32].

Expression of four genes was significantly downregulated in both Dicretin and Sema groups, but not in WM-treated mice (Supplementary Table 6). Aldehyde dehydrogenase (ALDH) 1 family member A1 and ALDH 3 family member A2 (*Aldh1a1* and *Aldh3a2*) were downregulated in both groups, albeit to a greater extent in Dicretin-treated compared to Sema-treated mice (log ratio −1.8 versus −0.9; −1.5 versus −0.8 respectively); ALDH 1 family member A7 (*Aldh1a7*) was also downregulated in both groups, although did not reach the prespecified cut off for statistical significance in Sema-treated mice (log ratio −1.4, q = 0.0001 for Dicretin; log ratio −1.2, q = 0.01 for Sema; and ns for WM). ALDH enzymes metabolize acetaldehyde to retinoic acid and ALDH2 knock out mice are resistant to hepatic steatosis[33]. Flavin containing dimethylaniline monoxygenase 1 (*Fmo1*) was also downregulated in Dicretin-treated mice and to a lesser extent Sema-treated mice (log ratio −1.1 versus −0.8). FMO1 converts trimethylamine (TMA) to trimethylamine-*N*-oxide (TMAO)[34]; we also observed higher hepatic levels of TMA in Dicretin-treated mice than vehicle mice (Fig. 4E). Higher levels of FMO1 and TMAO are implicated in the development of MASLD[35]. The leptin receptor gene (*Lepr*) was greatly upregulated in Dicretin-treated mice and to a lesser extent in Sema-treated mice (log ratio 6.9 versus 2.4).

Twenty-eight genes had altered transcription in Dicretin-treated mice and were either unchanged or altered in the reverse direction in the other treatment groups (Table 3). Of these, ten genes are expected targets of glucagon: for example, involved in the urea cycle (carbamoylphosphate synthetase, *Cps1,* and arginase, *Arg1*), the tricarboxylic acid cycle (fumarate hydratase, *Fh*), or gluconeogenesis (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Ppargc1a*, phosphoenolpyruvate carboxykinase 1, *Pck1*, and glucose-6-phosphatase, *G6pc1*). These changes are consistent with glucagon's role in hepatic amino acid catabolism and glucose mobilization [36].

Various genes were downregulated only by Dicretin; including three carboxylesterases, enzymes which govern hepatic lipid metabolism and are implicated in MASLD [37], and acyl-CoA synthetase short-chain family member 2 – downregulation of which prevents development of MASLD in mice fed a high fat diet [38]. Genes that were upregulated only in Dicretin-treated mice included insulin-like growth factor binding protein 1 (*Igfbp1*), upregulation of which has been shown to ameliorate MASLD [39]. The suppressor of cytokine signaling 3 (*Socs3*) gene was also upregulated, along with an observed reduction in phosphatidylcholine lipids incorporating longer length (20–22 C) and higher unsaturation level (4–6 double bonds) fatty acyl chains. The latter described chains (which included several lipids with the arachidonate 20:4 fatty acyl chain) are pivotally involved in inflammatory processes [31].This could indicate a global reduction in inflammatory processes in



E

Metabolite	chemical shift (ppm)	r	p	q
acetate	1.92	0.71084	0.000944	0.007117
glucose	5.24	-0.89854	4.09E-07	2.34E-05
glutamate	2.35	-0.61782	0.006289	0.037621
lactate	1.33	-0.84504	1.02E-05	0.000199
phosphocholine	3.22	0.94482	3.63E-09	1.02E-05
trimethylamine	2.88	0.69692	0.001309	0.009506
tyrosine	6.92	-0.60826	0.007398	0.043021

Fig. 4. Dicretin drives improvement in hepatic steatosis by downregulating hepatic metabolic pathways which are distinct from those altered by weight loss or semaglutide alone. A: 2-D PCA score plot of hepatic transcriptomic data from mice treated with vehicle (black), semaglutide (Sema; blue); Dicretin (red) or calorie restriction to match the weight of the other treatment groups (WM; green). B: Differential gene expression in livers of mice from the different treatment groups when compared with vehicle. Adjusted p value <0.05; Fold change <-1 or >1. C: PCA scores plot of ^{1}H NMR spectra of liver aqueous extracts from mice in the different treatment groups. Principal components 1 and 2 explain 45.8 % and 16.9 % variations, respectively: $R^2X = 0.169$. D: OPLS-DA cross-validated scores plot of ^{1}H NMR spectra of liver aqueous extracts from Dicretin- and Vehicle-treated mice. CVANOVA $p=1.57\times10^{-5}$, Q^2Y (predictivity of the model) =0.86, R^2X=63.4 %, R^2Y=92.9 %. E: Significantly different metabolites between the two groups from the CVANOVA model. r represents correlation coefficient values and a positive correlation indicates relatively higher levels of the metabolites in Dicretin compared to vehicle and *vice versa*. p represents p-value and q is Benjamini-Hochberg procedure corrected p-value. F,G: OPLS-DA cross-validated scores plot of LC-MS-based lipidomic data of liver extracts from Dicretin- and Vehicle-treated mice for positive (F; CVANOVA $p=3.68\times10^{-3}$, $Q^2Y = 0.76$, R^2X=51.3 %) and negative (G; CVANOVA $p=9.01\times10^{-4}$, $Q^2Y = 0.72$, R^2X=58.2 %) ionization modes.

Dicretin-treated mice.

4. Discussion

The weight loss effects of GLP1/GCGR co-agonists were first demonstrated in DIO mice using unimolecular preparations[15,23,40]. These initial studies demonstrated the efficacy of combining GCGR agonism with GLP1R agonism and leveraging glucagon-stimulated weight loss and increased energy expenditure [41,42]. Consistent with the known beneficial effects of glucagon receptor signaling on hepatic lipid pathways [18,43], pre-clinical reports of GLP1/GCGR co-agonists have subsequently also reported superior improvements in metabolic liver disease when compared to GLP1R agonism alone [12,16,22,44]. Clinical trials of the GLP1/GCGR co-agonists Efinopegdutide (NCT04944992) and Survodutide in people with MASLD have reported promising early results [45,46], as have those of the GLP1/GCGR/GIPR tri-agonist Retatrutide [47]. Despite this, weight-matched control studies for GLP1R and GCGR/GLP1R co-agonists, along with characterization of differentially activated metabolic pathways in the liver, are lacking.

Although higher relative activity at the GCGR is associated with superior weight loss[40], most GLP1/GCGR co-agonists studied have been much more potent at the GLP1R than the GCGR. This may be due to concerns that excessive GCGR agonism will cause glucose intolerance [40]. With a focus on the potential for a GCGR-biased co-agonist to treat hepatic steatosis, we investigated our Dicretin peptide which has greater activity at the GCGR compared to the GLP1R (approximately 10:1). Using a DIO-model of hepatic steatosis, the differential effects of Dicretin, Semaglutide and food restriction on hepatic steatosis were investigated using a weight-matched study paradigm.

Despite the relatively high GCGR potency of Dicretin, we found that the co-agonist confers improved glucose tolerance and insulin resistance in chronically treated DIO mice. In line with these findings, NMR demonstrated lower hepatic glucose and lactate in Dicretin-treated mice. This could suggest compensatory mechanisms downstream of glucagon-driven upregulation of G6Pase, PEPCK and other genes

Table 3
Genes significantly and uniquely altered in Dicretin mice. RNA-sequencing data comparing treatment groups with vehicle treatment (Qiagen IPA, 2000–2022). Sema = semaglutide; WM = weight-matched; q-value = false discovery rate. Filtered for log ratio <-1 or >1 in Dicretin group, with q<0.01. Significant changes (q<0.01) color-coded with heatmap for ease of viewing.

		Dicretin		Sema		WM	
Symbol	Entrez Gene Name	Log Ratio	q-value	Log Ratio	q-value	Log Ratio	q-value
Known Glucagon targets							
Arg1	arginase 1	1.443	2.76E-07	0.117	8.93E-01	-0.226	7.30E-01
Cps1	carbamoyl-phosphate synthase 1	1.571	4.78E-08	0.508	3.77E-01	0.85	2.38E-02
Pck1	phosphoenolpyruvate carboxykinase 1	1.596	1.92E-12	-0.151	8.20E-01	0.013	9.86E-01
G6pc1	glucose-6-phosphatase catalytic subunit 1	1.721	1.10E-07	-1.733	1.41E-06	1.067	8.69E-03
Fh	fumarate hydratase	2.369	1.24E-31	-0.016	9.85E-01	0.186	6.99E-01
Fgf21	fibroblast growth factor 21	2.669	6.29E-05	-0.907	5.47E-01	-0.451	7.75E-01
Nr4a3	nuclear receptor subfamily 4 group A member 3	5.444	1.54E-04	0.154	9.76E-01	-0.987	7.91E-01
Asns	asparagine synthetase (glutamine-hydrolyzing)	6.361	3.05E-31	1.576	1.08E-01	0.542	7.24E-01
Adcy1	adenylate cyclase 1	7.017	1.50E-07	4.419	1.45E-02	3.302	8.71E-02
Ppargc1a	PPARG coactivator 1 alpha	2.854	1.93E-37	-0.019	9.85E-01	0.364	3.91E-01
Other							
Mas1	MAS1 proto-oncogene, G-protein coupled receptor	-6.06	1.53E-04	-1.661	5.00E-01	-2.777	1.08E-01
Ces1g	carboxylesterase 1G	-4.413	2.01E-26	-1.105	1.03E-01	0.063	9.66E-01
Ces2c	carboxylesterase 2C	-4.11	3.42E-11	-1.473	1.32E-01	-1.187	2.08E-01
Slco1a1	solute carrier organic anion transporter family, member 1a1	-3.551	2.74E-15	3.351	1.20E-12	2.301	4.04E-06
Abcc3	ATP binding cassette subfamily C member 3	-2.519	2.33E-23	-0.584	2.03E-01	-0.7	5.32E-02
Gck	glucokinase	-2.193	3.40E-06	-0.199	8.89E-01	-0.036	9.82E-01
Adra1b	adrenoceptor alpha 1B	-1.796	2.30E-09	-0.395	5.71E-01	0.371	5.21E-01
Adrb3	adrenoceptor beta 3	-1.756	1.80E-04	0.828	2.78E-01	-0.33	7.38E-01
Ces2g	carboxylesterase 2G	-1.673	1.58E-04	-0.02	9.90E-01	0.075	9.57E-01
Acss2	acyl-CoA synthetase short chain family member 2	-1.645	2.66E-05	-0.748	2.96E-01	0.191	8.50E-01
Socs3	suppressor of cytokine signaling 3	1.99	7.00E-07	0.69	3.94E-01	0.051	9.72E-01
Acsl4	acyl-CoA synthetase long chain family member 4	2.949	2.57E-40	0.099	8.93E-01	0.386	3.30E-01
Sgk1	Serum glucocorticoid regulated kinase 1	3.325	1.71E-25	0.311	7.32E-01	0.695	1.79E-01
Sstr2	somatostatin receptor 2	3.651	8.75E-17	1.247	9.83E-02	1.091	1.26E-01
Tff3	trefoil factor 3	5.16	4.46E-05	0.148	9.72E-01	-0.05	9.91E-01
Igfbp1	insulin like growth factor binding protein 1	5.331	3.88E-28	0.096	9.57E-01	0.986	2.19E-01
Tg	thyroglobulin	9.515	6.20E-16	3.086	1.95E-01	0.671	8.87E-01
Ren	renin	10.262	6.76E-19	0.782	8.76E-01	0	1.00E+00

traditionally associated with increased gluconeogenesis, towards glycogenesis and away from glycolysis. Importantly, there was no deterioration in glycaemia which has been a concern with this drug class [40].

Although all treatment groups experienced improvement in hepatic triglyceride levels, consistent with a reduction in body mass and an improvement in insulin sensitivity[48], we observed additional weight loss-independent effects of Dicretin, but not Semaglutide, on steatosis resolution. Previous studies that have compared a GLP1/GCGR co-agonist to a GLP1R agonist demonstrate that the former is associated with greater reduction in food intake and body weight loss, corresponding to a greater reduction in hepatic steatosis [15,44]. In the present study, by using weight-matched controls, we confirm that mice treated with a GLP1/GCGR co-agonist exhibit enhanced steatosis resolution independent of body weight loss. Furthermore, compared to mice who were calorie-restricted or treated with Sema, those treated with Dicretin had a higher total food intake during the study, presumably due to glucagon-mediated energy expenditure. Although the trajectory of weight loss differed between the treatment groups, with mice treated with Dicretin losing weight more rapidly, the weights of mice in all groups stabilized for 4–5 days prior to tests and final cull. This study design was chosen to mitigate any metabolic differences due to being within a period of weight loss. Our finding of greater steatosis resolution despite similar weight loss corroborates those of Boland et al. using the GLP1/GCGR co-agonist Cotadutide in a mouse model with more advanced MASH-fibrosis and in the context of lesser weight loss (7–8 %) [17].

We did not observe any additional benefit on hepatic steatosis of GLP1 treatment compared to calorie-restricted weight-matched controls. Multiple studies have demonstrated that chronic GLP1R agonism leads to weight loss and improves obesity-associated liver disease in rodents[49–54]. In a randomized placebo-controlled trial, 12 weeks of daily liraglutide injections led to histological improvement in NASH in patients with obesity, alongside average BMI loss of 1.8 kg/m^2 [9]. Underlying possible mechanisms include increased hepatic and adipose tissue insulin sensitivity[55,56] and potentially adaptation of neural circuits[57]. A small number of studies have included a pair-fed group and these demonstrate that GLP1 agonism causes more weight loss than can be attributed to reduced food intake alone[56,57]. This corresponds to our finding of increased food intake in the Sema group (albeit not to the same degree as the Dicretin group) when compared to WM mice for the same degree of weight loss. This phenomenon is associated with an increase in resting energy expenditure (REE), which may relate to the metabolically higher contribution of lean mass than fat[11],however, in

man GLP1 agonism alone does not appear to increase REE [58].

Hepatic transcriptomic analysis demonstrated relative clustering of Vehicle-treated with WM and Sema mice and there were few differences between Vehicle-treated and WM or Sema mice hepatic small molecule and lipidomic composition. In contrast, we observed extensive transcriptomic and metabolomic changes in response to the Dicretin co-agonist (Fig. 4). Some of these were expected due to the known actions of glucagon, for example upregulation of genes involved in amino acid catabolism and hepatic glucose mobilization in Dicretin mice but not the other treatment groups. These include arginase 1 (*Arg*), carbamoyl phosphate synthase 1 (*Cps1)*, phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6p*). Other highly upregulated genes also included those implicated in the hepatic amino acid stress response, including fibroblast growth factor (*Fgf21*) and asparagine synthetase (*Asns*) [59,60]. Interestingly, adenylate cyclase 1 (*Adcy1*) was also highly upregulated in Dicretin-treated animals, and this could be a feedforward response to chronic GCGR pathway activation.

Our finding of increased hepatic expression of leptin receptor (*Lepr*) and *Fgf21* in mice treated with Dicretin is consistent with findings of Habegger et al.[61,62]. Leptin is an anorexigenic adipokine and was reduced to a similar extent in all treatment groups (Fig. 3B). Leptin directly affects the liver to decrease fat accumulation, by activating fatty acid oxidation and decreasing lipogenesis[63]. Furthermore, hepatic glucagon signaling has been shown to directly regulate the expression of *Lepr*[62]. These findings might indicate that increased leptin sensitivity contributes to improved steatosis in Dicretin-treated mice; however, it is unlikely to be the sole mediator[62]. FGF21 also decreases expression of hepatic genes related to fat synthesis; thus a Dicretin-induced increase in FGF21 expression could also contribute to improved steatosis [64].

Many of the hepatic gene changes observed in Dicretin-treated animals have also been observed in glucagon analogue-treated rodents[65], including renin, thyroglobulin and somatostatin receptor 2. The physiological implications of all of these changes are yet to be fully understood, although renin-angiotensin axis activity has been previously described in the liver, may be associated with MASLD[66], and, as in the kidneys, may be downstream to adenylate cyclase activation.

In keeping with the upregulation of amino acid catabolic pathways, total plasma amino acids were reduced in Dicretin-treated animals (Fig. 3D). Alanine, a major glucogenic amino acid, was also measured and found to be suppressed. These findings are consistent with preclinical and clinical reports of GCGR-targeted multi-agonists demonstrating a broad reduction in plasma amino acids, confirming GCGR receptor target engagement[67,68]. The reduction in plasma alanine along with the numerical reduction in fasting glucagon in the Dicretin group could also be related to altered activity of the liver-alpha cell axis [69]. In this context, the amino acid stimulus to glucagon secretion in Dicretin animals is reduced, which may reflect sustained pharmacological activity at the hepatic GCGR, in addition to improvements in hepatic glucagon sensitivity, secondary to an amelioration of hepatic steatosis[70]. Branched-chain amino acids (BCAAs) were reduced in response to both Dicretin and Semaglutide treatment. These amino acids are known to be less sensitive to the catabolic actions of hepatic glucagon receptor activation[65]. However, the reduction in plasma BCAAs was associated with reductions of the HOMA-IR marker of insulin resistance in both Dicretin- and Semaglutide- treated animals. These findings are in keeping with the known positive association between whole-body insulin resistance and circulating BCAAs[71,72].

With respect to lipidomic changes, co-agonists act on the hepatic GCGR to lower liver lipids, by modifying uptake, excretion and de novo synthesis, leading to a reduction in hepatic triglycerides, diglycerides and cholesterol esters[17,73]. Alongside an upregulation of ACSL4 and decrease of ACSS2, we observed changes in acetylcarnitine abundance in Dicretin-treated mice. This could reflect changes in beta-oxidation, a process known to affect propensity to MASLD in mice[74]. Changes in genes implicated in lipid metabolism were also noted including *Igfbp1* and *Socs3*, alongside a reduction in longer length phosphatidylcholine lipids, which could indicate a reduction in inflammatory processes in Dicretin-treated mice. There was also a higher abundance of sphingomyelins which could be related to a reduction in apoptosis in Dicretin-treated mice. Together, these results suggest that distinct pathways are affected by Dicretin, leading to a favorable hepatic lipid profile.

In conclusion, in this study we have demonstrated that chronic treatment with Dicretin, a GLP1/GCGR co-agonist with greater potency at the GCGR than at GLP1R, leads to weight-loss independent improvements in hepatic steatosis in DIO mice, without compromising on glucose tolerance outcomes. We also show that Dicretin downregulates specific hepatic metabolic pathways which are distinct from those altered by weight loss alone. GCGR-biased GLP1/GCGR co-agonists may be more suitable as a treatment for patients with MASLD and associated conditions than GLP1R monoagonists, benefiting from glucagon's direct actions to reduce hepatic fat accumulation. Further human studies are required to investigate the potential for a tailored pharmacotherapeutic strategy for those with MASLD using multi-agonists.

CRediT authorship contribution statement

Stephen R. Bloom: Writing – review & editing, Conceptualization. **David C.D. Hope:** Writing – review & editing, Writing – original draft, Investigation. **Tricia M-M Tan:** Writing – review & editing, Supervision, Conceptualization. **Iona Davies:** Writing – review & editing, Investigation. **Marian Dore:** Writing – review & editing, Formal analysis, Data curation. **Rob Goldin:** Writing – review & editing, Methodology, Investigation. **Panagiotis A. Vorkas:** Writing – review & editing, Methodology, Formal analysis. **Bernard Khoo:** Writing – review & editing, Investigation, Formal analysis. **David Carling:** Writing – review & editing, Conceptualization. **Emma Rose McGlone:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **James Minnion:** Writing – review & editing, Investigation. **Ben Jones:** Writing – review & editing, Investigation. **Zhigang Liu:** Writing – review & editing, Investigation. **Jia V. Li:** Writing – review & editing, Methodology, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TM-MT, JM and SRB declare that they are shareholders in and consultants for Zihipp Ltd., an Imperial College spin-out company that develops gut hormone analogues for the treatment of obesity and associated metabolic disorders.

Data Availability

RNA data generated from this study is openly available in Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/, reference number GSE243681.

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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.2024.116888.

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