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ORIGINAL ARTICLE

Plasma proteome profiles treatment efficacy of incretin dual agonism in diet-induced obese female and male mice

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Abstract

Aims: Unimolecular peptides targeting the receptors for glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (GLP-1/GIP co-agonist) have been shown to outperform each single peptide in the treatment of obesity and cardiometabolic disease in preclinical and clinical trials. By combining physiological treatment endpoints with plasma proteomic profiling (PPP), we aimed to identify biomarkers to advance non-invasive metabolic monitoring of compound treatment success and exploration of ulterior treatment effects on an individual basis.

Materials and methods: We performed metabolic phenotyping along with PPP in body weight-matched male and female diet-induced obese (DIO) mice treated for

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21 days with phosphate-buffered saline, single GIP and GLP-1 mono-agonists, or a GLP-1/GIP co-agonist.

Results: GLP-1R/GIPR co-agonism improved obesity, glucose intolerance, non-alcoholic fatty liver disease (NAFLD) and dyslipidaemia with superior efficacy in both male and female mice compared with mono-agonist treatments. PPP revealed broader changes of plasma proteins after GLP-1/GIP co-agonist compared with mono-agonist treatments in both sexes, including established and potential novel biomarkers for systemic inflammation, NAFLD and atherosclerosis. Subtle sex-specific differences have been observed in metabolic phenotyping and PPP.

Conclusions: We herein show that a recently developed unimolecular GLP-1/GIP co-agonist is more efficient in improving metabolic disease than either mono-agonist in both sexes. PPP led to the identification of a sex-independent protein panel with the potential to monitor non-invasively the treatment efficacies on metabolic function of this clinically advancing GLP-1/GIP co-agonist.

KEYWORDS
bariatric surgery, combinatorial pharmacology, incretins, obesity, plasma proteomics

1 | INTRODUCTION

Bariatric surgery is the most effective treatment option to correct severe obesity and non-alcoholic fatty liver disease (NAFLD), to improve type 2 diabetes (T2D) and to reduce cardiometabolic risk.[1–3] However, these surgeries are invasive and not applicable to the broad patient population. Hence, new drugs safely mimicking surgery-induced metabolic benefits are urgently needed. Unimolecular peptides targeting the glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptor (GLP-1/GIP co-agonist) are currently in clinical development for the treatment of obesity and diabetes.[4,5] Their efficacy to improve body weight, glucose handling and lipid metabolism translates from rodent models of obesity to non-human primates and humans outperforming best-in class GLP-1 monotherapies.[4–6]

Preclinical and clinical assessment of new drug candidates requires cost- and time-intensive metabolic testing and in case of metabolic diseases such as diabetes and NAFLD risky and highly invasive procedures such as hyperinsulinaemic-euglycaemic clamps and liver biopsies. Drug development would benefit from non-invasive biomarkers monitoring therapeutic efficacy and disease progression.[7] Particularly suitable for this approach is blood because of the fact that proteins from almost every cell and tissue are in circulation. Recent advances in mass spectrometry (MS)-based plasma proteome profiling (PPP) enabled system-wide analyses of plasma proteome changes under pathophysiological conditions.[8,9] In patients with obesity undergoing Roux-en-Y gastric bypass (RYGB), PPP revealed that systemic inflammation and lipid transport are the major remodelled processes by this weight loss intervention.[10] An in-depth evaluation of plasma proteome changes after treatment with clinically advancing incretin-based peptide mimetics has not been performed yet. This approach may point to unidentified mechanisms of action and identify new cardiovascular (CV) and metabolic health biomarkers, which are urgently needed to stratify patient groups and to assess drug efficacy early on in preclinical and early clinical stages.[7]

As approximately 80% of patients undergoing bariatric surgery are women,[11] we evaluated the metabolic efficacy of a GLP-1/GIP co-agonist in female diet-induced obese (DIO) mice in comparison with male DIO mice. This is of specific interest as some pharmacological treatments entail a different spectrum of efficacy in women.[12,13]

Herein, we use a recently developed long-acting acylated GLP-1/GIP co-agonist with balanced activity at the receptors for GLP-1 and GIP.[6] An α-aminoisobutyric acid residue at position 2 of the unimolecular co-agonist prevents dipeptidyl peptidase IV (DPP4) degradation, while the C-terminal extension improves solubility and pharmacokinetics.[6] We show that treatment with this GLP-1/GIP co-agonist lowers body weight and improves glucose handling and lipid metabolism to a larger extend than mono-agonists and with comparable efficacy in both sexes. MS-based PPP revealed that GLP-1/GIP treatment was more efficacious than either mono-agonist to reduce inflammatory proteins and lipid transport proteins similar to RYGB in humans. Despite sex-specific plasma proteome differences, we identified 10 proteins that are downregulated by GLP-1/GIP treatment in both sexes and correlate with systemic metabolic benefits. Thus, these proteins may be suitable to monitor non-invasively the drug responses in preclinical and clinical studies.

2 | MATERIALS AND METHODS

2.1 | Animals and diet

Eight-week old female C57BL/6J mice (Charles River Laboratories, Sulzfeld, Germany) were fed a high-fat, high-sugar diet (HFD)

comprising 58% kcal from fat (D12331; Research Diets, New Brunswick, NJ, USA) to induce DIO. For matching of body weight, age-matched male mice were switched from regular chow to HFD at the age of 33 weeks. This diet regimen was chosen as HFD-fed male mice more rapidly gain body weight relative to female mice.[14] Mice were double-housed and maintained at 22 ± 2°C, 55 ± 10% relative humidity and a 12-h light/dark cycle with free access to food and water. At the age of 37 weeks, DIO male (n = 30) and female mice (n = 28) were randomly assigned to treatment groups matched for body weight and fat mass.

All procedures were approved by the local Animal Use and Care Committee and the local authorities of Upper Bavaria, Germany in accordance with European and German animal welfare regulations.

2.2 | Compound synthesis

The synthesis, purification and characterization of the fatty-acylated GLP-1/GIP co-agonist as well as the pharmacokinetics-matched fatty-acylated (palmitic acid C16:0) GLP-1 and GIP mono-agonists was described previously in detail and was used without any further chemical modification or change in formulation.[6]

2.3 | Rodent pharmacological and metabolism studies

At the start of the study, body weight-matched obese male and female mice were randomized by body weight and fat mass to either vehicle, GIP, GLP-1 or GLP-1/GIP agonist treatment. Mice were treated with daily subcutaneous injections (5 μL/g body weight) in the middle of the light phase for 21 consecutive days at a dose of 10 nmol/kg/day. Body weight and food intake was measured daily. Whole-body composition (fat and lean mass) was measured via nuclear magnetic resonance technology (EchoMRI, Houston, TX, USA). Fasting blood glucose and intraperitoneal glucose tolerance (ipGTT) was determined after a 6-h fast at study day 14 and 20 h after the last injection. For the ipGTT, fasted animals were injected intraperitoneally with 2 g glucose per kg body weight. Blood glucose was subsequently measured at time points 0, 15, 30, 60 and 120 min using a handheld glucometer (FreeStyle).

2.4 | Biochemical analysis

Tail blood before ipGTT was collected after a 6-h fast using EDTA-coated microvette tubes (Sarstedt) and immediately chilled on ice. Mice were killed using CO_2 after a 4-h fast and at least 16 h after the last vehicle or compound injection. Sac blood was mixed with EDTA and immediately kept on ice. Plasma was separated by centrifugation at 5000 *g* at 4°C for 10 min. Levels of insulin (Crystal Chem, Elk Grove Village, IL, USA) were measured following the manufacturer's instructions. The homeostatic model assessment of insulin resistance (HOMA-IR) was used to determine insulin sensitivity. Total plasma cholesterol (Thermo Fisher Scientific, Waltham, MA, USA) was measured following the manufacturer's instructions. For lipoprotein separation, samples were pooled and analysed in a fast-performance liquid chromatography gel filtration as described previously.[15] Liver samples were fixed in 4% (w/v) neutrally buffered formalin, embedded in paraffin and cut into 3 μm slices for haematoxylin and eosin staining. Stained tissue sections were scanned with an AxioScan. Z1 digital slide scanner (Zeiss, Jena, Germany). Images were evaluated using the commercially available image analysis software Definiens Developer XD 2 (Definiens AG, Munich, Germany) as previously described.[16] A specific rule set was defined to detect and quantify the lipid vacuoles of the hepatocytes based on morphology, size, pattern, shape, neighbourhood and special colour features. The calculated parameter was the ratio of total area of lipid vacuoles per whole tissue section.

2.5 | Gene expression analysis

Gene expression profiling in the liver was performed in mice treated with compounds for 21 consecutive days. For tissue collection, mice were fasted for 4 h and treated with compounds at least 16 h before tissue collection. Total RNA was isolated using RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Liver gene expression was profiled with quantitative real-time reverse transcription-polymerase chain reaction using SYBR Green with validated primers. The relative expression of the selected hepatic genes was normalized to the reference gene peptidyl-prolyl *cis-trans* isomerase b (Ppib). The following primers were used: Abcg5 forward 5′-GTACATCGAGAGTGGCCAGA-3′, Abcg5 reverse 5′-CTGTGTATC GCAACGTCTCG-3′; Apoe forward 5′-GATCAGCTCGAGTGGCAAA G-3′, Apoe reverse 5′-TAGTGTCCTCCATCAGTGCC-3′; Cyp3a11 forward 5′-CTCTCACTGGAAACCTGGGT-3′, Cyp3a11 reverse 5′-TC TGTGACAGCAAGGAGAGG-3′; Cyp8b1 forward 5′-CAGCGGACAA GAGTACCAGA-3′, Cyp8b1 reverse 5′-TGGATCTTCTTGCCCGACTT -3′; Ldlr forward 5′-TCAGACGAACAAGGCTGTCC-3′, Ldlr reverse 5′-CCATCTAGGCAATCTCGGTCTC-3′; Lipc forward 5′-ATGTGGGG TTAGTGGACTGG-3′, Lipc reverse 5′-TTGTTCTTCCCGTCCATGG A-3′; Lrp1 forward 5′-AACCTTATGAATCCACGCGC-3′, Lrp1 reverse 5′-TTCTTGGGGCCATCATCAGT-3′; Pcsk9 forward 5′-CACCCTGG ATGCTGGTATCT-3′, Pcsk9 reverse 5′-GACCTCTTCCCTGGCTTCTT -3′; Ppib forward 5′-GCATCTATGGTGAGCGCTTC-3′, Ppib reverse 5′-CTCCACCTTCCGTACCACAT-3′; Sqle forward 5′-TGTTGCGGAT GGACTCTTCT-3′, Sqle reverse 5′-GAGAACTGGACTGGGGTTGA-3′.

2.6 | Statistical analyses

Statistical analyses were performed using GraphPad Prism8. The Kolmogorov-Smirnov test was used to assess for normality of

residuals. We used the Brown-Forsythe test to assess for the equality of group variances. Significance among different treatment groups and time points was determined by two- or one-way analysis of variances (ANOVA) followed by Tukey's post hoc analysis. Two-way ANOVA was used when the influence of time and treatment was examined. We used Kruskal-Wallis test followed by Dunn's multiple comparison test to determine statistical significance when normality of residuals was not given. In the case of only two groups, the unpaired Student two-tailed *t*-test was used to detect significant differences. A Grubbs test ($\alpha < .05$) was used to detect significant outliers, which were then excluded from the subsequent statistical analysis and figure drawing. $P < .05$ was considered statistically significant. All results are mean ± SEM unless otherwise indicated.

2.7 | Plasma proteome profiling

At the end of study, plasma was obtained from mice that were fasted for 4 h before sample collection and at least 16 h after the last vehicle or compound injection. Plasma sample preparation[8,17] and measurement with high-pressure liquid chromatography and MS as well as the analysis of MS raw files was reported previously.[17]

2.8 | Bioinformatics analysis

Bioinformatics analysis was performed in the Perseus platform[18] and R scripts. We filtered for proteins with 70% data completeness in at least one experimental group and replaced the missing values by drawing random samples.[17] From originally 591 identified peptides, we obtained a protein matrix of 563 peptides that were uniquely classified to a specific protein in male and female DIO mice, which was used for subsequent data analysis. Signal intensities were log2 transformed. A protein matrix with log2 imputed protein intensities for all samples can be found in Table S1. The study was assessed according to Geyer et al.[19] regarding individual sample quality (Figure S1) and significant bias in group comparisons (Figure S2). Because of overall low sample contamination, no sample was excluded from downstream analysis. We used the limma package[20] with its voom method[21] for linear modelling and to find differentially regulated proteins between two treatment groups. We used a *P*-value cut-off $P < .01$ for downstream analysis unless otherwise noted. Differentially regulated proteins were assessed with a global correlation matrix to test for erythrocyte lysis, platelet contamination or coagulation as their origin[19] (Figure S3). Importantly, only very few differentially regulated proteins cluster in one of these panels. This indicates that most highlighted proteins derive from a treatment effect. We used Enrichr[22,23] and manual search for pathway and functional allocation of the identified differentially regulated proteins. We filtered for proteins of the inflammation system by including the following keywords: complement, coagulation, chemotaxis, cytokine, inflammation, inflammatory, immune, immunity, defence response and membrane attack complex.

2.9 | Data availability

The MS proteomics data have been deposited with the ProteomeXchange consortium via PRIDE with the identifier PXD012056.

3 | RESULTS

3.1 | Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide decreases body weight and hyperglycaemia with equal efficiency in both sexes

Because of the slower progression of DIO in female mice, we delayed the onset of HFD feeding in male mice to achieve study cohorts matched for body weight and age for the adequate assessment of co-agonist-induced weight loss efficacy. Age-matched male and female mice were fed a HFD for 4 and 29 weeks, respectively. Despite similar body weight (Figure S4A), body fat mass was higher in female relative to male DIO mice (Figure S4B), whereas lean tissue mass was higher in male DIO mice (Figure S4C).

Similar to previous reports,[6] treatment with GLP-1/GIP decreased the body weight of male DIO mice (Figure 1A) primarily by reducing fat mass (Figure 1B) and to a greater extent relative to the activity matched GLP-1 and GIP mono-agonists. Inhibition of food intake was similar between male DIO mice treated with GLP-1 and GLP-1/GIP, suggesting that GLP-1/GIP also promotes weight loss via food-intake independent effects (Figure 1C). In females, the GLP-1/GIP-induced weight loss was similarly greater relative to treatment with either mono-agonist (Figure 1D). Interestingly, in female DIO mice the superior effect of GLP-1/GIP to induce greater body weight loss relative to GLP-1 alone emerged earlier relative to male DIO mice (day 15 vs. 19; Figure 1A and 1D). The weight loss in female DIO mice was mainly because of a loss of fat with only minor changes in lean tissue mass (Figure 1E). While food intake inhibition was similar between male DIO mice treated with GLP-1/GIP or GLP-1 (Figure 1C), we see in females a greater inhibition of food intake by GLP-1/GIP relative to treatment with GLP-1 (Figure 1F), although compound-induced weight loss was comparable in both sexes (Figure S5A,B). Treatment with the acylated GIP analogue alone at the given dose of 10 nmol/kg did not reduce body weight or food intake in both sexes (Figure 1A and 1C).

GLP-1/GIP additionally improved fasting glucose levels and glucose tolerance in male and female DIO mice (Figure S6A,B,E,F). Particularly in males, GLP-1 and GLP-1/GIP reduced fasting insulin levels and fasting HOMA-IR, indicating improved insulin sensitivity (Figure S6C,D). In females, only GLP-1/GIP but not the respective monotherapies improved HOMA-IR, with subtle effects on already low fasting insulin levels (Figure S6G,H). Chronic treatment with GIP improved glucose tolerance in both sexes (Figure S6A,B,E,F), without changes in fasting insulin levels (Figure S6C,G) or improving insulin sensitivity determined by HOMA-IR (Figure S6D,H). Collectively, GLP-1/GIP improves body weight and glucose handling with equal efficacy in both sexes and with superior potency relative to the respective single peptide mono-agonists.

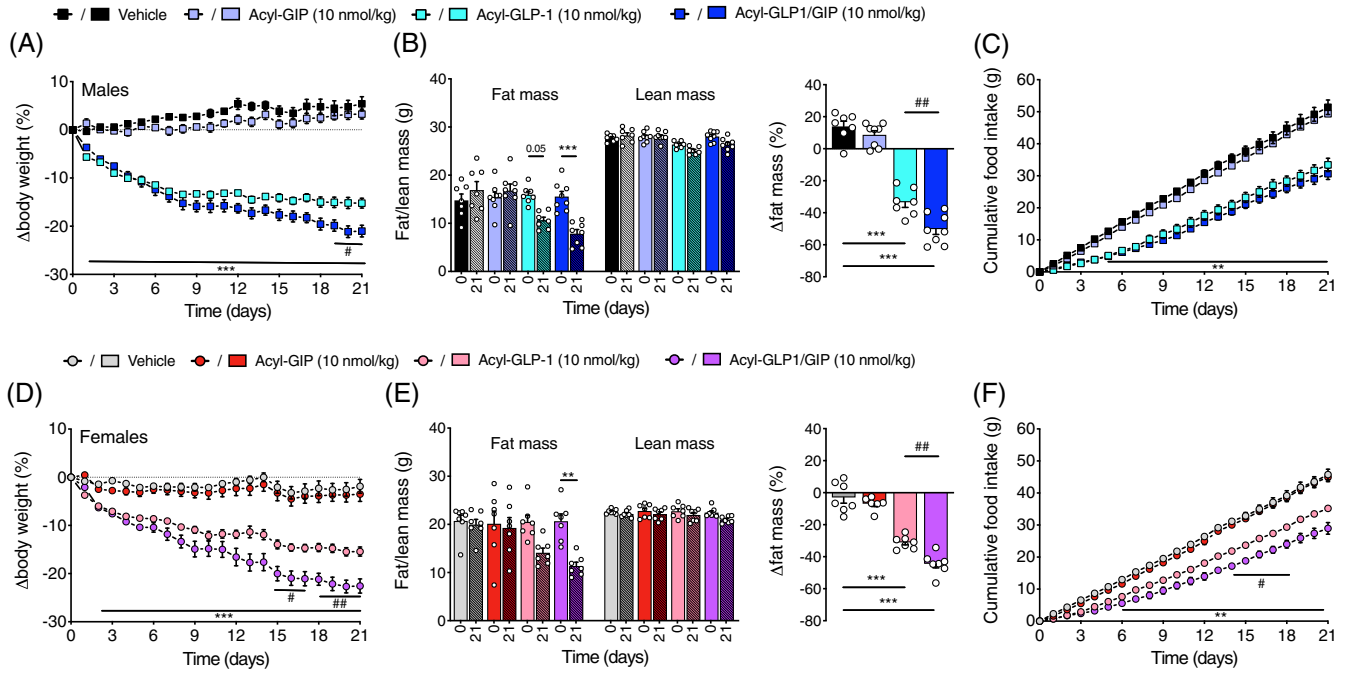

FIGURE 1 Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide (GLP-1/GIP) treatment reduces obesity to a larger extent relative to GLP-1 and GIP mono-agonists but with equal efficiency in diet-induced obese male and female mice. Male mice: A, percentage body weight; B, change of body composition; and C, cumulative food intake. Female mice: D, percentage body weight; E, change of body composition; and F, cumulative food intake. Mice were treated daily with either vehicle [n (female) = 7, n (male) = 7], acyl-GIP [10 nmol/kg; n (female) = 7, n (male) = 8], acyl-GLP-1 [10 nmol/kg; n (female) = 7), n (male) = 7] or acyl-GLP-1/GIP [10 nmol/kg; n (female) = 7, n (male) = 8] via subcutaneous injections for 21 consecutive days. Changes in body composition reflect changes from study day 0 to 21. Data represent means ± SEM. $^{**}P < .01$, $^{***}P < .001$, determined by two-way ANOVA comparing vehicle with the acyl-GLP-1/GIP co-agonist. $^{\#}P < .05$, $^{\#\#}P < .01$ determined by two-way ANOVA comparing acyl-GLP-1 with the acyl-GLP-1/GIP co-agonist. ANOVA was followed by Tukey post hoc multiple comparison analysis to determine statistical significance

3.2 | Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide treatment attenuates hypercholesterolemia and non-alcoholic fatty liver disease in both sexes

GLP-1/GIP co-agonism decreased fasting plasma cholesterol levels in male and female DIO mice more efficiently compared with either mono-agonist (Figure 2A and 2E). In males, the decrease in plasma cholesterol by GLP-1/GIP resulted primarily from a reduction in low-density lipoprotein (LDL) cholesterol, with only subtle changes of high-density lipoprotein (HDL) (Figure 2B). GLP-1 treatment also reduced LDL cholesterol in males, but to a lesser extent relative to treatment with the co-agonist (Figure 2B). In females, GLP-1/GIP but not the mono-treatments reduced plasma LDL levels, and both GLP-1 and GLP-1/GIP increased HDL (Figure 2F). Both, GLP-1 and GLP-1/GIP improved NAFLD in both sexes, as indicated by reduced liver weights and a decrease of the hepatic lipid area (Figure 2C-E,H-J). Moreover, hepatic expression of proteins regulating cholesterol- (e.g. Sqle) and bile acid (e.g. Cyp8b1) synthesis were downregulated after GLP-1/GIP treatment (Figure S7A and S7B). This effect was more pronounced in male DIO mice compared with female DIO mice potentially because of reduced baseline expression of these genes in female DIO mice compared with male DIO mice (Figure S7C). Thus, GLP-1/GIP improves hypercholesterolemia and NAFLD in both sexes and with greater potency relative to treatment with GIP or GLP-1 alone.

3.3 | Shotgun proteomics identifies sex-specific differences in plasma

We next compared the plasma proteome of vehicle-treated DIO mice to examine sex-specific differences in the untreated diseased (obese) state, a question that to our knowledge has not been addressed previously. One-dimensional principal components analysis was sufficient to separate plasma from male and female DIO mice demonstrating substantial differences between sexes (Figure S8A; a list of all proteins is given in Table S3). Sixty-two proteins were increased in male DIO mice relative to female DIO mice, of which 33 were associated with the complement system and inflammation (Figure S8B,C). Previously it was shown that female mice and women have significantly lower complement activity and plasma levels of complement components relative to their male counterparts by measuring specific proteins (e.g. C5, C6, C7, C8, C9).[24,25] These sex-specific differences were confirmed and extended in our study of DIO male and female mice matched for body weight by undirected and unbiased PPP. Apolipoproteins Apod and Apoa1, both constituents of the HDL

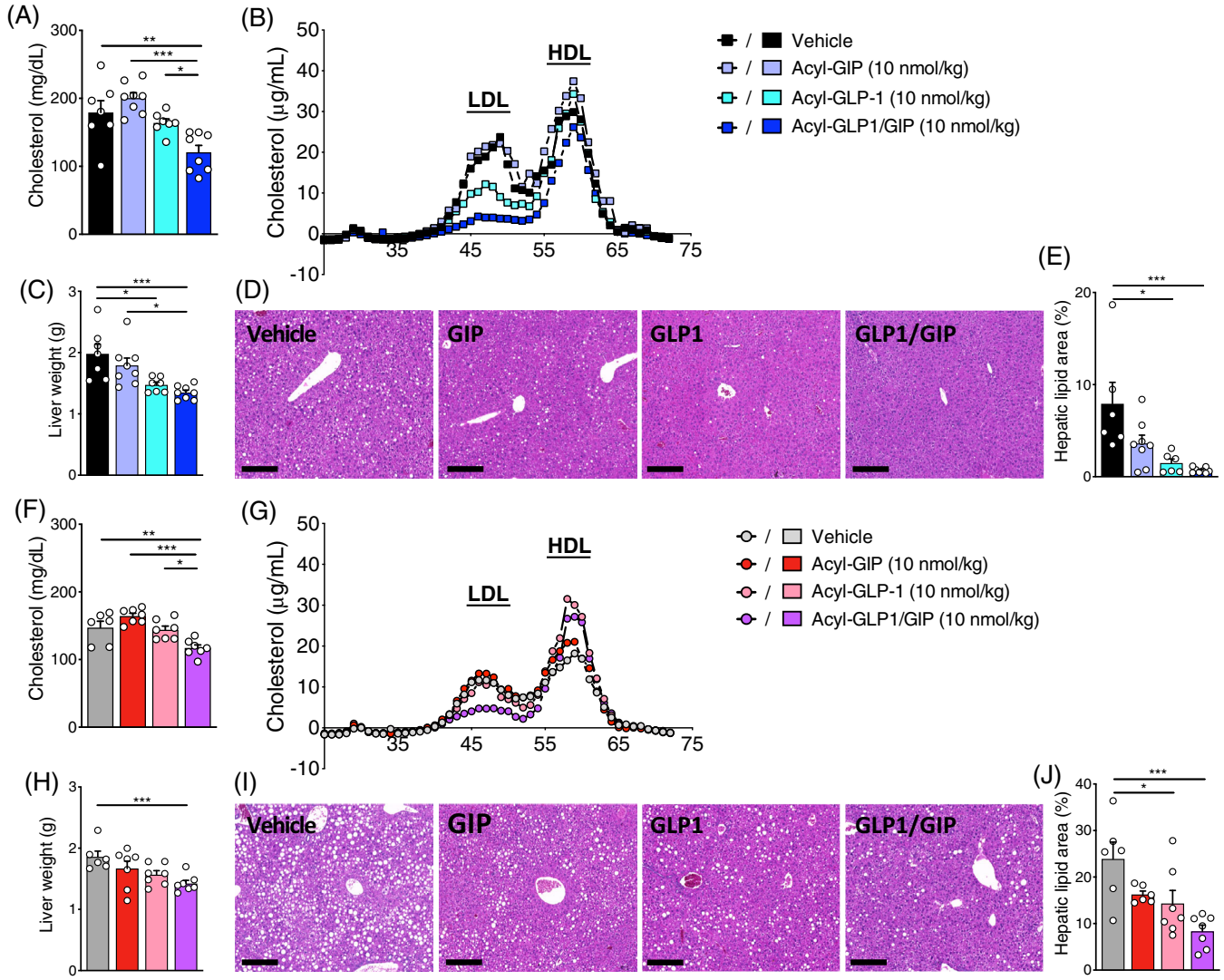

FIGURE 2 Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide (GLP-1/GIP) improves lipid metabolism with greater potency in diet-induced obese male and female mice relative to treatment with GIP or GLP-1 alone. A, Plasma cholesterol, B, plasma lipoprotein fractions, C, total liver weights, D, liver histology and E, lipid area within hepatic sections of male mice, and F, plasma cholesterol, G, plasma lipoprotein fractions, H, total liver weights, I, liver histology, and J, lipid area within hepatic sections of female mice treated daily with either vehicle [n (female) = 7, n (male) = 7], acyl-GIP [10 nmol/kg; n (female) = 7, n (male) = 8], acyl-GLP-1 [10 nmol/kg; n (female) = 7, n (male) = 7], or acyl-GLP-1/GIP [10 nmol/kg; n (female) = 7, n (male) = 8] via subcutaneous injections for 21 days. Blood lipids were determined from sac plasma in the end of the study. Data represent means ± SEM. A, E, F, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, determined by one-way ANOVA followed by Tukey post hoc multiple comparison analysis to determine statistical significance. D, $^{*}P < .05$, $^{***}P < .001$, determined by Kruskal-Wallis test followed by Dunn's multiple comparison test to determine statistical significance. HDL, high-density lipoprotein; LDL, low-density lipoprotein

particle, were increased in males (Figure S8C). Components of LDL particles, such as Apoc1, Apoc3, Apoc4 and LDL were slightly increased in male compared with female DIO mice ($P < .05$; Table S3). These results are consistent with the observation that total plasma cholesterol, HDL and LDL levels are increased in male relative to female DIO mice (Figure 2A, 2B, 2F and 2G). Among the differentially expressed proteins, we also observed high levels of major urinary proteins (Mup2, Mup3, Mup17) in male DIO mice (Figure S8C). Mup proteins are carrier of pheromones in the blood and secreted by urine with an increased abundance in male mice.[26] This shows that PPP captured inherent sex-specific differences between male and female DIO mice.

In plasma of female DIO mice, we found increased protein levels of aldolase B (Aldob), sorbitol dehydrogenase (Sord), aspartate aminotransferase [or glutamic-oxaloacetic transaminase (Got1)], serum amyloid P component (Apcs), Dpp4, leucine aminopeptidase 3 (Lap3), NADP-dependent malic enzyme (Me1) and glutamyl aminopeptidase (Enpep) (Figure S8D). Got1 is already used in the clinic as a regular determined biomarker for liver diseases in humans.[27] Aldob, Sord, Apcs, Dpp4, Lap3, Me1 and Enpep were recently described as novel markers for NAFLD in humans and heavily obese male mice.[17] These markers were upregulated in female DIO mice compared with weight-matched male DIO mice, suggesting that these markers translate to

both sexes. Hence, PPP implies a more pronounced NAFLD phenotype in females relative to body weight-matched male DIO mice. Liver histology revealed increased lipid content in female relative to male DIO mice, an observation that might be influenced by the different duration of HFD exposure (32 weeks in females vs. 7 weeks in males) (Figure 2D and 2I). Taken together, our data demonstrate that the plasma proteome markedly differs between both sexes with weight-matched male mice showing a greater level of markers indicative of inflammation and a less pronounced NAFLD phenotype relative to the females.

3.4 | Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide improves cardiovascular disease risk profile in male and female mice

To examine the contribution of each individual treatment, we compared the plasma proteome of vehicle-treated mice with that of GIP-, GLP-1- and GLP-1/GIP-treated mice. Because of the aforementioned sex differences, we analysed treatment effects on the plasma proteome in a sex-dependent manner.

In male DIO mice, we observed a stronger effect of downregulation than upregulation of plasma protein levels after compound treatment (Figure 3A; a complete list is given in Table S4). There was no overlap among the upregulated proteins by the different treatments (Figure 3A). Of note, GLP-1/GIP treatment increased plasma levels of complement C1q C chain (C1qc) and pyruvate kinase (Pkm), both of which are implicated in the polarization of anti-inflammatory macrophages.[28,29] In line with the observation that treatment with GIP alone had no effect on body weight (Figure 1A and 1B) and lipid metabolism (Figure 2A-2E), we see no major changes in the plasma proteome of male mice treated with GIP relative to vehicle controls (Figure 3A, 3B and 3D).

We observed the downregulation of only 10 plasma proteins by GLP-1 treatment compared with 33 proteins that decreased by GLP-1/GIP treatment (Figure 3A). Thus, the co-agonist treatment induced a far broader spectrum of plasma protein changes than GLP-1 pointing to additive, so far unknown constructive effects by this combinatorial pharmacology approach. Six proteins overlapped between GLP-1 and GLP-1/GIP treatment in male DIO mice (Figure 3A and 3C). Among the overlapping proteins, four proteins [histidine-rich glycoprotein (Hrg), serum amyloid A1 (Saa1), alpha-1-acid glycoprotein 1 (Orm1),

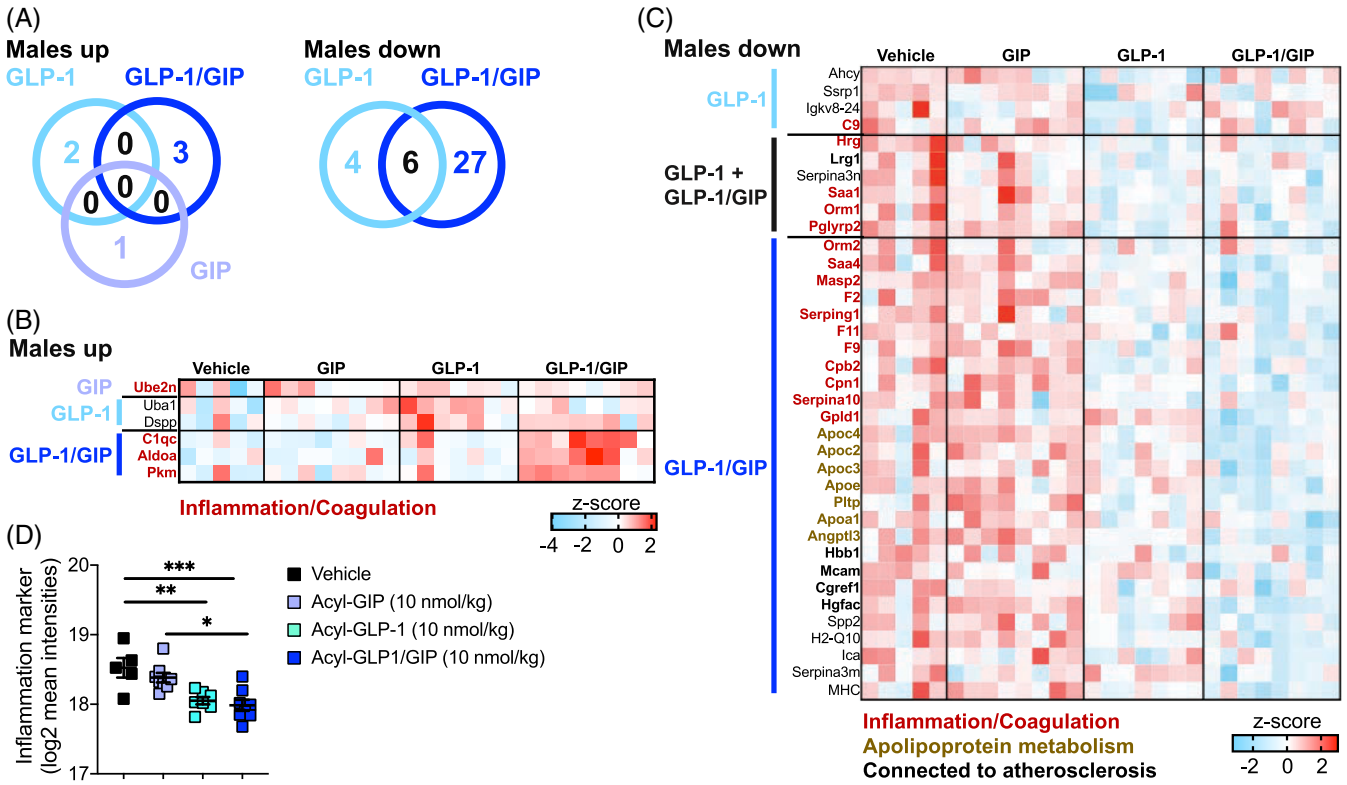

FIGURE 3 Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide (GLP-1/GIP) resolves systemic inflammation in male mice. A, Venn diagram of upregulated (left) and downregulated (right) proteins in the plasma of GIP-, GLP-1- and GLP-1/GIP-treated male diet-induced obese (DIO) mice compared with vehicle-treated male DIO mice ($P < .01$). B, Heatmap of upregulated and C, downregulated proteins. Intensities of proteins were log2-transformed and *Z*-scored to normalize across samples. Proteins involved in different biological processes or belonging to different classes are indicated by colour. D, Changes of the inflammation status of male DIO mice after treatment with vehicle, GIP, GLP-1 and GLP-1/GIP. Inflammation status is calculated as the mean of the 16 inflammation proteins that changed after GLP-1/GIP treatment (including Lrg1). Vehicle, n = 5; GIP, n = 8; GLP-1, n = 7, GLP-1/GIP, n = 8

peptidoglycan recognition protein 2 (Pglyrp2)] were associated with inflammatory processes (Figure 3C). Furthermore, Saa1 belongs to the newly identified NAFLD biomarker panel.[17] Interestingly, GLP-1 and GLP-1/GIP treatment reduced plasma levels of Lrg1, a recently suggested biomarker for obesity, systemic inflammation and atherosclerosis.[30,31]

Remarkably, among the 27 proteins more potently reduced exclusively by the GLP-1/GIP co-agonist, we found 11 additional markers of known implication in inflammatory processes or the complement system (Figure 3C).

We calculated the mean protein level of all downregulated plasma proteins by GLP-1 and/or GLP-1/GIP (Figure 3C) as a surrogate of anti-inflammatory effectiveness. This analysis revealed an anti-inflammatory effect of GLP-1 and GLP-1/GIP treatment with an additional effect of the co-agonist to reduce the systemic inflammation state compared with vehicle and GIP treatment (Figure 3D).

Specifically, co-agonist treatment reduced plasma levels of serum amyloid A4 (Saa4) and alpha-1-acid glycoprotein 1 (Orm2) (Figure 3C). Both proteins are associated with an acute phase reaction upon inflammation.[32,33] Blood coagulation factors II (F2), IX (F9) and XI (F11) as well as plasma regulators of the blood coagulation cascade such as for example the complement component 1 (C1) inhibitor (Serping1), serpin family A member 10 (Serpina10), carboxypeptidase B2 (Cpb2), carboxypeptidase N (Cpn1) and mannan-binding lectin-associated serine proteases (Masp2) were also lower in the GLP-1/GIP-treated male mice (Figure 3C). These findings are of interest because obesity has been associated with increases in components of the fibrinolytic and haemostatic system in circulation potentially contributing to the increased CV risk in these individuals.[29,34] In addition, GLP-1/GIP treatment decreased plasma levels of recently suggested biomarkers for CV disease (CVD): haemoglobin subunit beta-1 (Hbb1), melanoma cell adhesion molecule (Mcam), cell growth regulator with EF hand domain protein 1 (Cgref1) and phosphatidylinositol-glycan-specific phospholipase D (Gpld1).[31,35–38] Together with a reduction of the LDL particle constituents Apoc2, Apoc3 and Apoe (Figure 3C), these findings highlight the additional, potentially beneficial effects in treating cardiometabolic disease by the GLP-1/GIP co-agonist.

Similar to male DIO mice, we observed also in females that the majority of affected proteins are decreased rather than increased after compound treatment (Figure 4A; a complete list is given in Table S5). GIP monotherapy increased circulating lactate dehydrogenase A (Ldha) levels, a monomeric subunit of lactate hydrogenase, which is a rate-limiting enzyme in anaerobic glycolysis (Figure 4B). GLP-1 monotherapy increased complement and blood coagulation associated proteins such as coagulation factor XIII B chain (F13b), coagulation factor XIII A chain (F13a1) and protein Hc (Hc, also known as α1-microglobulin) (Figure 4B). The first two proteins belong to the plasma factor XIII tetramer that catalyses the last step of the blood coagulation pathway. Among the GLP-1/GIP-regulated proteins, we found increased plasma levels of platelet factor 4 (Pf4, also known as C-X-C motif chemokine 4, Cxcl4) and thrombospondin 1 (Thbs1) (Figure 4B). Thbs1 has been associated to obesity and the metabolic syndrome, particularly in women.[39]

GIP, GLP-1 and GLP-1/GIP treatment reduced one, nine and 19 plasma protein levels respectively relative to vehicle treatment

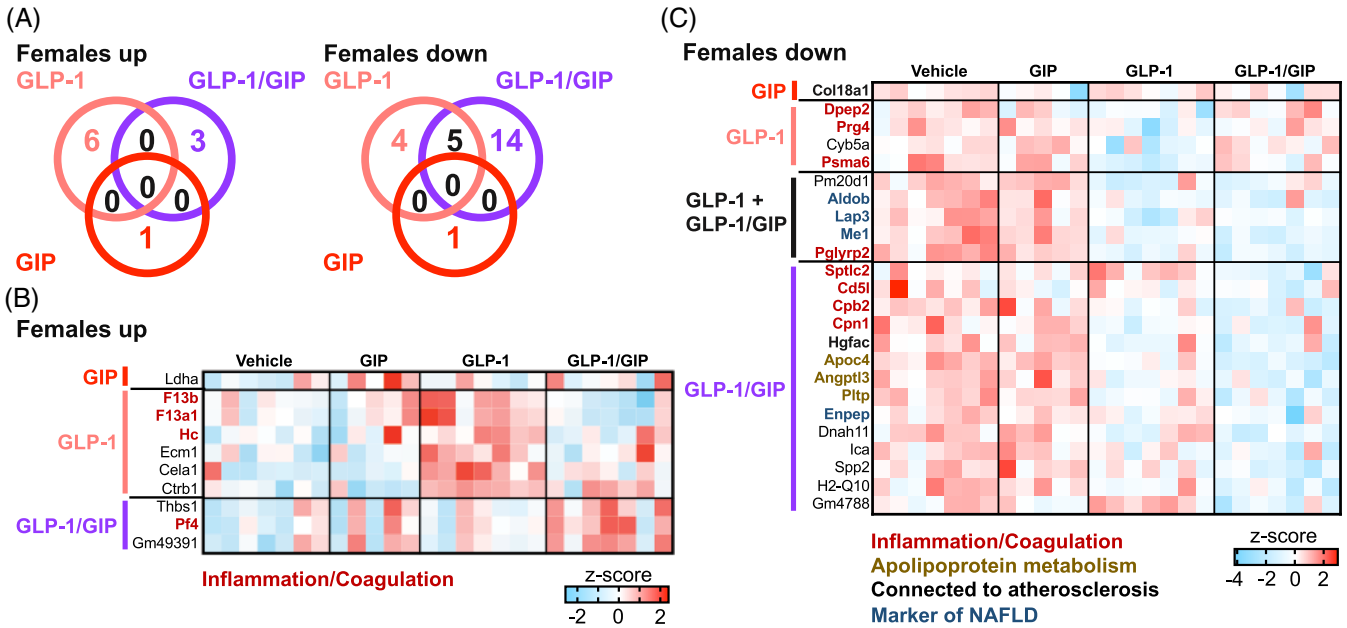

FIGURE 4 Glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide (GLP-1/GIP) improves metabolic state in female diet-induced obese (DIO) mice. A, Venn diagram of upregulated (left) and downregulated (right) proteins in the plasma of GIP-, GLP-1- and GLP-1/GIP-treated DIO mice compared with vehicle treated DIO mice ($P < .01$). B, Heatmap of upregulated and C, downregulated proteins. Intensities of proteins were log2-transformed and *Z*-scored to normalize across samples. Proteins involved in different biological processes or belonging to different classes are indicated by colour. Vehicle, n = 7; GIP, n = 5 GIP; GLP-1, n = 7; GLP-1/GIP, n = 7 GLP-1/GIP

(Figure 4A). GIP decreased plasma protein levels of collagen type XVIII alpha 1 chain (Col18a1), a novel potential biomarker for CVD (Figure 4C).[40] GLP-1 treatment reduced the inflammation-associated proteins dipeptidase 2 (Dpep2), proteoglycan 4 (Prg4) and proteasome subunit alpha type-6 (Psma6) (Figure 4C). Five of the GLP-1-regulated proteins overlapped with proteins downregulated by GLP-1/GIP treatment (Figure 4A and 4C). Three of these five proteins were associated with an improvement of NAFLD (Aldob, Lap3, Me1) (Figure 4C). An additional 14 proteins pertinent to protein classes associated with inflammation, coagulation, lipid transport and to novel biomarkers for NAFLD and CVD were further reduced exclusively by GLP-1/GIP treatment (Figure 4A and 4C).

3.5 | Sex-independent plasma protein panel downregulated by glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide treatment correlates to systemic metabolic benefits

Although PPP responses after mono-agonist treatment were mainly different between sexes (Figure S9), we detected 10 plasma proteins that were decreased in DIO male and female mice after co-agonist treatment (Figure 5A and 5B). Among these we found: (i) phospholipid transfer protein (Pltp) and angiopoietin-like protein 3 (Angplt3), which are both modulators of lipoprotein metabolism, and high plasma levels are associated with obesity, T2D and CVD in humans[41,42]; (ii) plasma apolipoprotein levels of Apoc4 in line with the further decreased LDL fraction after co-agonist but not mono-agonist treatments (Figure 2B, G); and (iii) hepatocyte growth factor activator (Hgfac), a serine protease that converts Hgf to its active form, of which increased levels have been associated with a more severe progression of atherosclerosis indicated by increased numbers of carotid plaques in humans.[43]

By correlating each of the sex-independent downregulated 10 proteins to physiological parameters determined in this study, we aimed to find biomarkers that broadly reflect systemic metabolic benefits after weight loss such as after treatment with a clinically advancing GLP-1/GIP co-agonist. Expectedly, and in agreement with similar weight-lowering efficacy of GLP-1/GIP treatment in both sexes, each of the 10 proteins significantly correlated to body weight loss in male and female mice (Figure 5C). Particularly in male mice, most of these 10 proteins also correlated to improvements in glucose and insulin homeostasis, lipid metabolism and liver fat area. Apoc4, Hgfac, Angptl3 and Pltp depicted the strongest overall correlation to GLP-1/GIP-mediated metabolic benefits (as assessed by the mean across all

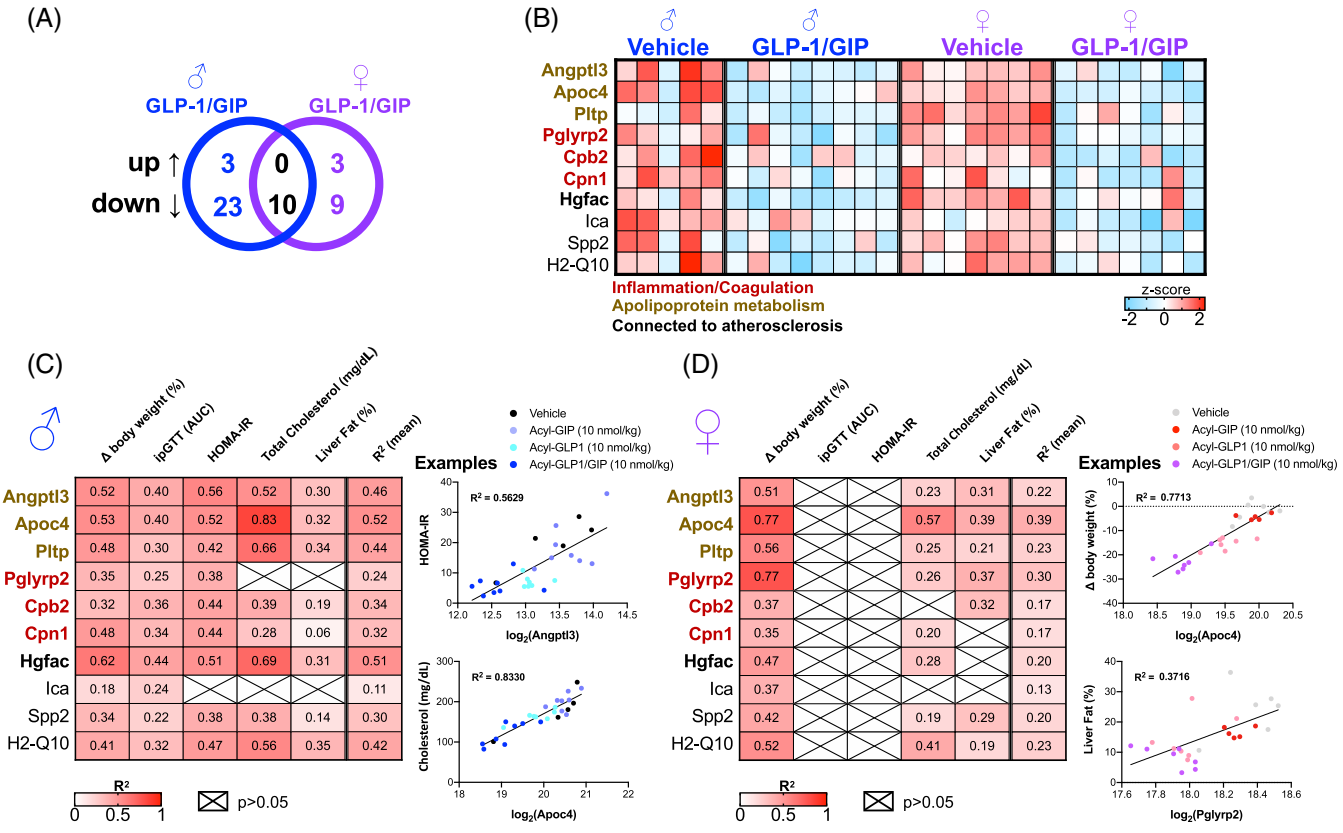

FIGURE 5 Sex-independent plasma proteins correlate with glucagon-like peptide-1/glucose-dependent insulinotropic polypeptide (GLP-1/GIP)-induced metabolic benefits. A, Venn diagram showing the differentially up- and downregulated plasma proteins of diet-induced obese male and female mice after GLP-1/GIP treatment. B, Heatmap of commonly downregulated proteins in male and female mice after GLP-1/GIP treatment. Correlation matrix of the 10 sex-independent downregulated proteins to physiological parameters determined in this study of C, male, and D, female, mice. HOMA-IR, homeostatic model assessment of insulin resistance; ipGTT, intraperitoneal glucose tolerance

correlation coefficients) (Figure 5C). The correlation coefficients in female mice were in general lower with no significant correlations to improvements in glucose and insulin homeostasis (Figure 5D). This is possibly because of the overall superior metabolic state of female compared with male mice (Figure S6). As for male mice, Apoc4 had the strongest overall correlation coefficient in female mice (Figure 5D).

4 | DISCUSSION

To the best of our knowledge, here we show for the first time that a balanced GLP-1/GIP monomeric peptide co-agonist has a superior treatment efficacy and induces a broader spectrum of changes in plasma proteome relative to treatment with GLP-1 or GIP alone in both sexes of DIO mice. PPP revealed that co-agonist treatment improved plasma protein markers suggestive of systemic inflammation more potently than either monotherapies, particularly in male DIO mice. Furthermore, our combination of pharmacology and PPP potentially revealed sex-independent plasma biomarkers that might enable minimal-invasive monitoring and uncover, ulterior, underlying mechanisms of systemic metabolic improvements after weight loss interventions such as the clinically advancing GLP-1/GIP co-agonists.

GLP-1/GIP lowered body weight with comparable potency in both sexes. In females, the superior weight loss of GLP-1R/GIPR co-agonism relative to treatment with GLP-1 was accompanied by an increased anorectic effect of the co-agonist. However, in male DIO mice we found that the additional weight-lowering efficacy of the co-agonist compared with the GLP-1 mono-agonist cannot be explained solely by reduced energy intake alone. Finan et al. showed that GLP-1/GIP treatment in DIO male mice does not increase energy expenditure in male DIO mice, but exceeds pair-feeding induced weight loss.[6] Together, these findings suggest that other mechanisms could contribute to the body weight-lowering effect of the GLP-1/GIP co-agonist in male DIO mice, and interestingly, this may differ for female mice. We found that the ulterior body weight loss of the co-agonist versus the GLP-1R mono-agonist was slightly greater in female DIO mice compared with male DIO mice [3.1 g (7.2%) vs. 2.9 g (5.8%)]. This translated to a mean additional fat mass lost by GLP-1/GIP co-agonist over GLP-1 mono-agonist treatment of 2.4 and 2.9 g for male and female mice, respectively. As Benz et al. have recently shown that female DIO mice respond to weight-lowering interventions with an enhanced lipolytic activity in adipose tissue compared with male DIO mice,[44] we cannot exclude that this inherent sex difference accounts for the slightly enhanced weight loss in female DIO mice we observed after GLP-1/GIP treatment. However, as female DIO mice exhibited a greater fat mass than male DIO mice at the start of treatment start is also possible that direct effects of GIP on adipocytes[45] contributed to the greater separation because of the additional adipose tissue available. Future studies are warranted to dissect GIP-dependent mechanisms on adipose tissue in detail. As reported previously,[6] the body weight neutral dose of acyl-GIP applied herein improves glucose metabolism in male DIO mice and, as shown here, in female DIO mice.

The comparison of vehicle treated male and female DIO mice showed substantial differences in the plasma proteome. We detected markedly increased plasma levels of the complement cascade in male relative to female DIO mice. This is in line with literature describing a decreased abundance and activity of complement proteins in the plasma of female rodents and women compared with male counterparts.[24,25] Whether a differentially regulated complement system contributes to the protection of female mice against DIO-mediated complications remains uncertain. Accordingly, we did not observe a comparably pronounced effect of GLP-1/GIP treatment in reducing proteins involved in inflammatory processes and immunological responses in female as compared with male DIO mice. In male DIO mice, GLP-1/GIP treatment resolved the mild obesity-associated systemic inflammation more potently than both mono-agonists. Although we cannot exclude that the additional anti-inflammatory phenotype after GLP-1/GIP co-agonist treatment results from a higher degree of weight loss, it is conceivable that the direct immunoregulatory effects of GLP-1 and/or GIP might have contributed.[46] In future studies it would be of specific interest to investigate plasma proteomic changes induced by caloric restriction and pharmacological treatment that are matched for body weight loss (e.g. pair-feeding) to test for compound-specific changes.

In addition to the clinical pilot trial of a PEGylated unimolecular dual incretin[6] and to the phase 2 clinical study with LY3298176, a novel dual GLP-1/GIP receptor co-agonist from Eli Lilly,[5] we showed in a 12-week proof-of-concept study in humans that a dose of 1.8 mg/day of a sustained-acting fatty-acylated GLP-1/GIP co-agonist (NNC0090-2746) with balanced activity at each receptor (GLP-1R EC_{50} = 5pM; GIPR EC_{50} = 3pM) reduced body weight and improved glycaemic control compared with placebo in T2D.[4] This safety study was not designed to assess the body weight- and glucose-lowering potential of NNC0090-2746 in patients with obesity and/or with T2D. Clearly, to assess completely the metabolic efficacy of this balanced GLP-1/GIP co-agonist more studies are needed, including for example a dose-finding trial, a refined escalation schedule and importantly, a trial with longer treatment duration. However, NNC0090-2746 reduced total plasma cholesterol compared with the placebo control group, whereas the GLP-1 control (liraglutide; 1.8 mg with a 2-week dose escalation) had no effect despite similar weight loss compared with NNC0090-2746.[4] Here, we show that only GLP-1/GIP co-agonist treatment significantly reduced fasting plasma cholesterol levels in male and female DIO mice. This effect was superior to both mono-agonists and was accompanied by a striking decrease of LDL lipid fractions in both sexes. The GIPR is widely expressed on adipocytes. GIP has been shown in the past to affect white adipose tissue indirectly by increasing adipose tissue blood flow and directly by modulating adipocyte metabolism.[45] The effect of GIP to increase white adipose tissue perfusion is blunted in obese humans, but can be reverted by weight loss.[47] GLP-1-induced weight loss could thus prime GIP action on lipid metabolism contributing to the superior benefits of the GLP-1/

GIP co-agonist compared with its respective mono-agonists. However, the pharmacological potential of GIP analogues to treat dyslipidaemia and/or target adipose tissue directly in vivo has not been entirely tested yet and requires future studies.[48] These findings are further supported by a more potent reduction of plasma apolipoproteins pertinent to LDL particles by GLP-1/GIP compared with GLP-1 in male and female DIO mice as determined by PPP. This underlines the power of PPP to detect multiple lipid particles in more depth. The advantage of the proteomic approach is that less plasma volume is required as for conventional assays and thus makes the comparison of multiple parameters in preclinical pharmacological studies possible. Besides apolipoprotein fractions, PPP revealed additional markers of CVD risk that were reduced particularly after GLP-1/GIP treatment in male (e.g. Hbb1, Mcam, Cgref1, Hgfac and Gpld1) and female (e.g. Hgfac) DIO mice. Collectively, the GLP-1/GIP co-agonist treatment more potently improved the CV risk profile of male and female DIO mice compared with mono-agonist treated mice. It is of specific interest to explore potential novel underlying mechanisms and efficacy of GLP-1R/GIPR co-agonism or either mono-agonism in future studies in this regard.

NAFLD is the hepatic manifestation of metabolic disease and shares almost identical risk factors.[49] There is no approved pharmacological therapy to treat the disease. Therefore, predictive biomarkers of disease risk and therapy response are urgently needed.[49] Niu et al. recently applied PPP to identify novel biomarkers of NAFLD in mice and humans, which potentially discriminate between reversible NAFLD and irreversible liver cirrhosis.[17] Treatment of female DIO mice with GLP-1 and GLP-1/GIP reduced plasma levels of Aldob, Lap3 and Me1 with an additional effect of GLP-1/GIP to reduce Enpep accompanied by a decreased hepatic lipid content and an improved hepatic histology. Furthermore, we found decreased levels of Cd5l in the plasma of DIO female GLP-1/GIP-treated mice. Plasma Cd5l is higher in women than in men and pathologically increased Cd5l levels were found in patients with liver cirrhosis and proposed as a potential biomarker for assessing liver fibrosis.[50] Altogether, our results not only demonstrate the potential of these new markers to characterize liver diseases, but also highlight the potential application of the GLP-1/GIP co-agonist to treat NAFLD[51] and the power of PPP to access treatment responses cross-species in a systemic way.

Bariatric surgery is still the most effective intervention to correct obesity and its comorbidities. Combining pharmacology and MS-based unbiased PPP, we herein show that GLP-1R/GIPR co-agonism is more effective than either the mono-agonist to reduce body weight, to improve glucose tolerance, to ameliorate systemic inflammation, to improve dyslipidaemia, to reduce the CVD risk profile and to reverse liver steatosis in male and female DIO mice. In particular, PPP revealed superior GLP-1/GIP-mediated downregulation of inflammatory and lipid-particle associated proteins compared with either mono-agonist in male and to a lesser extent in female mice. Inflammatory proteins were the most significantly downregulated proteins indicative of long-term RYGB success to reduce body weight in patients with obesity.[10] While comparing the similarities of the anti-inflammatory effect after GLP-1/GIP treatment in mice and RYGB-treatment in humans, we detected eight overlapping proteins. Three of these, Saa1, Orm1 and Pglyrp2 were downregulated by both GLP-1 and GLP-1/GIP co-agonist treatment. However, the levels of the other five plasma proteins Orm2, Saa4, Masp2, F2 and Serping1 were significantly reduced exclusively by GLP-1/GIP treatment. In contrast to male DIO mice, only Pglyrp2 of the RYGB-associated inflammation markers was decreased in plasma of female DIO mice after GLP-1 and GLP-1/GIP treatment, highlighting previously mentioned sex-specific differences in the DIO-associated inflammation status (Figure S8). Taken together our results point to ulterior benefits of our novel unimolecular co-agonist beyond its respective mono-agonists. Collectively, our results support the rationale of integrating multiple hormones to close the gap to metabolic benefits usually achieved only by surgical intervention.

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AUTHOR CONTRIBUTIONS

S.S. generated, analysed and interpreted experimental data and co-wrote the article; L.N., S.J., M. K., P.G., K.S, and N.W.A. generated and helped interpret experimental data; A.F. evaluated histopathological hepatic data; M.B. supervised mouse studies; B.F., R.D.D., M.H.T. and M.M. advised study concept and critical revision of the article; T.D.M. oversaw the in vivo experiments, interpreted experimental data and co-wrote the article; S.M.H. headed the lipoprotein profile measurements and analysis, oversaw the in vivo experiments, interpreted experimental data and co-wrote the article. T.D.M. and S.M.H. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTERESTS

B.F. and R.D.D. are current employees of Novo Nordisk. Novo Nordisk has licensed from Indiana University intellectual property pertaining to this report. Novo Nordisk has had no role in the writing of or the cases reported in the present manuscript. M.H.T. serves as an SAB member of ERX Pharmaceuticals, Inc., Cambridge, MA. The Institute for Diabetes and Obesity receives research support from Novo Nordisk. The remaining authors declare that they have no competing interests.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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