Research Paper

CMPF, a Metabolite Formed Upon Prescription Omega-3-Acid Ethyl Ester Supplementation, Prevents and Reverses Steatosis

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A R T I C L E   I N F O

A B S T R A C T

Prescription ω-3 fatty acid ethyl ester supplements are commonly used for the treatment of hypertriglyceridemia. However, the metabolic profile and effect of the metabolites formed by these treatments remain unknown. Here we utilized unbiased metabolomics to identify 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) as a significant metabolite of the ω-3-acid ethyl ester prescription Lovaza™ in humans. Administration of CMPF to mice before or after high-fat diet feeding at exposures equivalent to those observed in humans increased whole-body lipid metabolism, improved insulin sensitivity, increased beta-oxidation, reduced lipogenic gene expression, and ameliorated steatosis. Mechanistically, we find that CMPF acutely inhibits ACC activity, and induces long-term loss of SREBP1c and ACC1/2 expression. This corresponds to an induction of FGF21, which is required for long-term steatosis protection, as FGF21KO mice are refractory to the improved metabolic effects. Thus, CMPF treatment in mice parallels the effects of human Lovaza™ supplementation, revealing that CMPF may contribute to the improved metabolic effects observed with ω-3 fatty acid prescriptions.

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1. Introduction

Excessive caloric intake combined with increasingly sedentary lifestyles is producing an epidemic of overweight and obesity, affecting nearly 40% of Americans (Cameron et al., 2004). The cluster of metabolic disturbances associated with increased adiposity, termed metabolic syndrome (MetS), confers a 1.6-fold increased risk of mortality (O'Neill and O'Driscoll, 2015), attributed to consequential risk for diabetes (5-fold), stroke (2- to 4-fold), myocardial infarction (3- to 4-fold) and cancer in MetS individuals (Eckel et al., 2005; Masters et al., 2013a; Masters et al., 2013b; Micucci et al., 2016; O'Neill and O'Driscoll, 2015). The underlying pathophysiology of MetS is primarily based in dysregulated lipid metabolism, resulting in aberrant lipid storage in the liver and muscle, hypertriglyceridemia, increased insulin resistance, and altered circulating lipoprotein levels (Avramoglu et al., 2006; Bergman and Ader, 2000; Cao et al., 2008; Ginsberg, 2006).

Fish oil (FO) is mainly composed of ω-3 fatty acids (ω-3 FA) and its administration consistently demonstrates beneficial metabolic effects that include lowering of plasma triglycerides (TG) in a dose-dependent manner (Imaichi et al., 1963; Kinsell et al., 1961; Shearer et al., 2012) which results in long-term beneficial effects on many aspects of MetS (Koski, 2008). Lovaza™ is a prescription ω-3-acid ethyl ester supplement (comprised of approximately 55.1% EPA, 44.9% DHA) approved for the lowering of TG in patients with plasma TG levels ranging between 500 and 2000 mg/dl (Koski, 2008). Clinical studies at the recommended dose of 4 g/day of Lovaza™ for 6–16 wks resulted in an average drop of 42% in TG levels compared to placebo (Harris et al., 1997; Koski, 2008; Pownall et al., 1999). There are several proposed mechanisms

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underlying this phenomenon (Hotamisligil et al., 1995; Rustan et al., 1993; Strissel et al., 2007; Yoshikawa et al., 2002) including reduced hepatic VLDL synthesis and secretion, enhanced lipid oxidation in muscle and liver and increased uptake of TG from VLDL and chylomicrons through increased lipoprotein lipase activity (Harris and Bulchandani, 2006; Vedala et al., 2006). Importantly, however, while these processes are associated with ω-3 FA supplementation, the mechanisms and active components mediating these effects remain poorly characterized.

Herein we identify the metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) as an abundant urinary and plasma metabolic product of Lovaza™ administration in humans using an unbiased metabolomics approach. Consistent with previous studies showing CMPF enhances lipid metabolism in the islet (Liu et al., 2016; Prentice et al., 2014), we now present the beneficial effects of CMPF on whole-body metabolic homeostasis, particularly in the prevention and reversal of insulin resistance and hepatic steatosis, *via* inhibition of acetyl CoA-carboxylase (ACC) and induction of FGF21.

2. Materials and Methods

2.1. Human Study Design

To study the effects of dietary fatty acids on the formation of active components mediating beneficial metabolic effects, biospecimens were sourced from the human study "Effects of Fish Oil and Red Wine on Oxidative Stress Biomarkers" registered in clinicaltrials.gov as NCT00682318. The clinical study protocol and written informed consent were approved by the Institutional Review Board of the University of Pennsylvania prior to starting subject enrollment. Authorization by the FDA (IND#79,750) to administer Lovaza™ ω-3 FA prescription supplements exceeding the maximum recommended daily dose was also obtained by C.S. prior to initiating study activities.

2.1.1. Study A: High-Dose Study

In an open single-arm design, healthy volunteers, n = 12 (7 females, 58%), 30.8 ± 11.6 years of age, were supplemented with 7 capsules of Lovaza™ three times daily for 24.2 ± 2.3 days (Fig. 1), which delivered a total of 17.6 g/day ω-3 PUFA, consisting of 55.1% EPA (9.7 g/day) and 44.9% DHA (7.9 g/day). Study inclusion criteria were 21–55 years of age, non-smokers, not pregnant and abstained from the use of high-dose vitamins, NSAIDs, and illicit drugs examined by cotinine (Craig Medical, Vista, CA) and pregnancy tests, history, platelet aggregometry (Pedersen and FitzGerald, 1985), and a urine drug screen (RDI, Poteau, OK), for at least two wks before enrollment and throughout the study. Study exclusion criteria comprised administration of an experimental drug or experimental medical device within the past 30 days, a blood donation of ≥ one pint within the past 8 wks, signs of a coagulation, bleeding or blood disorder. Since high doses of fish oil supplements increase the exposure to contaminant heavy metals including mercury, participants were asked, per FDA recommendation, to refrain from additional fish foods during study enrollment. Routine medical history, physical exam, and laboratory work (hematology, biochemistry, and urinalysis) at time of screening and on completion of the study assessed health status and safety. Compliance was assessed i) in real-time using text messages or emailing the intake of Lovaza™ capsules, ii) weekly by capsule count in the Clinical and Translational Research Center (CTRC) of the University of Pennsylvania, iii) by the change of lipid ratios in red blood cell membranes (Skarke et al., 2015) and iv) by the diversion from AA-derived isoprostanes and epoxides toward EPA-/DHA-derived species (Skarke et al., 2015).

2.1.2. Study B: Low-Dose Study

Using the human study conditions as specified above for study A, and adjusting the study inclusion to 21–60 years of age, n = 7 healthy participants (4 females, 57%), 38.4 ± 13.2 years of age, were all started on 2 capsules Lovaza™ for 41.9 ± 0.4 days (Fig. 2), which delivered a total of 1.7 g/day ω-3 PUFA, consisting of 930 mg/day EPA and 750 mg/day DHA, followed by randomizing n = 4 (2 females, 50%), 40.3 ± 13.1 years of age, to continue on 4 capsules Lovaza™ three times daily for 27.8 ± 0.5 days (Fig. 2), which delivered a total of 10.1 g/day ω-3 FA, consisting of 930 mg/day EPA and 750 mg/day DHA, and randomizing n = 3 (2 females, 66.7%), 36 ± 15.6 years of age, to continue on 4 capsules SOLGAR® safflower oil three times daily for 28.3 ± 0.6 days (Fig. 2), which delivered a total of 9.8 g/day of linoleic acid as an ω-6 fatty acid source.

2.2. Animal Study Design

All experiments were approved by the Animal Care Committee at the University of Toronto and animals were handled according to the Canadian Council of Animal Care guidelines. Seven-wk-old male CD1 mice were purchased from Charles River and allowed to acclimate for one week prior to the beginning of experiments. For obese studies, 8 wk old CD1 mice were placed on either a 60% kcal from fat high fat diet (HFD; OpenSource diets D12492, Research Diets Inc., USA) or a sucrose-matched control (Chow) diet (OpenSource diets D12450J, Research Diets Inc., USA) for 6 wks prior to CMPF injection. Seven-week-old leptin-deficient male *ob/ob* mice were purchased from Jackson Labs and allowed to acclimate for one week prior to initiation of injections. Mice were injected with either 6 mg/kg CMPF or vehicle control (70% ethanol) at 24 h intervals for 14 days while maintained on diet, as described previously(Liu et al., 2016). For long-term studies mice were maintained on standard chow diet while being injected intraperitoneally (i.p.) with 6 mg/kg CMPF or vehicle control (70% ethanol) at 24 h intervals for 7 days, as previously described (Prentice et al., 2014). 24 h following the final injection, mice were switched to either HFD or a Chow diet for 4–5 wks. Mice were also monitored weekly for individual body weights and whole-cage food consumption.

Six to nine wk old male FGF21KO mice were maintained at Taconic (Hudson, NY) and obtained through Eli Lilly along with age-matched c57 controls. Six to nine wk old male mice were allowed to acclimate for one week and randomized to treatment groups prior to the beginning of the treatment period. Mice were injected with 6 mg/kg/day CMPF for 7 days and changed to HFD and matched sucrose control as above. Mice were maintained on the diet for 6 wks prior to evaluation. FGF21 deletion was confirmed by PCR.

2.3. Sample Preparation and LC-MS/MS Analysis

Details can be found in the Supplementary material. Full dataset can be found on the XCMS website, job #1094729.

2.4. LC-MS/MS Quantitation of CMPF in Human Samples

CMPF and its corresponding metabolites were analyzed by LC-ESI-MS/MS. CMPF extracts were resolved for quantitation purposes using a reversed phase Luna C18(2) HPLC column (2 × 100 mm, 5 μm, Phenomenex, Torrence, CA) with a gradient solvent system consisting of solvents (A): H_2O containing 0.1% acetic acid and (B): acetonitrile containing 0.1% acetic acid, at a 0.65 ml/min flow rate. Samples were applied to the column at 15% B and eluted with a linear increase in B (15%–85% from 0.3 to 9.7 min) that reached 100% at 10 min and was held for 3 min before returning to initial conditions for 3 min of equilibration. Analyte quantification was performed in multiple reaction monitoring mode (MRM) using an API5000 triple quadrupole or an API4000 QTrap mass spectrometer (Sciex; San Jose, CA) equipped with an electrospray ionization source. The following transitions were used to monitor CMPF (m/z 239 ➔ 195 and 239 ➔ 151) and CMPF-$_{d5}$ (m/z 244 ➔ 200 and 244 ➔ 156). A synthetic CMPF standard and the CMPF-$_{d5}$ internal standard were used to develop a calibration curve for quantitation. The following MS parameters were used, electrospray voltage was −4.5 kV, declustering potential −80, entrance potential −10, collision
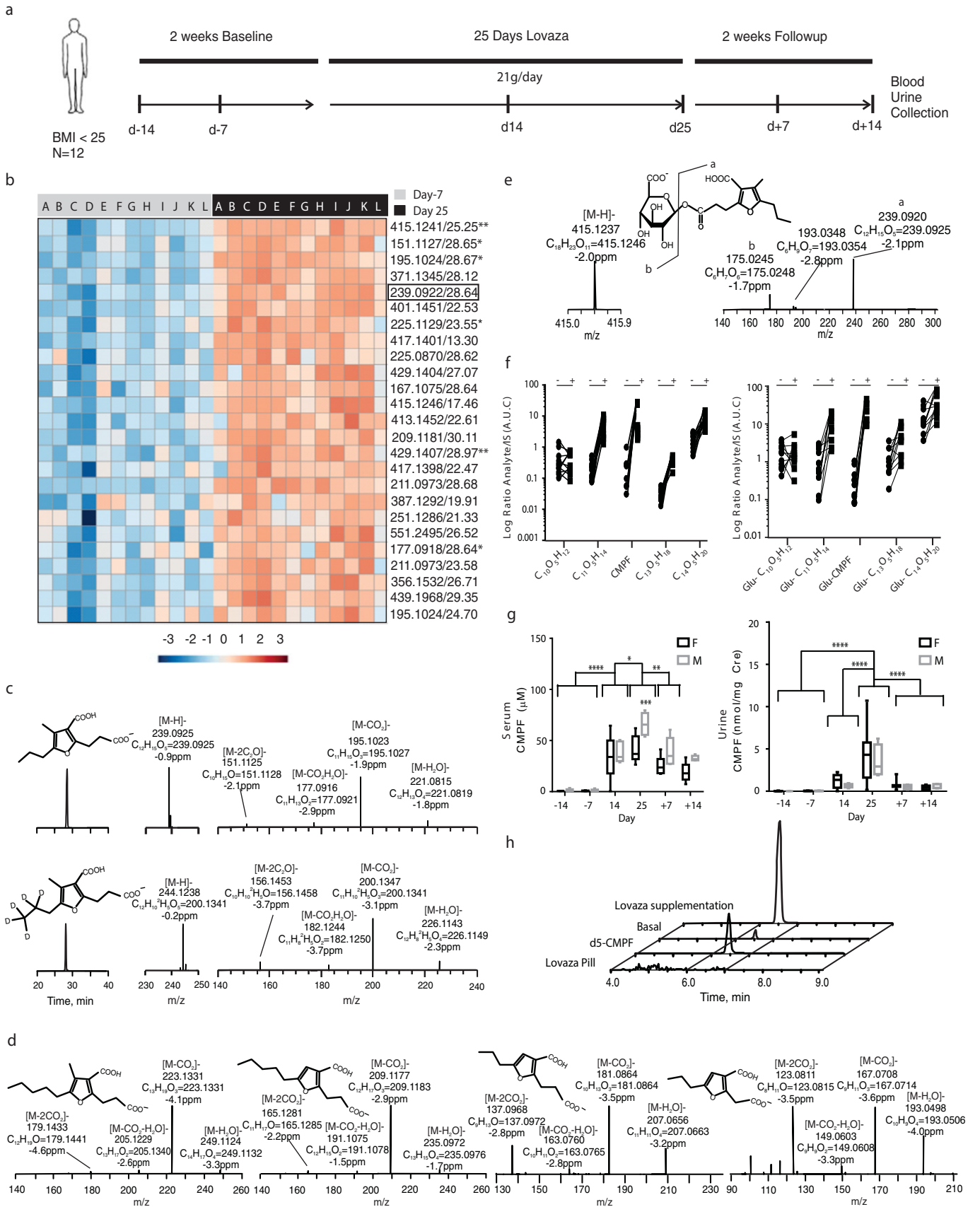
energy −25, gas1 55 and gas2 50 and the source temperature was set at 650 °C.

2.5. CMPF Pharmacokinetic Study in Mice

Briefly, mice were injected with 6 mg/kg CMPF intraperitoneally, as described. Details on CMPF preparation can be found in the Supplementary Material. Blood was collected at time 0, 2, 24 h and 4 wks following final injection. CMPF standards (1–500 ng) and samples were spiked with 25 ng of CMPF-d_5 internal standard. A surrogate matrix of 4% BSA in PBS was used for standards. Plasma (20 μl) and standards (20 μl matrix surrogate) were diluted with 480 μl of ultrapure water. 20 μl of 80% phosphoric acid was added, mixed by vortexing then 1.5 ml of ethyl acetate (EtOAc) was added. Samples were chilled on ice and then centrifuged. The upper EtOAc layer was collected and re-extracted with another 1.5 ml of EtOAc. Samples were vortexed, chilled on ice, and centrifuged. The upper EtOAc layers were removed and combined with the previous extract. The combined EtOAc layers were dried and residues were reconstituted in 500 μl of acetonitrile and analyzed by LC-MS/MS using an Agilent 1200 HPLC with an API 4000 mass spectrometer (Sciex). For the liver samples, the same extraction protocol was used, except the liver was homogenized in 1:1 water:ethanol at a concentration of 100 mg/ml and the equivalent of 20 mg was extracted (200 μl). 300 μl water was added instead of 480 μl. The curve range for the liver was 0.5–100 ng and the curve was calculated from control liver.

2.6. In Vivo Analysis of Mice

Details regarding the *in vivo* analysis of mice, including MRI, CLAMS, and insulin tolerance testing can be found in the Supplementary material. Array data is MIAME compatible and available on the GEO database under accession GSE106639.

2.7. Statistics

Statistical significance was assessed using either the Student's *t*-test or a two-way ANOVA for repeated measures followed by a Bonferroni post-test comparison where required. $P < 0.05$ was considered significant. All data are presented as mean ± SEM unless otherwise specified.

3. Results

3.1. Identification of CMPF as a Metabolite of Lovaza™ Supplementation

Arachidonic acid bioactive species and its more stable and polar metabolic products can be detected in urine. Examples of these include the leukotrienes, prostaglandins, thromboxanes, prostacyclins and isoprostanes, dinor and tetranor beta-oxidation products and cysteine and glucuronide adducts (Mesaros et al., 2009; Rockwell et al., 2016; Sasaki et al., 2015; Yan et al., 2010). Thus, to identify metabolites of Lovaza™ that may represent the active components mediating its beneficial effects, we sourced biospecimens from an open-label, single-arm study that enrolled 12 healthy volunteers for 25d of high-dose Lovaza™ (21 g/d; Fig. 1a) to allow for the identification of low abundance urinary metabolites. Volunteers with BMI < 25 were selected to minimize confounding effects of dyslipidemia on metabolism.

Untargeted metabolomics was performed on urine samples, a less complex matrix compared to plasma, collected from participants 7d

prior to initiation of supplementation (d-7) and at day 25 (d25). Of the 7186 features identified, the top 25 differentially abundant features between d-7 and d25 were selected as features of interest based on the lowest p-value, retention time window, highest fold change, and manual curation to remove isotopes and non-existent peaks (Fig. 1b). The feature with an m/z of 239.0916 had both the highest fold change (31.9) and lowest p-value (3.99×10^{-5}). Database searching prospectively identified the ion as CMPF, which was confirmed by comparing retention time and high-resolution mass spectrometry product ion spectra to the deuterated internal standard, CMPF-d_5 (Fig. 1c).

Closer analysis of the top 25 metabolites revealed the presence of CMPF product ions (Fig. 1c; single star in Fig. 1b), related furandicarboxylic acid metabolites (single star; Fig. 1d), the glucuronide of CMPF (m/z 415.1242; Fig. 1d; top metabolite in Fig. 1b) and glucuronide derivatives of CMPF-related furandicarboxylic acid metabolites. The newly detected furandicarboxylic acid metabolites displayed the sequential losses or additions of methyl or methanediyl groups (14 m/z) to the parent mass of CMPF (Supplementary Fig. 1a–d; two stars in Fig. 1b). Overall, furandicarboxyilic acids including CMPF and their glucuronides increased up to 100-fold after Lovaza™ supplementation (Fig. 1f). Corresponding glucuronides were characterized by high-resolution mass spectrometry and their identity further confirmed by incubation with β-glucuronidase to form unconjugated species (Supplementary Fig. 1).

CMPF levels were quantified in serum and urine throughout the study to evaluate its temporal formation (Fig. 1g). Serum levels of CMPF were significantly increased at d14 and remained elevated through d25. Interestingly, the concentration of CMPF in urine and serum did not return to baseline following the cessation of supplementation (+ d7, + d14). Furthermore, serum levels of CMPF at d25 were significantly elevated in men compared to women, suggesting sex-specific differences in CMPF generation and/or clearance (Fig. 1g, Supplementary Tables 1 and 2). Importantly, lipid analysis of Lovaza™ revealed that CMPF is not present in the prescription formulation and therefore is generated *de novo* from a precursor molecule found in Lovaza™ *in vivo* (Fig. 1h).

3.2. Clinical Dose Lovaza™ Supplementation Increases CMPF

A second study was initiated in which half the clinical dose of Lovaza™ (2 g/day) was administered to lean subjects for 40d to evaluate if CMPF is generated under standard pharmacological conditions (Fig. 2a). Similar to the high dose study, the increase in CMPF concentration was much greater in plasma than in urine at both d33 and d40 (Fig. 2b–e). After 40d of supplementation, subjects were randomized into 2 groups receiving either an increased dose of Lovaza™ (10 g/day) or 10 g/day of ω-6 rich safflower oil for 28d to monitor dose-dependent alterations in CMPF abundance and clearance (Supplementary Table 3). The 5-fold increase in Lovaza™ supplementation resulted in a corresponding 5-fold increase in plasma CMPF levels (Fig. 2b, Supplementary Table 4) and a 4-fold increase in urinary CMPF levels (Fig. 2d, Supplementary Table 5) on d56, suggesting dose-dependency. In contrast, the rise in plasma CMPF levels until d56 was less pronounced in those administered safflower oil (Fig. 2b–c, Supplementary Tables 4,6). While plasma CMPF levels did not return to baseline by the completion of the study, urinary levels did, suggesting continued generation or release of CMPF beyond the end of supplementation (Fig. 2, Supplementary Tables 4–7).

Fig. 1. Discovery and characterization of the Lovaza™ -derived metabolite CMPF. a) High-dose Lovaza™ (ω-3 FA prescription) study design (n = 12). b) Top 25 features (m/z/RT (min)) upregulated with high dose Lovaza™ administration. Single asterisk is a CMPF fragment and double asterisk are CMPF-related metabolite glucuronide species. Boxed is CMPF. c) Elution profile, HR-MS and HR-MS/MS of CMPF and structural comparison to the CMPF-d_5 standard. d) HR-MS characterization of CMPF-related metabolites. e) HR-MS and HR-MS/MS characterization of CMPF glucuronide conjugate. f) CMPF, CMPF-related metabolites and corresponding glucuronide conjugates increase after Lovaza™ supplementation. g) Serum and urine CMPF concentrations over the study time course, stratified by gender (n = 7 F, n = 5 M). h) Elution profile of CMPF-d_5 internal standard, serum CMPF pre- and post-Lovaza™ supplementation and absence of CMPF in the Lovaza™ pill, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All error bars SD. Complete statistical analysis can be found in Supplementary Tables 1 and 2.

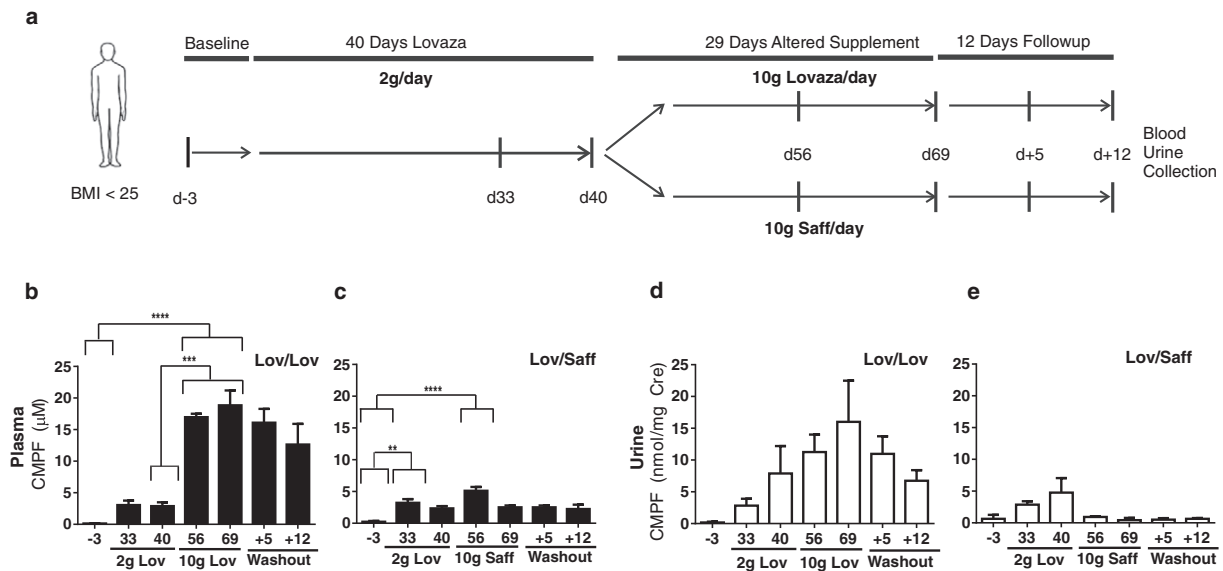**Fig. 2.** CMPF concentration dose dependently increases with pharmacological levels of prescription FO. a) Low dose Lovaza™ study design (n = 7) Plasma CMPF concentrations over the study time course with switch to b) high-dose Lovaza™ (10 g/day, n = 4) or c) switch to safflower oil supplementation (10 g/day, n = 3). Urine CMPF concentrations over the study time course with switch to (d) high-dose Lovaza™ and (e) switch to safflower oil supplementation. *P < 0.05, ** P < 0.01, ***P < 0.001, ****P < 0.0001. All error bars SEM. Complete statistical analysis can be found in Supplementary Tables 4–7.

3.3. CMPF Treatment Reverses Insulin Resistance and Steatosis in Obese Mice

Given the high concentration of CMPF achieved in circulation with Lovaza™ supplementation, we sought to determine if CMPF represents an active component of ω-3-acid ethyl ester treatment that contributes to its effects on whole-body metabolism and TG lowering. To determine the role of CMPF independently, we used two obese, insulin resistant mouse models and administered CMPF for 2 wks (Fig. 3a). In the diet-induced obese (DIO) model, 8 wk old male CD1 mice were fed a HFD for 6 wks to induce obesity and insulin resistance prior to CMPF treatment. The DIO mice were then treated for 2 wks with CMPF (6 mg/kg/day) or vehicle while maintained on the HFD, for a total of 8 wks HFD feeding. Additionally, 8 wk-old leptin-deficient male *ob*/*ob* mice were treated for 2 wks with either CMPF (6 mg/kg/day) or vehicle. At this dose of CMPF, plasma concentrations peaked at 10 min post-injection and declined thereafter, returning to baseline within 24 h of each injection (Fig. 3b). To compare the CMPF exposure in humans supplemented with Lovaza™ to levels achieved upon administration to mice, the respective area under the curve (AUC) were established. Ip injection of 6 mg/kg CMPF to mice (reported rat β half-life ($t_{1/2}β$) = 6 h) resulted in an exposure characterized by an AUC of 506 μM/h over 24 h, which is within the range of AUC levels observed in our clinical studies (910.8, 407.04, and 73.44 μM/h over 24 h for high (21 g/d), mid (10 g/d), and low-dose (2 g/d) Lovaza™ studies respectively).

CMPF treatment had no effect on body weight in either model (Fig. 3c,d). At the end of the injection period, DIO animals were placed in metabolic cages (CLAMS) and assessed for changes in whole-body metabolism. There were no differences in food or water consumption (Supplementary Fig. 2a,b), though DIO-CMPF mice had reduced activity during the light cycle (Fig. 3e). DIO-CMPF exhibited a significant reduction in RER compared to both DIO-Control and Chow-Control

mice (Fig. 3f), suggesting an increased reliance on fatty acids as their primary fuel source.

Despite no difference in body weight, both DIO-CMPF and *ob*/*ob*-CMPF animals exhibited improved insulin sensitivity compared to respective controls at the end of the treatment period (Fig. 3g,h). In fact, DIO-CMPF mice had comparable insulin sensitivity to Chow-Controls following only 2 wks of treatment (Fig. 3g). This difference in *in vivo* insulin sensitivity corresponded to significantly enhanced hepatic pAKT in response to an intravenous bolus of insulin as compared to DIO-controls (Fig. 3i). Interestingly, there was no difference in pAKT in the skeletal muscle samples, suggesting a specificity of the effect of CMPF for improving hepatic insulin sensitivity (Supplementary Fig. 2c). Consistent with this, and the effect of Lovaza™ supplementation in humans (Scorletti et al., 2014), CMPF treatment was associated with reductions in hepatic TG accumulation as determined by gross visualization and Oil red O staining in both DIO CD1 mice and *ob*/*ob* animals (Fig. 3j,k). Importantly, this was not accompanied by any signs of fibrosis or necrosis, as confirmed by histological examination. This is further corroborated by no evidence of hepatocellular injury or change in serum AST in either model compared to DIO and *ob*/*ob*-Controls respectively, as well as a reduction in ALT in DIO-CMPF compared to DIO-Controls (Fig. 3l). Quantification of hepatic TG levels showed DIO-CMPF treated animals were comparable to Chow-Controls and significantly improved compared to DIO-Controls (Fig. 3m). Furthermore, *ob*/*ob*-CMPF mice were also improved compared to controls (Fig. 3n).

3.4. Hepatic Lipid Metabolism is Altered with CMPF Treatment In Vivo and In Vitro

To evaluate the mechanism through which CMPF improves hepatic insulin sensitivity we performed global microarray and metabolomics analysis on livers isolated from DIO-CMPF mice and DIO-Controls at

Fig. 3. CMPF Reverses Steatosis and Improves Insulin Sensitivity in Obese Mice. a) Schematic of the treatment protocol for diet-induced obese (DIO) and *ob*/*ob* mouse treatment with CMPF for 2 wks. b) Plasma CMPF concentration following ip injection (n = 4–6). Change in body weight before and after injection period in (c) DIO (n = 8/group) and (d) *ob*/*ob* models (n = 8–14/group). e) Activity over 24 h period in x, y, and z planes (n = 2 for chow controls as reference; n = 6–8 for DIO groups). f) Respiratory exchange ratio (RER) calculated as VCO$_2$/VO$_2$ over 24 h period (n = 2 for chow controls as reference; n = 6–8 for DIO groups). Insulin tolerance tests in (g) DIO (n = 8/group) and (h) *ob*/*ob* (n = 4–7/group) models. (i) Western blots and quantification of *in vivo* insulin signaling in liver from DIO mice (n = 4/group). Representative liver images with Oil red O and H&E staining from (j) DIO (n = 8/group), and Oil red O staining from (k) *ob*/*ob* (n = 8–14/group) models. Scale bars for Oil red O are 50 μm, for H&E are 100 μm. l) Serum ALT and AST quantification (n = 4–5) and TG quantification in liver (n = 4–7) in (m) DIO and (n) *ob*/*ob* models. *P < 0.05, **P < 0.01, ***P < 0.001 All error bars ± SEM.
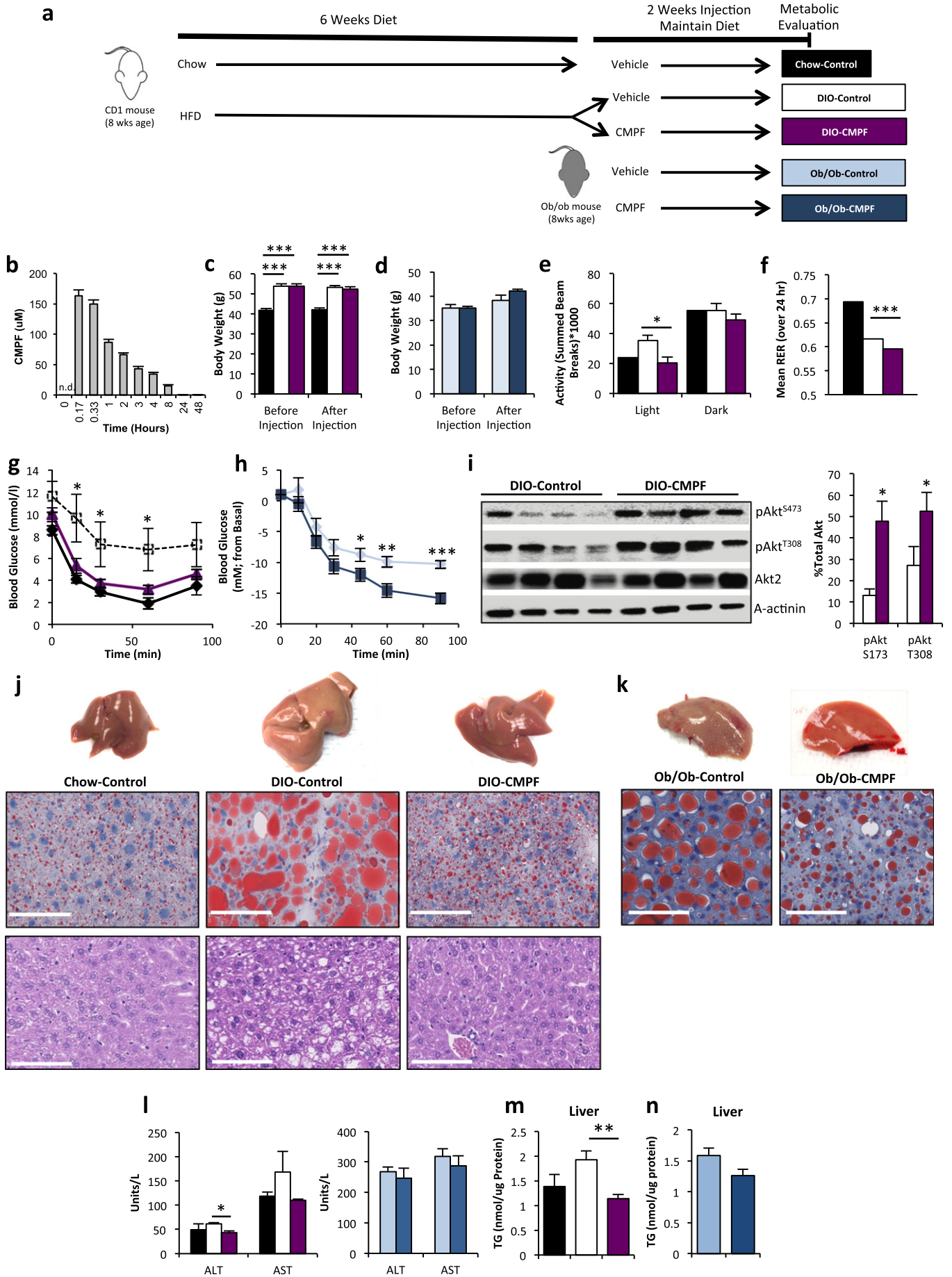
the end of the 2 wk treatment period. Consistent with reduced TG accumulation, and previous observations of the effect of CMPF in the islet (Prentice et al., 2014), the most significantly changed pathways were those regulating lipid biosynthesis and metabolism (Fig. 4a–c Supplementary Table 8). Microarray analysis revealed reduced expression of several genes involved in *de novo* lipogenesis and fatty acid elongation, including ELOVL1, ELOVL5, ELVOL6, and FADS2 (Fig. 4a, Supplementary Table 8). Consistent with reduced expression of these genes, metabolomics revealed significantly reduced levels of several long-chain fatty acids, and unsaturated lipid species that require these genes for their synthesis (Fig. 4b). Indeed, of the 304 metabolites quantified, 50 were significantly (P < 0.05) or showed a strong trend toward being changed (P < 0.10) (Fig. 4b), with the majority of significantly reduced metabolites being lipids. Interestingly, several carbohydrate and amino acid species were increased in the livers of DIO-CMPF mice as compared to DIO-Controls (Fig. 4b). Thus, CMPF could reduce steatosis through prevention of *de novo* lipogenesis, and altering substrate utilization to preferentially utilize lipids over carbohydrates, resulting in reduced TG accumulation.

Alterations in hepatic metabolism with CMPF treatment *in vivo* could be due to direct or indirect activity of CMPF on the liver. Therefore, we utilized primary hepatocytes from lean CD1 mice to examine the mechanism underlying the reduction in lipid accumulation with CMPF treatment. 24 h incubation with CMPF resulted in a significant increase in beta-oxidation (Fig. 4d), consistent with the *in vivo* alteration in this pathway. Specificity of the assay for measuring beta-oxidation was confirmed through co-treatment with the CPT1a inhibitor etomoxir, which blocked stimulation (Fig. 4d). TOFA (5-(tetradecyloxy)-2-furoic acid), an allosteric inhibitor of acetyl-CoA carboxylase (ACC) 1 and 2, the master regulatory proteins of beta-oxidation and lipogenesis, increased beta-oxidation in isolated hepatocytes (Fig. 4d). Based on the structural similarity between TOFA and CMPF, and their common effect on fatty acid utilization, we hypothesized that CMPF may act through a related mechanism, *via* ACC inhibition (Supplementary Fig. 3). 24 h treatment with either CMPF or TOFA stimulated beta-oxidation in the absence of an increase in the activating phosphorylation of ACC (Fig. 4e), suggesting direct inhibition. This is further supported by modulation of AMPK activity with the inhibitor Compound C, or the activator AICAR, where the effect of CMPF remained uninfluenced by AMPK manipulation, as evidenced by no change in the fold increase over control with CMPF treatment (Fig. 4f). Direct biochemical assessment of CMPF on ACC1 and 2 activities revealed a significant 54.2% reduction in ACC1 activity and 8.4% reduction in ACC2 (Fig. 4g) with ACC inhibitor CP-640186 used as a positive control. Thus, CMPF may act through ACC inhibition to reduce lipid synthesis and enhance metabolism, contributing to the reversal of steatosis.

3.5. CMPF Treatment Causes Long-Term Alterations in Fat Deposition and Utilization

Given the direct role of CMPF in enhancing beta-oxidation and reducing lipid biosynthesis, we next wanted to investigate whether CMPF could prevent the development of steatosis and insulin resistance. To test this, 8 wk old male CD1 mice were treated for 7 days with 6 mg/kg/day CMPF, as previously described. Mice were then placed on a HFD for 4–5 wks to induce steatosis and insulin resistance (Fig. 5a). No differences were observed in body weight throughout the injection period (Fig. 5b). Interestingly, however, the CMPF-HFD group gained less weight on HFD over time than the Control-HFD group, a difference that was significant by the end of the observational period (Fig. 5b). This did not correlate with significant differences in food or water consumption at any point during the study (over 48 h at the end of diet period shown; Supplementary Fig. 4a,b). To determine if the difference in body weight was due to altered body composition, mice underwent MRI analyses (Fig. 5c). CMPF-HFD mice had significantly lower total adipose area than Control-HFD mice due to a significant reduction in

subcutaneous adipose area (Fig. 5d). Despite this reduction, there were no differences in adiponectin or free fatty acid levels compared to Control-HFD mice, with a small decrease in circulating leptin levels (Fig. 5e–g). Mice were placed in metabolic CLAMS for 48 h to further explore these metabolic differences. CMPF-HFD mice had activity levels comparable to the Control-Chow animals during the light phase, which was significantly greater than Control-HFD mice (Supplementary Fig. 4c). Interestingly, the CMPF-HFD cohort also exhibited a significantly reduced RER over a 24 h period when compared to both controls, suggesting preferential utilization of fatty acids over carbohydrates (Supplementary Fig. 4d), as observed in the obese models previously (Fig. 3f).

3.6. CMPF Prevents High-Fat Diet-Induced Steatosis and Insulin Resistance

Importantly, and consistent with the effects of Lovaza™ supplementation in humans, CMPF-HFD mice had significantly lower hepatic TG levels compared to Control-HFD animals (Fig. 5h). Strikingly, livers from the CMPF-HFD mice appeared nearly identical to Control-Chow livers, with no evidence of fibrosis or inflammation in H&E stained sections (Fig. 5i,j). Reduced lipid deposition was confirmed by Oil red O staining, which revealed little intracellular lipid accumulation in the CMPF-HFD animals (Fig. 5j). CMPF-HFD mice were remarkably protected against HFD-induced insulin resistance during IpITT (Fig. 5k). Indeed, the rate of glucose clearance was equal between the Control-Chow and CMPF-HFD groups. To determine if this effect of CMPF is specific or a general effect of furan dicarboxylic acids, we utilized 2,5-furandicarboxylic acid (FDCA) as a control according to the same protocol. FDCA-HFD mice had no difference in insulin sensitivity compared to Control-HFD animals, implying that insulin sensitization is not common among this class of molecules (Fig. 5l). To further quantify the improvement in insulin sensitivity, tissues were isolated from mice following an intravenous bolus injection of insulin, and insulin signaling was evaluated. Enhanced Akt phosphorylation was observed in both liver and skeletal muscle of the CMPF-HFD mice compared to Control-HFD mice (Fig. 5m, Supplementary Fig. 4h), consistent with improved insulin sensitivity. Importantly, there was no difference in serum ALT concentration (Fig. 5n, Supplementary Fig. 4e), though a significant reduction in serum AST in CMPF-HFD mice compared to Control-HFD animals (Fig. 5n), suggesting the reduction in hepatic TG accumulation was not related to liver injury. Furthermore, CMPF treatment was associated with a significant reduction in hepatic TUNEL positive staining compared to Control-HFD (Supplementary Fig. 4f), though no difference in TUNEL from subcutaneous fat sections, suggesting that reduced adiposity is not due to increased cell death (Supplementary Fig. 4g).

3.7. Altered Hepatic Lipid Metabolism Persists Following CMPF Clearance from the Liver and Circulation

CMPF accumulates in the liver rapidly following ip administration, peaking within 2 h of injection (Supplementary Fig. 4i). Notably, however, within 24 h there is no longer any detectable CMPF in either the liver or in the circulation (Fig. 3b, Supplementary Fig. 4i). Thus, while direct ACC inhibition by CMPF may contribute to early increases in beta-oxidation during the injection period, CMPF must induce long-term changes to the liver to provide persistent protection against HFD feeding. To investigate this mechanism, we performed microarray analysis on livers from CMPF-HFD and Control-HFD mice at the end of the 4 wk diet period. Of note, there were significant reductions in both ACC1 and 2 and Scd1, with increased expression of Cpt1a, supporting an increase in beta-oxidation and reduced lipogenesis, as observed in the acute treatment model (Fig. 6a,b). Furthermore, CMPF treatment was associated with a reduction in expression of genes required for glucose utilization including pyruvate kinase (*pklr*), glucokinase (*gck*), and glucose-6-phosphatase (*g6pc*) (Fig. 6a,b). Given the potential role for ACC1 and 2 inhibition in the acute effect of CMPF treatment by both
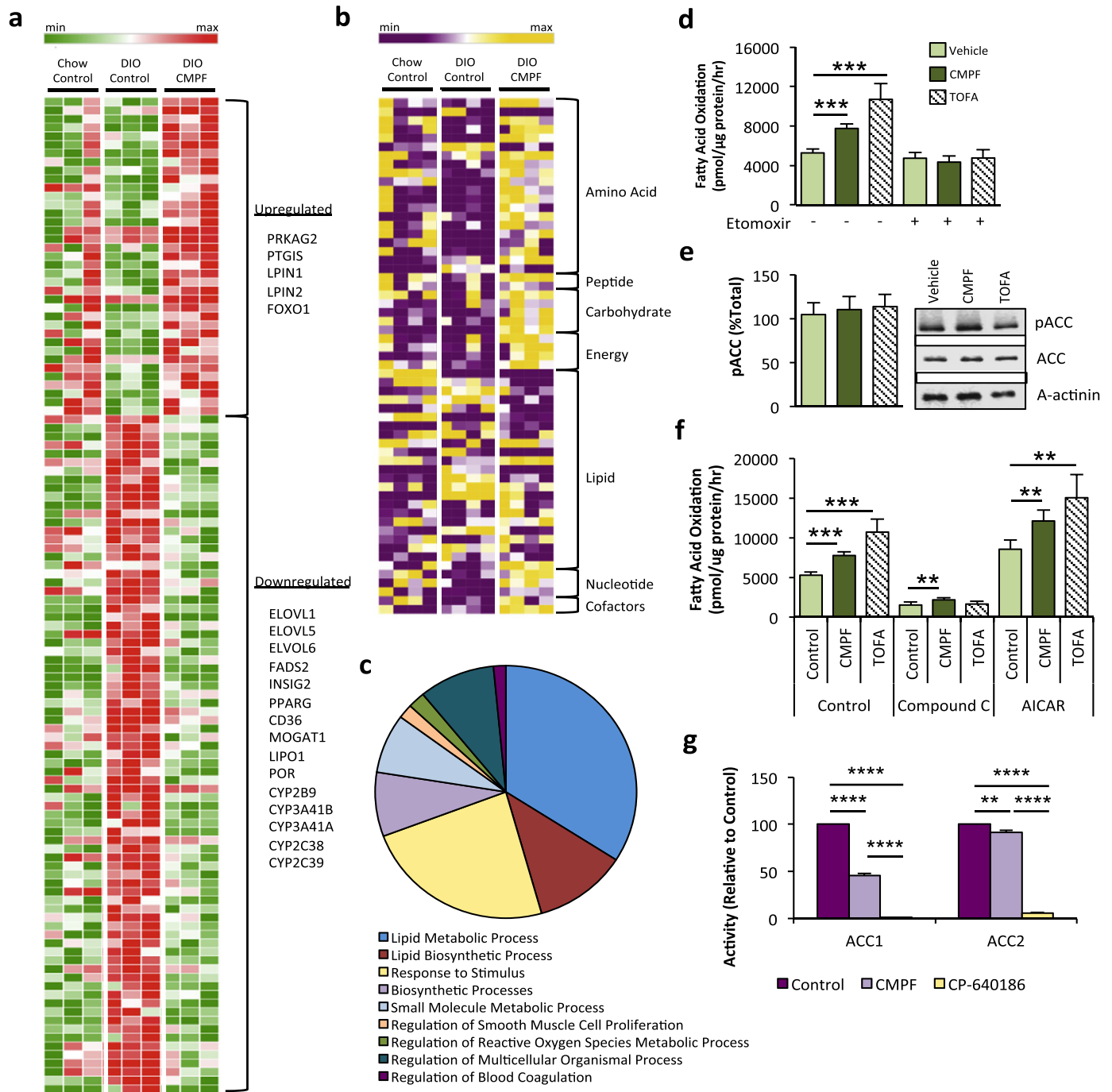
Fig. 4. CMPF treatment alters hepatic lipid metabolism. a) Significantly altered genes in livers from DIO-CMPF mice as compared to DIO-Controls measured by microarray analysis (n = 3/group). b) Significantly altered metabolites in livers from DIO-CMPF mice as compared to DIO-Controls clustered by metabolite super pathway (n = 5/group). c) Significantly altered genes by microarray analysis clustered by gene ontology (n = 3/group). d) Fatty acid oxidation per hr. in isolated hepatocytes following 24 h treatment with CMPF or ACC-inhibitor TOFA, and blockage with etomoxir co-treatment (n = 4/group). e) Western blot and quantification of ACC phosphorylation in isolated hepatocytes treated for 24 h with CMPF or TOFA (n = 3/group). f) Fatty acid oxidation rate in isolated hepatocytes treated for 24 h with AMPK activation and inhibition (n = 3/group). g) Activity assay of ACC1 and 2 with CMPF treatment or CP-640186 as control (n = 3/group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All error bars SEM.

expression and biochemical inhibition, we further evaluated ACC abundance in livers from CMPF-HFD and Control-HFD mice. There was a striking reduction in total ACC abundance with CMPF treatment, resulting in a relative increase in phosphorylated ACC, suggesting inhibition of existing protein (Fig. 6c). This suggests CMPF may be potentiating lipid metabolism through persistent inactivation of this pathway. Interestingly, both induction of beta-oxidation and treatment with ω-

3 FA are known to reduce expression of SREBP1c, downstream of mTOR, activating a feedback loop to further decrease expression of ACC 1 and 2 (Georgiadi and Kersten, 2012). Importantly, pathway analysis of the microarray data demonstrates a significant alteration in the mTOR pathway in CMPF-HFD animals, and SREBP1c expression, particularly the active nuclear fraction, is also reduced with CMPF treatment (Fig. 6a,b,d,e).

FGF21 is known to attenuate hepatic steatosis through regulation of lipogenesis and increasing beta-oxidation, with downstream targets including SREBP1c and ACC1 and 2 (Fisher et al., 2011). Expression of FGF21 was found to be strongly induced in CMPF-HFD livers compared to controls (Fig. 6a,b). This was further validated in the plasma, where circulating FGF21 was significantly elevated in CMPF-HFD mice (Fig. 6f). Closer examination of the gene profiling revealed the anticipated directions of change associated with increased FGF21 activity including potent changes in key FGF21 target genes such as increased expression of Lepr, PCK1 and CPT1a, and decreased Scd1, SREBP1c, ACC 1 and 2, Gck, and Cyp7a1 (Fig. 6a,b). Therefore, FGF21 is likely a key regulator of increased beta-oxidation and diminished lipogenesis in the livers of CMPF-HFD mice.

To determine when FGF21 expression is increased with CMPF treatment, we performed a time-course experiment during the CMPF treatment period with FDCA as a control. After three days of injections, plasma levels of FGF21 were significantly increased with CMPF compared to both vehicle and FDCA controls, and remained elevated at least one week after the last CMPF injection (Fig. 6g). To confirm that FGF21 is coming from the liver, and associated with CMPF treatment, we examined hepatic FGF21 mRNA following 24 h *in vitro* and 7d *in vivo* CMPF treatment. In both conditions FGF21 was significantly increased (Fig. 6h,i). Importantly, expression of key genes regulating lipogenesis and glucose metabolism including ACC 1 and 2 and Gck were also significantly reduced following 7d of CMPF injection before HFD, indicating that these alterations in gene expression are the direct result of CMPF treatment, and not a consequence of exposure to the HFD (Fig. 6i).

3.8. FGF21KO Mice are Resistant to CMPF Treatment

To confirm FGF21 is necessary for the long-term prevention of steatosis, we administered CMPF to 6-9 wk old male mice with global deletion of FGF21 (FGF21KO) and age-matched C57bl/6 controls (WT) for 7d, followed by 6 wks of HFD feeding to induce insulin resistance (outlined in Fig. 7a). Interestingly, both WT-HFD and FGF21KO-HFD treated with CMPF gained less weight than controls, suggesting the difference in body weight is FGF21 independent (Fig. 7b). At the end of the protocol insulin sensitivity was evaluated. Consistent with what was observed in the CD1 mice, WT-CMPF-HFD C57bl/6 mice had significantly improved insulin sensitivity compared to HFD controls (Fig. 7c). Paradoxically, however, FGF21KO-CMPF-HFD mice had poorer insulin sensitivity (Fig. 7c), which corresponded to both worsening of fasting hyperinsulinemia (Fig. 7d) and no difference in hepatic TG content in FGF21KO-CMPF-HFD mice compared to FGF21KO-HFD controls (Fig. 7e,f). CMPF treatment significantly reduced TG content in HFD-fed WT control mice, as observed previously in the CD1 strain (Fig. 7e,f). These observations indicate that the long-term protection against steatosis and insulin resistance induced by CMPF occurs through an FGF21-dependent mechanism in this model.

4. Discussion

Our studies propose CMPF as bioactive metabolite formed *de novo* from prescription fish oil. Targeted mass spectrometric identification shows definitive dose-dependent formation in healthy volunteers with a corresponding renal elimination profile. This confirms earlier observations made in patients with GDM and T2D that elevated formation of CMPF correlates with ω-3-fatty acids levels (Prentice et al., 2014). The identification of a CMPF glucuronide is also consistent with previous

findings demonstrating the presence of a CMPF glucuronide in mice following CMPF administration, suggesting hepatic processing of CMPF is conserved among species (Nagy et al., 2017). Notably, CMPF disposition consistently exhibits sex specific differences, which adds a new perspective to the sexual dimorphic effects reported for fish oil supplements (Lohner et al., 2013). Despite the limited sampling to define the pharmacokinetic profile of CMPF disposition, our data suggest that CMPF is bioavailable long after cessation of Lovaza™ supplementation in humans, likely reflecting the enrichment of cell membranes with precursors. For the clinical setting, however, other factors, such as status of organic anion transporters (OATs) responsible for CMPF clearance (Deguchi et al., 2004; Prentice et al., 2014) or the high affinity binding of CMPF to albumin (Sakai et al., 1995), might need to be considered. Studies with labeled precursor fish oils can address this further *in vivo*.

Treating obese, insulin-resistant mice with CMPF at doses comparable to the levels observed in humans after Lovaza™ supplementation improved systemic, and notably hepatic, insulin activity. We find that many of the lipid-lowering effects of Lovaza™ supplementation are mimicked by those of CMPF treatment including reduced hepatic lipids, particularly triglycerides, and altered ACC activity (Alwayn et al., 2005a; Alwayn et al., 2005b; Woodman et al., 2002), the latter suggesting a decrease in *de novo* lipogenesis and increased fatty acid oxidation. While ω-3 FA intake strongly correlates with reduced plasma TG levels (Alwayn et al., 2005a; Alwayn et al., 2005b), many studies have shown relatively weak or no correlation between absolute serum EPA and DHA concentrations and serum TG (Bonaa et al., 1992a, 1992b; Friday et al., 1989; Zheng et al., 2016). CMPF, in contrast, might provide a stronger predictive relationship to TG levels in humans, a hypothesis strongly supported by observations made in two recent studies in Chinese patients with type 2 diabetes which demonstrate a significant negative correlation between CMPF and plasma TG (Zhang et al., 2017; Zheng et al., 2016). This relationship warrants closer examination as previous studies suggest that CMPF is not derived directly from the ω-3 FA in fish oil supplements, but instead from furan fatty acids (F-acid) (Nagy et al., 2017). F-acids are acquired from numerous sources, notably the liver and testis of fish, and are known to be present in fish oil supplements (Vetter et al., 2012). This raises the possibility that different fish oil-derived supplements have different levels or types of F-acids, making it imperative to control for them in animal preclinical work and human clinical trials. In this regard, the characterization and quantification of F-acid levels in Lovaza™ and other ω-3 FA supplements remain an important avenue of future research.

The role of ω-3 FAs in glycemic control remains controversial. ω-3 FA supplementation in non-diabetic individuals has been shown to increase glycemia, decrease insulinemia following glucose load, and reduce whole-body carbohydrate utilization while increasing beta-oxidation (Delarue et al., 1996). Similar effects have also been observed in diabetic populations, including increases in fasting blood glucose (reviewed in (Chen et al., 2015; Friedberg et al., 1998)). Interestingly, many studies report no significant effect on HbA1c, circulating insulin levels, or BMI (Chen et al., 2015; Friedberg et al., 1998). However, recent studies have shown alterations in body composition associated with supplementation including decreased fat mass (Noreen et al., 2010). Importantly, in both human and rodent studies, ω-3 FA supplementation is associated with significant decreases in hepatic fat accumulation, though effects on markers of fibrosis and liver damage, as measured by serum ALT and AST are less clear. Many studies observe significant improvements in AST, without impacting ALT (Parker et al., 2012), thus the effect of ω-3 FA supplementation is likely beneficial to early

Fig. 5. Acute treatment with CMPF induces persistent changes in whole-body metabolism. a) Schematic of the treatment protocol with initial CMPF administration followed by high fat diet (HFD). b) Weekly weight gain through injection period and following placement on HFD (n = 20/group). c) MRI scan images and (d) quantification of fat distribution (n = 4/group). e) Fasting plasma leptin, (f) adiponectin, and (g) free fatty acid (FFA) levels 4 wks following final injection (n = 8/group). h) Hepatic TG levels 4 wks following final injection (n = 8/group). i) Representative liver morphology of isolated livers from mice 4 wks following final injection (n = 8/group). j) H&E and Oil red O staining of liver sections (n = 8/group; scale bars: top 100 μm, middle 200 μm, H&E 100 μm). k) Blood glucose levels during Ip insulin tolerance test 4 wks following final CMPF injection (n = 8/group) or (l) 2, 5-Furandicarboxylic acid (FDCA) (n = 4/group). (m) Western blots and quantification of *in vivo* insulin signaling in liver (n = 3/group). n) Serum ALT and AST concentration at the end of the diet period (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.001. All error bars SEM.
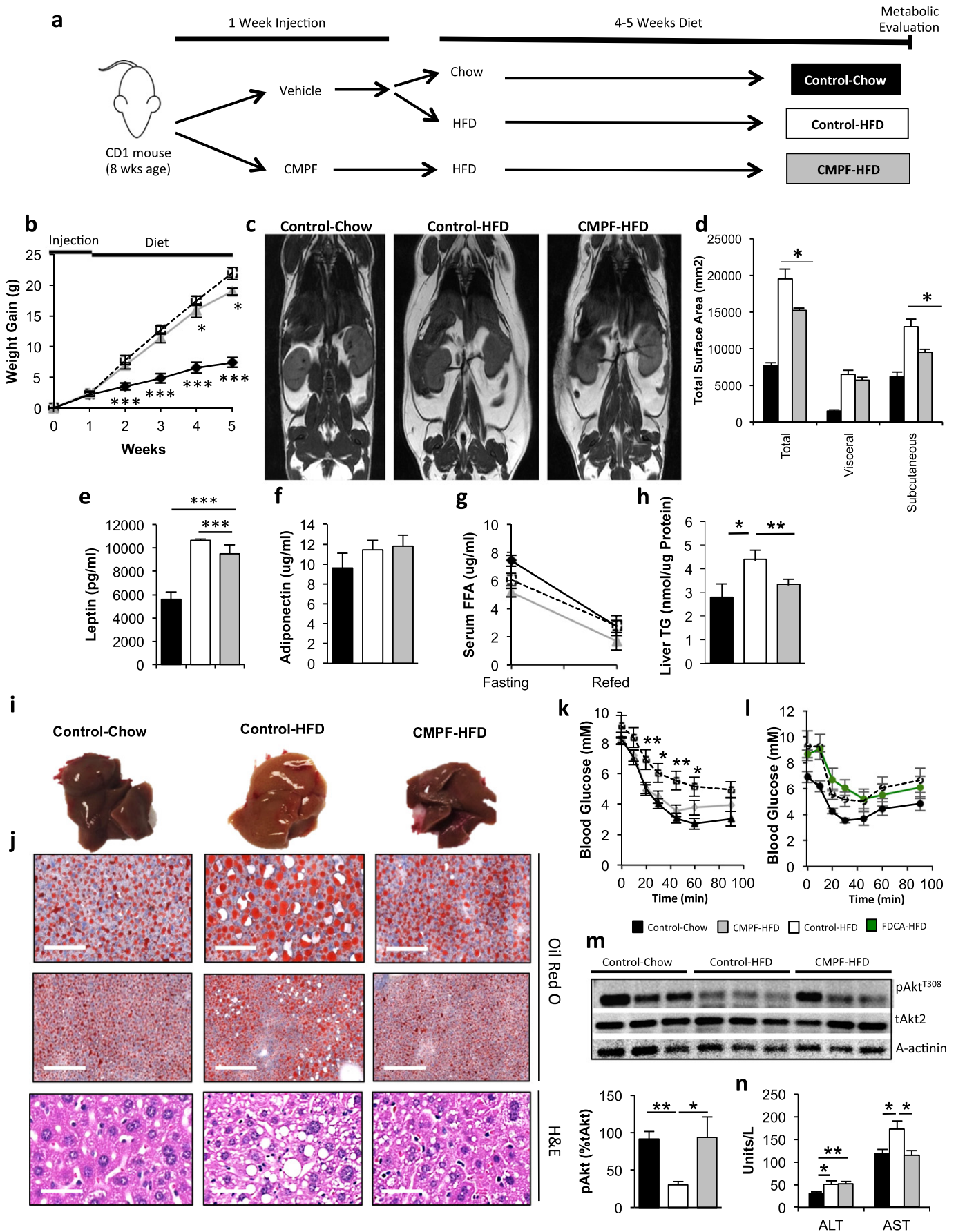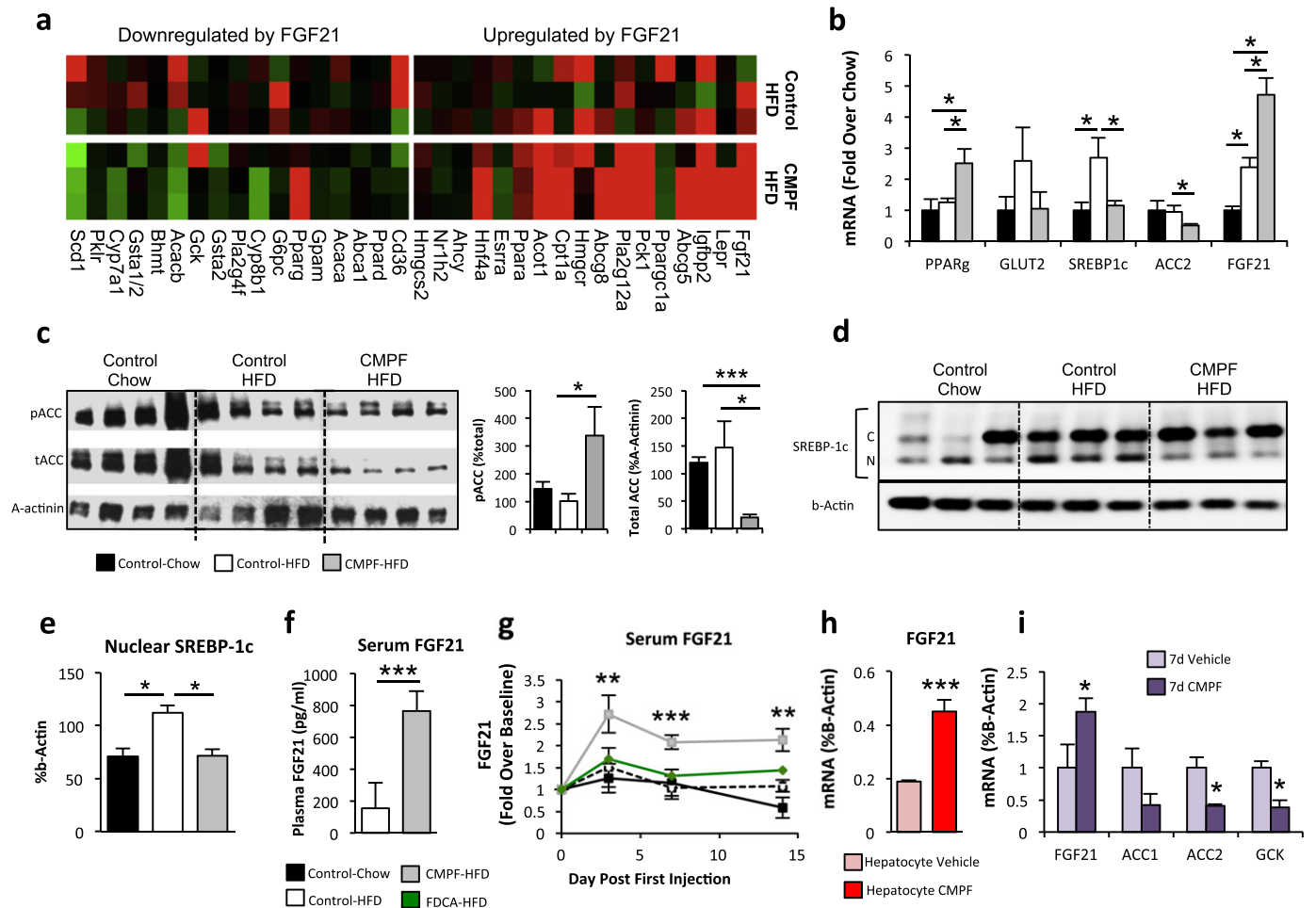
Fig. 6. Persistent effect of CMPF is associated with decreased ACC abundance and increased FGF21 abundance. a) Heat map showing significantly altered genes associated with FGF21 activity in livers from mice isolated 4 wks following treatment as determined by microarray (n = 3/group). Red increased, green decreased. b) Quantitative PCR validation of differential gene expression (n = 8/group). c) Western blot showing total ACC1/2 and pACC1/2 expression in livers from Control-Chow, Control-HFD, and CMPF-HFD mice (n = 4/group). d) Cytosolic (C) and nuclear (N) SREBP1c levels in livers from Control-Chow, Control-HFD, and CMPF-HFD mice (n = 3/group), and (e) quantification. f) FGF21 levels in serum from mice following 4 wks of dietary intervention (n = 8/group). g) Serum FGF21 levels in the days following first injection of CMPF, vehicle, or 2,5-Furandicarboxylic acid (n = 4/group). h) FGF21 mRNA expression isolated hepatocytes treated for 24 h with CMPF (n = 4/group). i) FGF21 and target gene mRNA expression in liver isolated from mice following 7 days of treatment (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.001. All error bars SEM.

stages of steatosis and NAFLD, with less impact through NASH progression. This correlates with the observed effects of CMPF treatment on reducing hepatic lipid accumulation, with minor increases in fasting blood glucose, no change in fasting plasma insulin, decreased serum AST, and decreased fat mass.

We found that CMPF induces FGF21, an endocrine member of the fibroblast growth factor family. FGF21 expression is normally elevated under fasting or nutrient restrictive conditions (Galman et al., 2008). Treatment with CMPF may mimic this status in the liver of chow-fed mice through inhibition of ACC 1 and 2 and the subsequent increase in beta-oxidation, the primary fuel source during fasting, as well as by inhibiting glucose utilization. Indeed, the underlying mechanism of CMPF action in all tissue types we have examined appears to be *via* the induction of fatty acid metabolism while reducing glucose

utilization (Liu et al., 2016; Prentice et al., 2014). Importantly, this "fasting-like state" is induced directly, rapidly, and independently of FGF21, as increases in fatty acid oxidation are observed during *in vitro* treatment of isolated hepatocytes from FGF21KO animals (data not shown). This potent stimulation of beta-oxidation is likely to stimulate FGF21 expression and secretion. Interestingly, FGF21 is also known to be elevated in the serum of patients with prediabetes and T2D, which is consistent with periods of elevated CMPF (Chen et al., 2011; Liu et al., 2016; Zhang et al., 2017). Increased FGF21, combined with a persistent drive toward beta-oxidation induced by CMPF over the 7-day treatment period may activate a feedback loop that continues to perpetuate after CMPF is eliminated (Fig. 7g). FGF21 activates AMPK, resulting in inhibition of SREBP1c, and reduced expression of ACC 1 and 2 (Potthoff et al., 2009). These enzymes are rate-limiting for the production of

Fig. 7. FGF21 knockout mice are resistant to CMPF-mediated improvements in insulin resistance and TG accumulation. a) Schematic of the treatment protocol with initial CMPF administration followed by high fat diet (HFD) in FGF21KO mice and c57 controls (WT). b) Weight gain in FGF21KO and WT controls through the injection and follow up periods (n = 4–7/group). c) Blood glucose during IpITT and (d) plasma insulin following 14 h fast (n = 4–7/group). e) Representative photographs and Oil red O staining of liver sections (n = 10–13/group) and (f) quantification of liver TG content (n = 10–13/group). g) Proposed model for the mechanism of CMPF action in liver under acute and long-term recovery conditions. *P < 0.05, **P < 0.01, ***P < 0.001. In panel (c) * indicates significance between Chow and HFD controls, # indicates significant between CMPF-HFD and Control-HFD groups. All error bars SEM.
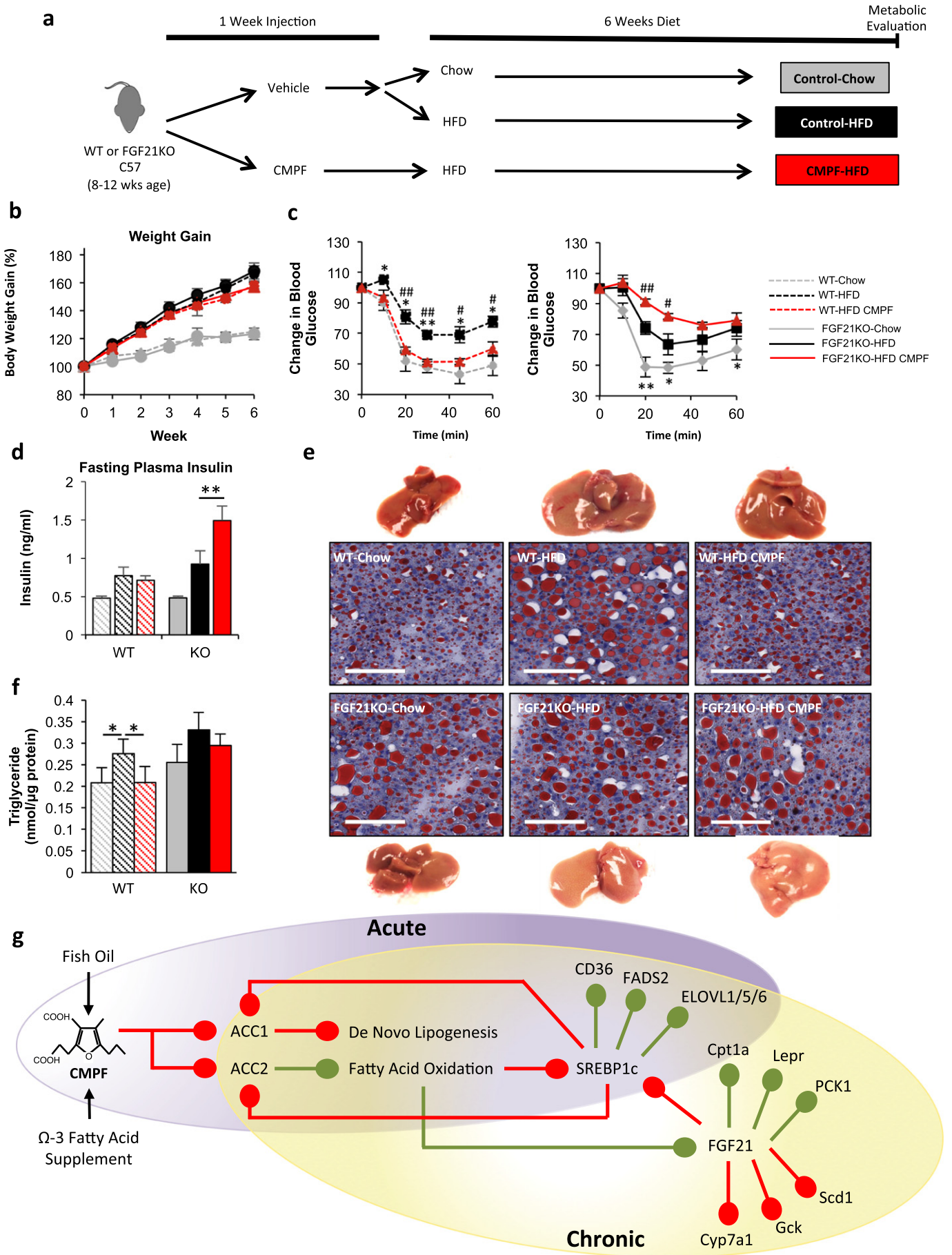
malonyl-CoA, which is required for both TG synthesis, as well as inhibition of beta-oxidation (Abu-Elheiga et al., 2001). Livers isolated from mice treated with CMPF have a significant reduction in total ACC and SREBP1c levels, consistent with this hypothesis. Thus, CMPF mediates reduction and prevention of steatosis through ACC-dependent mechanisms; acutely through direct inhibition, and chronically through induction of FGF21 resulting in a loss of SREBP1c and ACC expression.

It is possible that the effect of CMPF is concentration-dependent, with moderate levels being beneficial for the liver and systemic metabolism via induction of fatty acid oxidation and FGF21, whereas chronically high levels are detrimental to beta cells, contributing to diabetes development (Prentice et al., 2014). This is supported by observations that the improvement in insulin sensitivity occurs prior to the onset of beta cell dysfunction in obese mice treated acutely with CMPF (Liu et al., 2016; Prentice et al., 2014). Furthermore, this hypothesis is consistent with human observations, which interestingly demonstrate correlations between elevated CMPF and beta cell dysfunction together with decreased circulating TG (Liu et al., 2016; Zhang et al., 2017; Zheng et al., 2016). Overall, these results suggest that high-dose prescription ω-3 FA supplementation, while beneficial for the prevention of steatosis, may be deleterious for those with T2D due to impaired beta cell function. Furthermore, it could relate to literature indicating that prescription ω-3 FA supplementation in T2D results in worsened fasting blood glucose (Djousse et al., 2011; Glauber et al., 1988; Woodman et al., 2002).

Conflicts of Interest

ACA, TBD, CDH, and KWS are employees of Eli Lilly and Company and may own company stock or possess stock options. These authors report no other potential conflicts of interest relevant to this article. All other authors have no conflicts of interest to declare.

Author Contributions

KJP, SGW, YL, FJS, CS, and MBW designed the study. KJP and SGW researched the data and wrote/edited the manuscript. YL, JAE, SRS, ACA, XSW, DW, HM, SLB, TBD, and CDH researched the data and reviewed/edited the manuscript. GAF and KWS reviewed/edited the manuscript and contributed to the discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2017.12.019.

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