Received: 24 May 2018 | Revised: 24 July 2018 | Accepted: 26 July 2018

DOI: 10.1111/dom.13483

WILEY

ORIGINAL ARTICLE

3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) prevents high fat diet-induced insulin resistance via maintenance of hepatic lipid homeostasis

Haneesha Mohan PhD[1*] | Sydney L. Brandt MSc[1*] | Ja Hyun Kim MSc[1] | Frances Wong Hon. BSc[1] | Mi Lai PhD[2] | Kacey J. Prentice PhD[1] | Dana Al Rijjal Hon. BSc[1] | Lilia Magomedova PhD[3] | Battsetseg Batchuluun PhD[1] | Elena Burdett MSc[1] | Alpana Bhattacharjee PhD[1] | Carolyn L. Cummins PhD[3] | Denise D. Belsham PhD[1] | Brian Cox PhD[1] | Ying Liu PhD[2†] | Michael B. Wheeler PhD[1,2†]

[1]Department of Physiology, University of Toronto, Toronto, Canada

[2]Department of Advanced Diagnostics, Toronto General Hospital Research Institute, University Health Network, Toronto, Canada

[3]Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Canada

Correspondence
Michael B. Wheeler PhD, 1 King's College Circle, Medical Science Building, Room: 3352, Toronto, Ontario, M5S 1A8, Canada.
Email: michael.wheeler@utoronto.ca

Funding information
This study was funded by a CIHR operating grant to M. B. W. (FDN-143219). H. M. and S. L. B. were supported by a BBDC Postdoctoral Fellowship and a BBDC Graduate Studentship, respectively. A CIHR Doctoral Research Award supported K. J. P. Some of the equipment used in this study was supported by the 3D (Diet, Digestive Tract and Disease) Centre, funded by the Canadian Foundation for Innovation and the Ontario Research Fund, project numbers 19442 and 30961, respectively.

Aim: Omega-3 fatty acid ethyl ester supplements, available by prescription, are common in the treatment of dyslipidaemia in humans. Recent studies show that 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), a metabolite formed from fish oil supplementation, was able to prevent and reverse high fat diet (HFD)-induced fatty liver in mice. In the present study, we investigated the underlying molecular mechanisms responsible for CMPF's hepatic lipid-lowering effects.

Materials and Methods: CD1 male mice were i.p. injected with CMPF (dosage, 6 mg/kg) for 7 days, followed by 5 weeks of a 60% HFD to induce a fatty liver phenotype. Metabolic parameters, liver morphology, lipid content, protein expression and microarray analysis were assessed. We also utilized primary hepatocytes, an in vitro model, to further investigate the direct effects of CMPF on hepatic lipid utilization and biosynthesis.

Results: CMPF-treated mice display enhanced hepatic lipid clearance while hepatic lipid storage is prevented, thereby protecting against liver lipid accumulation and development of HFD-induced hepatic insulin resistance. Mechanistically, as CMPF enters the liver, it acts as an allosteric acetyl-coA carboxylase (ACC) inhibitor, which directly induces both fatty acid oxidation and hepatic production of fibroblast growth factor 21 (FGF21). A feed-back loop is initiated by CMPF, which exists between ACC inhibition, fatty acid oxidation and production of FGF21. As a consequence, an adaptive decrease in Insig2/SREBP-1c/FAS protein expression results in priming of the liver to prevent a HFD-induced fatty liver phenotype.

Conclusion: CMPF is a potential driver of hepatic lipid metabolism, preventing diet-induced hepatic lipid deposition and insulin resistance in the long term.

KEYWORDS

CMPF, fatty acid oxidation, fatty liver, FGF21, lipid

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of disorders characterized by an impairment of lipid metabolism within the liver, resulting in excessive accumulation of intrahepatic lipid content.[1] Within the western world, approximately 30% of the general population is affected by NAFLD.[2] There are two stages in the pathological development of NAFLD, the earlier fatty liver stage and the later non-alcoholic steatohepatitis (NASH) stage. Increased fat accumulation within the hepatocytes, with little or no inflammation or liver cell damage, can be observed in the fatty liver stage of NAFLD. In

*Co-first author

†Ying Liu and Michael B. Wheeler contributed equally to this study.

contrast, the hepatocellular injury that is associated with the NASH stage of NAFLD, which includes inflammation, liver cell damage, fibrosis and scarring, puts an individual at high risk of developing liver cirrhosis and eventual liver failure.[3] Importantly, the presence of NAFLD contributes to the development of hepatic insulin resistance, leading to impaired glucose homeostasis and, ultimately, promoting the pathogenesis of type 2 diabetes (T2D).[4,5] At this time, there are no official treatments designed specifically to reduce hepatic fat accumulation,[6,7] apart from the emerging use of fish oil supplementation. Fish oils are often prescribed to reduce hyperlipidaemia, a condition often seen in obese individuals.[8]

In a very recent study, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) was shown to be a major metabolic by-product resulting from the consumption of Lovaza, (GlaxoSmithKline, Research Triangle Park, NC, U.S.A) a fish oil (FO) supplementation available by prescription.[9] Despite its previously reported negative effects on pancreatic beta cell function,[10,11] CMPF was found to reverse and prevent high fat diet (HFD)-induced hepatic steatosis in mouse models.[9] This observation is in line with the reported beneficial effects of Lovaza on regulation of lipid profiles in humans,[12,13] suggesting that CMPF may be an active component of FO, contributing to the beneficial metabolic effects seen in humans. Interestingly, human studies have also revealed a strong correlation between elevated circulating CMPF and reduced plasma TG levels, associating CMPF with a role in regulating glycolipid metabolism.[14–16]

Mechanistically, CMPF was found to be an inhibitor of acetyl-coA carboxylase (ACC), the master regulator of fatty acid metabolism, as well as having the ability to induce the production of fibroblast growth factor 21 (FGF21).[9] CMPF's long-term preventive effect on HFD-induced hepatic lipid accumulation is absent in FGF21KO mice, suggesting that the long-term beneficial effect of CMPF on metabolism is FGF21 dependent.[9] Whether this observed hepatic lipid-lowering effect is the result of CMPF's direct action on hepatocytes or a result of its indirect effects conveyed through other metabolically responsive tissues remains to be determined. In addition, acute CMPF treatment (2 weeks treatment via i.p. injection) reduced intrahepatic lipid accumulation in diet-induced obese (DIO) and ob/ob mouse models without an elevation in circulating FGF21, suggesting that additional intrahepatic mechanisms exist and are yet to be identified.[9] In the present study we investigated the underlying molecular mechanisms that are responsible for CMPF's intrahepatic lipid-lowering effects and its ability to prevent NAFLD-associated fatty liver development by employing both in vitro and in vivo mouse models.

2 | MATERIALS AND METHODS

2.1 | Animal study design

All animal procedures were approved by the Animal Care Committee at the University of Toronto and were undertaken according to guidelines of the Canadian Council of Animal Care. Male CD1 mice (7 weeks) were utilized for all in vivo studies (Charles River USA). Male C57BL/6 mice (7-9 weeks) were utilized for all in vitro studies (Jackson Laboratories USA). For the chronic model, mice received intraperitoneal injections of sterile saline containing either vehicle (70% ethanol) or CMPF at a concentration of 6 mg/kg for 7 days, while a standard CHOW diet was maintained. Following the final injection, mice received either a diet with 60% kcal from fat or a sucrose-matched control diet for an additional 5 weeks. Further details concerning the diet are provided in Appendix S1.

2.2 | In vivo and in vitro assessments

We evaluated body weight and blood parameters and we performed three metabolic tests, glucose, insulin and lipid tolerance. MRI was performed to examine body fat distribution. Liver lipid content and protein expression were also quantified. The direct effects of CMPF on hepatocyte function in vitro was conducted to measure fatty acid uptake and oxidation, lipogenesis, glucose uptake, gluconeogenesis, and FGF21 expression and secretion. More information concerning detailed methods can be found in Appendix S1.

2.3 | Microarray

Total RNA from isolated murine livers was processed for microarray analysis at the Princess Margaret Genomics Centre, Toronto, Canada (www.pmgenomics.ca). The R/Bioconductor (www.R-project.org) packages oligo and limma were used for data normalization, quality assessment and statistical analysis.[17,18] Gene set enrichment analysis (GSEA) using the Mouse Go Pathways, annotated by the Bader Lab (http://download.baderlab.org/EM_Genesets/current_release/Mouse/symbol/; November 1, 2017 release), was applied for pathway enrichment analysis.[19] All GO sets with 10-1000 members were analysed. The recommended number of permutations (1000) was performed using the less stringent (gene set) permutation type. GO sets with a P value less than 0.05 and an FDR Q value cut-off of 0.25 were reported as significant. Pathway enrichments were visualized with cytosape, using the enrichment map[19] to visualize networks with a P value cut-off of 0.05, a corrected false discovery rate (FDR) q value cut-off of 25% and an overlap coefficient of 0.5.

2.4 | Statistical analysis

Statistical significance was assessed by Student's t-test or two-way ANOVA for repeated measures, followed by Bonferroni analysis when applicable. For all assessments, $P < 0.05$ was considered significant. Unless otherwise stated, all data are expressed as MEAN $\pm$ SEM.

3 | RESULTS

3.1 | CMPF exposure prior to HFD feeding protected mice against HFD-induced weight gain and insulin resistance

To examine the effect of CMPF on hepatic lipid accumulation, mice were injected with vehicle or CMPF for 1 week prior to 5 weeks of dietary intervention (Con-HFD vs CMPF-HFD) (Figure 1A). In line with data previously reported by Prentice et al. (2017), CMPF-HFD mice gained significantly less weight than their diet-matched controls

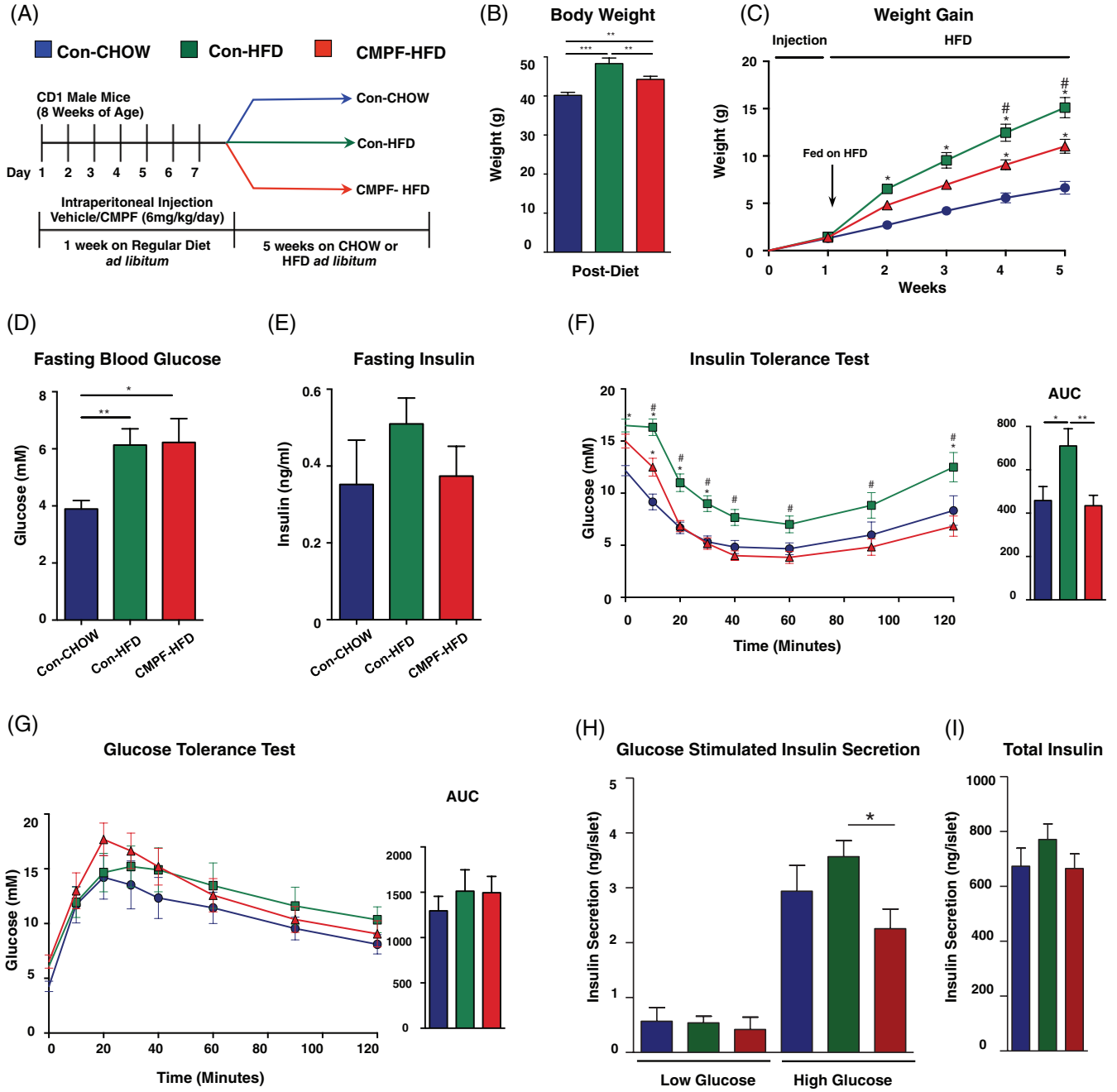

FIGURE 1 CMPF prevents HFD-induced insulin resistance while impairing beta cell function. A, Treatment design to determine preventative effects of CMPF. B, Murine body weight at completion of the 5-week dietary intervention (n = 17–20). C, Body weight gain over the course of study (n = 17–20). Terminal D) blood glucose (n = 11–12) and E) circulating insulin levels (n = 8–10) following overnight fast. F, Intraperitoneal insulin tolerance test and respective area under the curve (n = 8–14). G, Intraperitoneal glucose tolerance test and respective area under the curve (n = 11–14). H, Glucose-stimulated insulin secretion on islets isolated from mice following study completion (n = 8–10). I) Total insulin content within isolated islets (n = 8–10). *P < 0.05, **P < 0.01. In panel (C) and (E) * indicates significance relative to CHOW and # indicates significance between Control-HFD and CMPF-HFD groups. All error bars, SEM

(Figure 1B and C and Figure S1A), with no significant changes in accumulated food intake over 24 hours (Figure S1C), fasting blood glucose (Figure 1D) and fasting insulin levels (Figure 1E). Importantly, this inhibition of HFD-induced weight gain corresponded to significant prevention of HFD-induced insulin resistance, causing insulin sensitivity to remain at a level that was comparable to that with a diet of Con-CHOW (Figure 1F). Interestingly, this prevention of HFD-induced insulin resistance did not translate into improved glucose homeostasis (Figure 1G). This finding could be a result of CMPF's negative effect on beta cell function as previously reported, in which acute CMPF administration lead to reduced insulin biosynthesis and secretion.[10,11] Indeed, even at 5 weeks post exposure to CMPF, this impairment of beta cell function remained, shown here through significant down-regulation of glucose-stimulated insulin secretion during high-glucose incubation (Figure 1H) within islets isolated from CMPF-HFD mice, with no change in total insulin (Figure 1I).

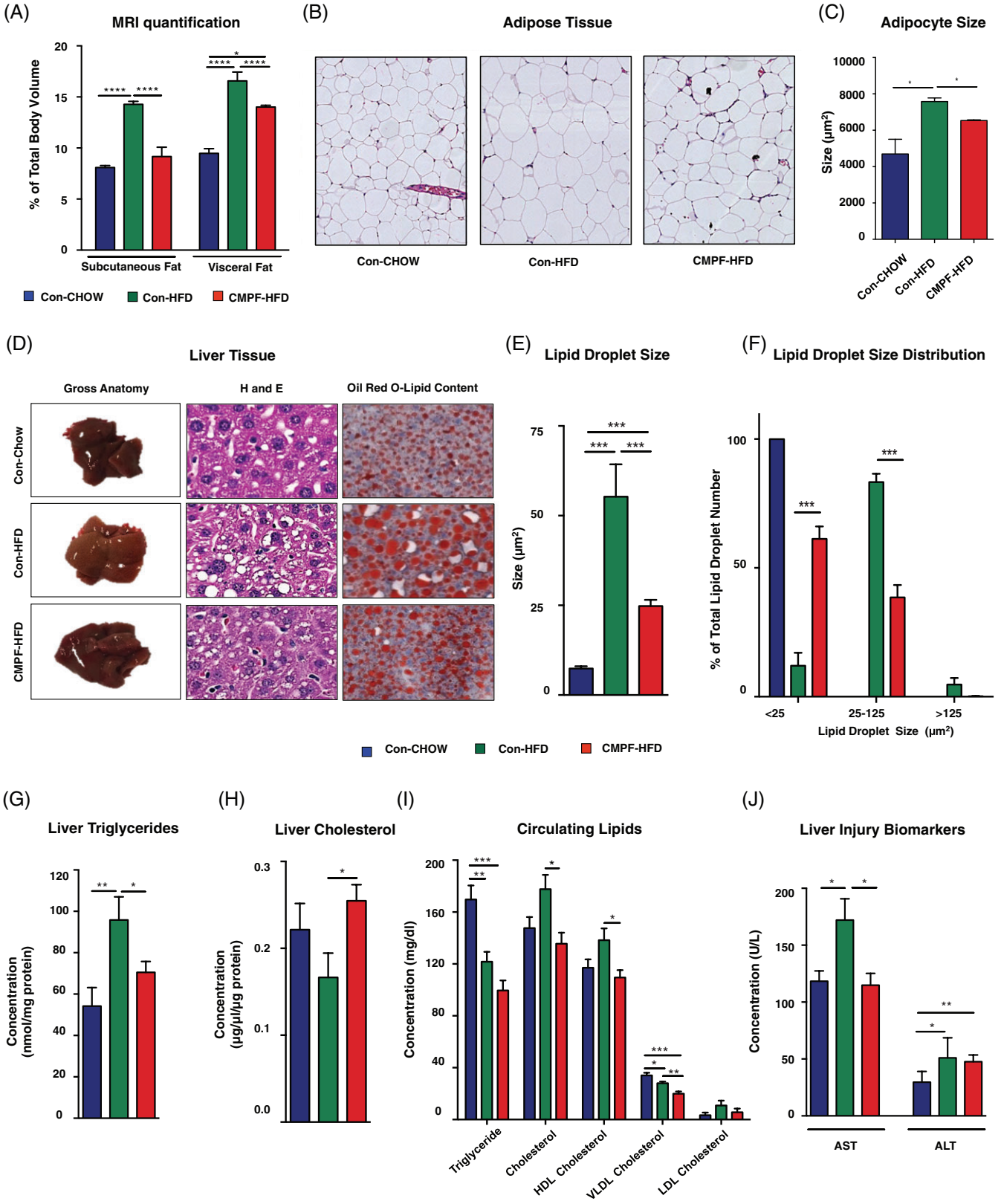

FIGURE 2 CMPF prevents triglyceride accumulation in liver and adipose tissue. A, Quantification of fat distribution in mice determined by MRI scan (n = 4). B, Representative adipose image with H&E staining. C, quantification of adipocyte size in white adipose tissue (n = 3). D, Representative liver images with H&E, and Oil Red O staining. E, Quantification of lipid droplet area (n = 5–9). F) Lipid droplet size distribution within H&E stained liver sections (n = 5–9). Quantification of liver G, triglycerides (n = 13–16) and H) total cholesterol (3–5). I, Serum lipid profile following overnight fast (n = 5–6). J, Circulating levels of AST and ALT (n = 5–6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P, 0.0001. All error bars, SEM

3.2 | Pre-exposure with CMPF prevents HFD-driven lipid deposition in the liver and adipose tissue in vivo

To investigate the reduced weight gain in mice exposed to CMPF prior to HFD feeding, we first utilized magnetic resonance imaging (MRI) to evaluate body fat distribution. A significantly smaller percentage of both subcutaneous and visceral fat mass over total body mass was observed in CMPF-HFD mice when compared to Con-HFD mice (Figure 2A and Figure S1A). Importantly, the percentage of subcutaneous fat mass within CMPF-HFD mice was similar to that of Con-CHOW mice (Figure 2A). These observations corresponded to a significantly smaller adipocyte size within the subcutaneous fat of CMPF-HFD mice compared to that of Con-HFD (Figure 2B and C). Assessment of gross liver morphology, in combination with Oil Red O staining (Sigma-Aldrich, Ontario, Canada), revealed a reduction in hepatic lipid storage (Figure 2D). In addition, the livers of CMPF-HFD mice had a significantly reduced lipid droplet area, with a higher number of medium-to-small sized lipid droplets rather than the primarily large lipid droplets observed in the livers of Con-HFD mice (Figure 2E and F). Hepatic TG and cholesterol quantification revealed that mice that had received CMPF pre-treatment prior to dietary challenge maintained hepatic lipid content at a level similar to that of Con-CHOW mice (Figure 2G and H), leading to a significantly lower level of aspartate aminotransferase (AST) (Figure 2J), one of the biomarkers of hepatic liver injury. Despite CMPF's ability to prevent hepatic lipid accumulation, no difference was observed in circulating TG between Con-HFD and CMPF-HFD mice. However, CMPF-HFD mice exhibited complete prevention of HFD-induced elevation in circulating cholesterol, HDL and LDL, with a further decrease in the level of VLDL, allowing these mice to maintain a Con-CHOW-like phenotype (Figure 2I). Thus, a relatively short-term and temporal exposure to high levels of CMPF primes the body and defends against diet-induced lipid deposition in both the liver and adipose tissue.

Organic anion transporter 2 (OAT2) was highly expressed in both whole liver tissue and isolated primary mouse hepatocytes, suggesting that OAT2, the most abundantly expressed OAT isoform across liver tissue, and primary hepatocytes, can be one of the functional transporters that facilitates the entry of CMPF into hepatocytes. No intracellular CMPF was detected in adipose tissue, suggesting that the in vivo effect of CMPF on prevention of lipid deposition in adipose tissue may be an indirect metabolic consequence (Figures S2 and S3).

3.3 | CMPF treatment causes prolonged negative regulation of lipid biosynthesis and positive regulation of lipid utilization

To elucidate how short-term exposure to CMPF leads to a prolonged effect on hepatic lipid content, a gene microarray followed by GSEA was performed on livers isolated from mice post after 7 days of CMPF in the absence of dietary stress (Con vs CMPF) and from mice after 7 days of CMPF following 5 weeks of dietary stress (Con-HFD vs CMPF-HFD). Following statistical analysis of the enriched gene sets, using a *P* value less than or equal to 0.25, as suggested by the results of GSEA, we identified 372 significantly enriched pathways within Con-HFD livers, and 740 significantly enriched pathways within CMPF-HFD livers, with no enrichment of gene sets found between Con and CMPF (Figure S5). From these significantly enriched pathways, we next identified those specifically related to lipid metabolism (Figure 3).

In Con-HFD mice, diet alone led to the enriched expression of genes involved in pathways of lipid bioprocess, packaging and transport, including fatty acid derivative biosynthetic processes, cholesterol biosynthesis and lipoprotein particle binding and receptor activity, thus promoting lipid storage. As expected, a HFD alone also led to enriched expression of genes in pathways that have been associated with diet-induced inflammation.[20] In contrast to the HFD-induced lipid storage mode, mice that received CMPF pre-treatment prior to a HFD (CMPF-HFD) exhibited significantly enriched gene expression within pathways that prevent lipid storage, including lipid catabolic processes and the negative regulation of fatty acid biosynthesis (Figure 3). Furthermore, CMPF pre-treatment enriched genes of pathways involved in acetyl-coA biosynthesis and oxidative phosphorylation, both of which support increased mitochondrial substrate oxidation to promote lipid clearance. As a net result, CMPF pre-treatment enriched regulation of the mTOR and insulin receptor signalling pathways, which corresponded to the improved hepatic insulin sensitivity observed in our in vivo studies.[9] Interestingly, we also observed gene enrichment in pathways involved in the cellular response to glucose starvation and the negative regulation of gluconeogenesis, further supporting our previous findings that CMPF can induce a metabolic switch resulting in decreased glucose utilization.[10,11] Essentially, short pre-exposure to high levels of CMPF prevented diet-enriched gene sets from promoting lipid storage (Figure 6).

3.4 | CMPF treatment causes a prolonged and significant reduction in fatty acid synthase

We further analysed the impact of CMPF pre-treatment on protein expression of the major transcription factors, enzymes and kinases that are involved in regulating hepatic lipid metabolism. Comparable to the gene array data in which we found no enrichment of gene sets between Con and CMPF, we did not observe changes in the protein expression of ACC1 and ACC2 (Figure 1G), the two major enzymes involved in the regulation of lipid metabolism, when mice were intraperitoneally injected with CMPF for 1, 3 and 7 days in the absence of dietary stress. Interestingly, in CMPF-HFD mice, we observed a prolonged significant reduction in ACC2 expression which is involved in the inhibition of fatty acid oxidation, further supporting the notion of CMPF's enrichment of the genes involved in the mitochondrial oxidation pathway, including acetyl-coA biosynthetic processes and oxidative phosphorylation (Figure 4). Additionally, as major genes activated by SREBP are involved in fatty acid and cholesterol production, we found that both the inactive full-length and active cleaved nuclear form of sterol regulatory element-binding protein-1c (SREBP-1c), the transcription factor that is responsible for regulating the genes required for lipogenesis, were lower in CMPF-HFD mice compared to Con-HFD mice. This could be the result of a higher expression level of insulin-induced-gene 2 (Insig2), the negative regulator of SREBP-1c[21] (Figure 4). Upon activation, the nuclear form of SREBP-1c is primarily

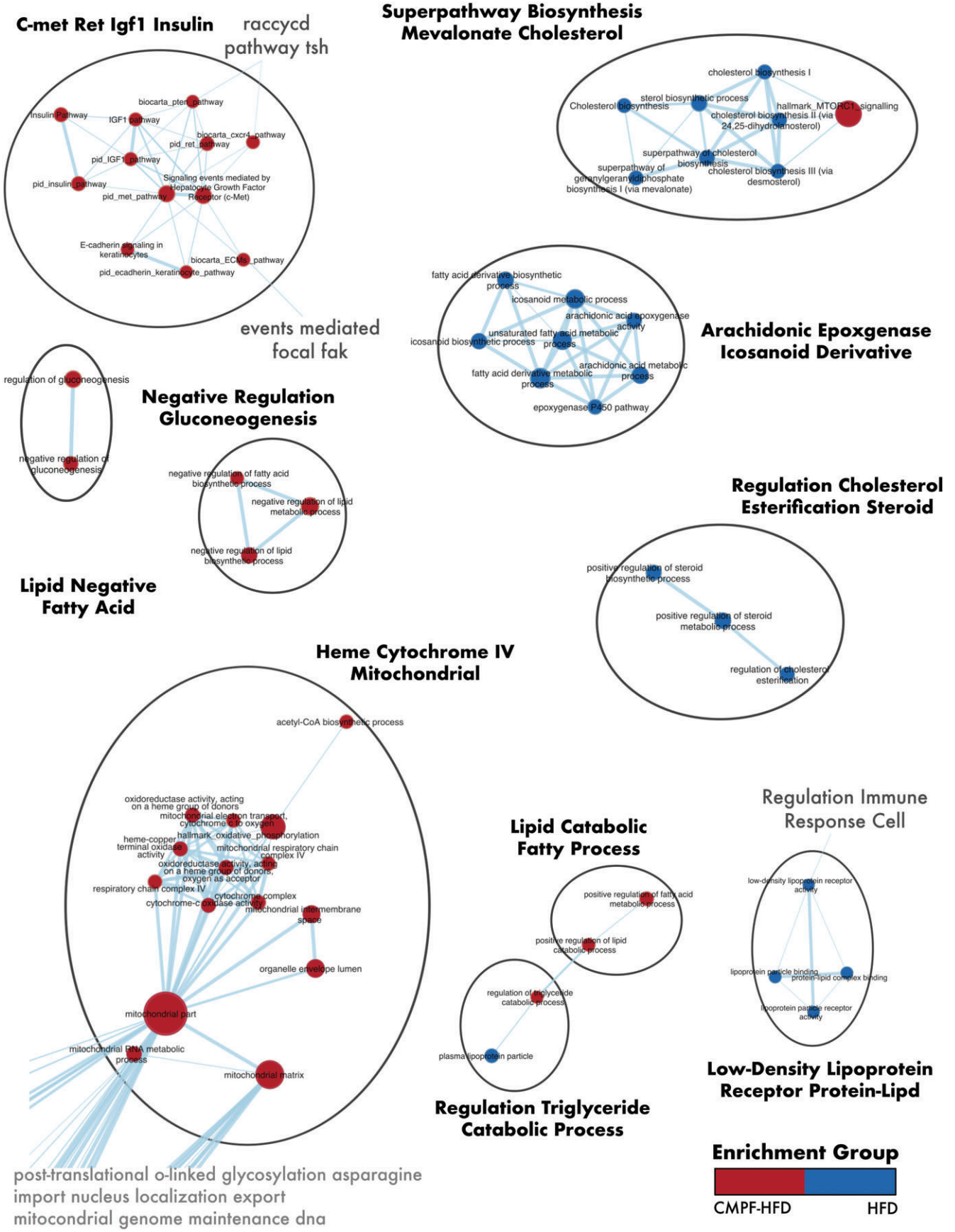

FIGURE 3 CMPF prevents development of the HFD phenotype Microarray data showing lipid pathways enriched in mice fed an HFD (n = 3) and enriched in mice treated with CMPF prior to an HFD (n = 3). Red represents enriched pathways, while blue represents depleted pathways

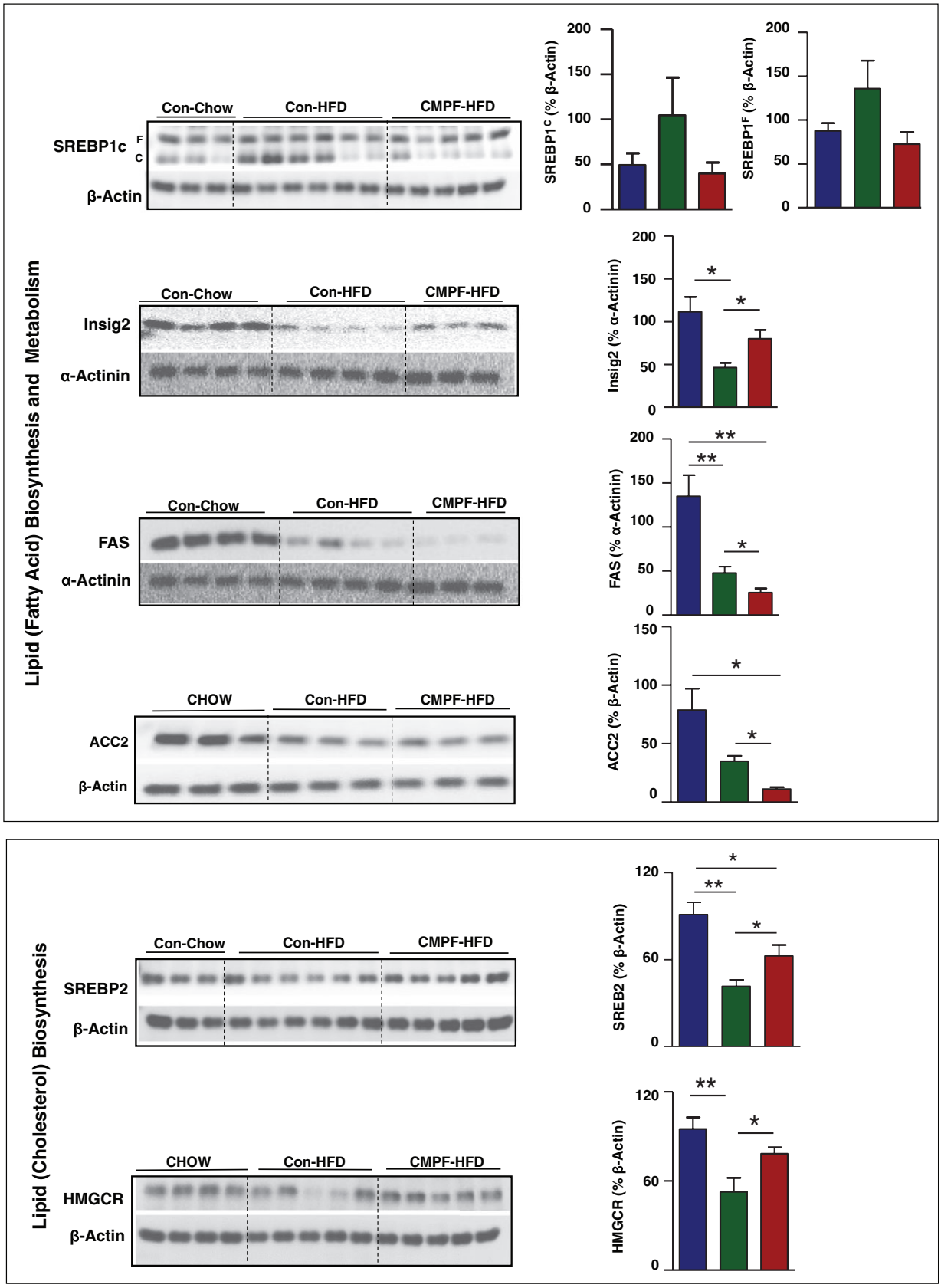

FIGURE 4 CMPF chronically decreases SREBP activity leading to reduced total ACC2 expression. Representative western blot and quantification of full-length and cleaved SREBP-1c (n = 4–5), Insig2 (n = 3–4), ACC2 (n = –4) and FAS (n = 6–7) involved in fatty acid biosynthesis and metabolism. Representative western blot and quantification of SREBP2 (n = 4–5) and HMGCR (n = 8–10) involved in cholesterol biosynthesis. All expression measured through densitometry and expressed as a percentage of alpha-actinin/ beta-actin. *P < 0.05, **P < 0.01, ***P < 0.001. All error bars, SEM

responsible for controlling the expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), the major regulatory proteins involved in fatty acid biosynthesis. Among them, the expression of FAS was significantly reduced, further supporting an inhibition of fatty acid synthesis upon CMPF pre-treatment. However, SCD1 and ACC1 expression were unchanged between mice with and without CMPF pre-treatment. Interestingly, with CMPF pre-treatment, we observed significantly increased expression of the transcription factor SREBP2 and its downstream-targeted protein 3-hydroxy-3- methylglutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in the regulation of cholesterol biosynthesis.

3.5 | CMPF enhances fatty acid oxidation and cholesterol production in primary hepatocytes in vitro

Given CMPF's prolonged effect towards preventing development of the HFD phenotype in the liver, we next wanted to investigate how CMPF pre-treatment primes the liver to better handle the incoming fat load. Following 7 days of CMPF intraperitoneal injection, lipid clearance was significantly improved during a lipid tolerance test (Figure 5A), suggesting that CMPF regulates the processes of hepatic lipid turnover prior to dietary intervention, despite negligible detection of CMPF in circulation after 24 hours (Figure S1B). To further elucidate the direct hepatic effect of CMPF, primary hepatocytes were isolated from untreated mice and cultured for 24 hours with CMPF at a concentration comparable to that of the in vivo injection.[11] CMPF did not directly alter hepatic fatty acid uptake (Figure 5B). However, similar to the increase in fatty acid oxidation previously observed within the islets,[10] CMPF treatment significantly increased hepatic fatty acid oxidation (Figure 5C). Unexpectedly, despite CMPF's ability to act as an allosteric inhibitor of ACC1,[9] in vitro CMPF treatment also significantly increased hepatic lipogenesis (Figure 5D). As our measurement of hepatic lipogenesis cannot determine whether the de novo generated lipids originated from TG or cholesterol biosynthesis, we specifically measured the concentration of hepatic cholesterol. Indeed, following acute CMPF treatment, we observed an increase in cholesterol production, shown through an elevation in intracellular and secreted cholesterol content (Figure 5E and F). This parallels our observations in which CMPF up-regulates protein expression of the major transcription factor (SREBP2) and rate-limiting enzyme (HMGCR) involved in cholesterol biosynthesis (Figure 4). Importantly, we observed an opposite regulation on cholesterol biosynthesis between CMPF (promote) and TOFA (inhibit), the general pharmacological ACC inhibitor; therefore, we suggest that CMPF up-regulates cholesterol biosynthesis independently of its previously reported allosteric ACC1 inhibitory properties.[9] As a net result of CMPF's impact on hepatic lipid metabolism, and consistent with our results in vivo (Figure 1F and Figure 4), CMPF treatment corrected lipid-induced insulin resistance, shown here as a significant enhancement of (fat-load impaired) insulin-stimulated AKT phosphorylation relative to fat-supplemented controls (Figure 5G). In addition, CMPF treatment did not directly alter hepatic glucose uptake or production (Figure 5H and I), nor glycogen content determined via PAS staining in CMPF-HFD mice (Figure 1E), suggesting that the effect of CMPF within the liver occurs primarily through the regulation of lipid metabolism.

3.6 | CMPF increases FGF21 expression and secretion from primary hepatocytes via ACC inhibition in vitro

We also investigated the cellular mechanism behind CMPF's ability to drive hepatic FGF21 production.[9] Within isolated hepatocytes, treatment with CMPF increased both the expression (Figure 5K) and secretion of FGF21 (Figure 5I). The expression of FGF21 can be initiated through activation of the transcription factor PPARα.[22] However, CMPF itself did not act as a ligand to activate PPARα activity, as measured by luciferase reporter assay (Figure S2B, S2C). Many studies have indicated a positive correlation between hepatic fatty acid oxidation and FGF21 expression.[23,24] Given our observation that CMPF can stimulate fatty acid oxidation in the absence of upstream AMP-activated protein kinase (AMPK) or ACC phosphorylation (Figure 5J), we tested whether CMPF's inhibition of ACC activity occurs upstream of its initiation in FGF21 expression. A series of known allosteric ACC inhibitors, TOFA,[25] S-2E[26] and PF-05175157,[27] along with CMPF, were used here to treat primary hepatocytes. All three ACC inhibitors increased both mRNA expression (Figure 5K) and secretion of FGF21 (Figure 5I) from isolated hepatocytes. Similarly, inhibition of ACC via phosphorylation that was induced by activation of its upstream kinase, AMPK, also led to an increase in hepatic FGF21 production (Figure 5I). This indicates that inhibition of ACC, via either allosteric inhibition or upstream kinase phosphorylation, can stimulate hepatic FGF21 production.

4 | DISCUSSION

CMPF is an active metabolite of FO and is independent of its mother compound (FO); both demonstrate protective effects on hepatic lipid metabolism. In the present study, we utilized both in vivo and in vitro models and undertook detailed mechanistic lipid research to gain an understanding of how CMPF protects against the development of fatty liver.

Close examination of lipid accumulation within CMPF-HFD-treated livers revealed a greater number of small-sized lipid droplets, opposed to the large-sized droplets more characteristic of liver steatosis.[28] These data suggest that the lipid accumulation occurring within the liver as a result of high-fat diet feeding is significantly reduced following transient CMPF treatment. However, the presence of small lipid droplets suggests that excessive absorption of free fatty acids would further contribute to CMPF's prolonged protective effect against HFD-induced hepatic steatosis, thus maintaining hepatic insulin sensitivity.[29,30] Although CMPF prevented diet-enriched genes in promoting hepatic lipid storage, microarray data shows no significant changes in genes of enzymes that are involved in the regulation of hepatic lipid production, which includes glycerol-3-phosphate acyltransferase (GPAT), phosphatidate phosphatase (PAP) and diglyceride acyltransferase (DGAT). Further studies focusing on CMPF's effect on the activity of enzymes involved in hepatic lipid production are

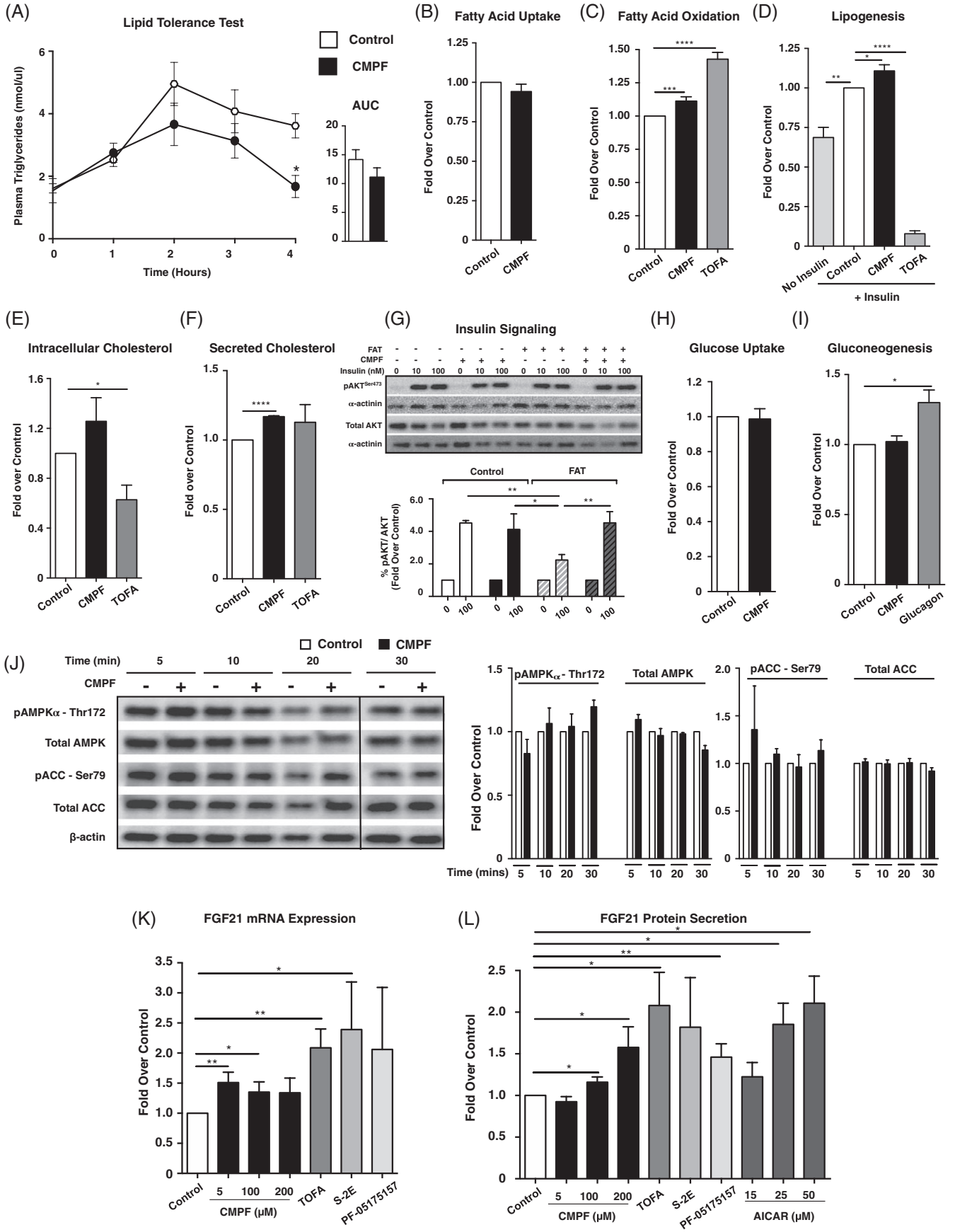

FIGURE 5 Legend on next page.

required. We believe that enrichment of genes in the lipid biosynthesis pathway are a consequence of CMPF's defense against HFD stress, resulting from early activation of the ACC inhibtion-FGF21 production-Beta oxidation loop. In addition, the prolonged effect of CMPF could potentially be the result of epigenetic changes, leading to alteration in long-term gene expression, that are yet to be determined. Fatty liver is usually associated with elevated levels of ALT and AST, but it is surprising to observe no change in ALT, while AST levels are increased. However, studies suggest that ALT levels can fluctuate; therefore, measurement at a single time point is insufficient to reach conclusions concerning the normalcy of ALT.[31]

In our studies, we consistently found that there is no change in circulating TG levels despite a reduction in hepatic TG in the various diet-induced dyslipidaemic models upon CMPF treatment. We believe our study model (1 week of CMPF prior to 5 weeks of an HFD) is limited in its ability to determine only the preventive effects of CMPF on the early stages of NAFLD, the fatty liver stage, as the more advanced pathological characteristics of NAFLD, which include inflammation and fibrosis, begin to appear only after at least 18 weeks of a HFD intervention.[32] Additionally, male C57BL/6 mice were treated with CMPF for 1 week prior to a 3-week period of a low-methionine, choline-deficient diet (MCD), which is the most widely used diet to induce NAFLD/NASH. We found no significant differences in liver TG content (Figure S1H) and serum TG levels (Figure S1I), suggesting that CMPF treatment cannot prevent severe liver damage and progressive fibrosis (Figure S1H). Therefore, we believe that CMPF's effect on regulation of hepatic lipid metabolism can only prevent and treat early stages of NAFLD, the fatty liver stage, and the effect of CMPF on lowering hepatic TG cannot be translated into an effect on circulating TG.

Our study revealed that CMPF can accumulate in liver tissue but not in adipose tissue; thus, we propose that OAT2 facilitates CMPF's entry into the hepatocyte and exerts its direct effect. Because of the absence of OATs expression, we believe adipose tissue is unlikely to be the primary target tissue for CMPF. Studies have shown that OAT2 is capable of transporting a wide range of substrates including dicarboxylates such as CMPF,[33,34] and it is significantly expressed within both whole liver and isolated hepatocytes. Once CMPF has entered the hepatocyte, it enhances fatty acid oxidation via its inhibitory effect on ACC[35] and directly induces FGF21 production, all of which could contribute to the improvement observed in the acute lipid tolerance test (Figure 5A). However, CMPF might have an effect on fat absorption, chylomicron release or lipid particle clearance that would affect the lipid turnover rate, which requires further investigation.

We also observed, paradoxically, an increase in lipid production in isolated hepatocytes. Further analysis suggests that an increase in cholesterol biosynthesis upon CMPF treatment may account, in part, for the unexpected increase in lipid production. This increase in hepatic cholesterol production aligned with the significant increase observed in hepatic cholesterol secretion. In particular, high-density lipoprotein (HDL) could bring excess cholesterol back to the liver via reverse cholesterol transport for elimination.[36] Indeed, we observed a significant increase in circulating HDL particles in ob/b mice treated acutely with CMPF (data not shown). Although the exact mechanism leading to this increase in cholesterol production remains unclear, we know that it occurs independently of CMPF's inhibitory effect on ACC1, as 5-tetradecyloxy-2-furoic acid (TOFA), the general pharmacological ACC inhibitor, induces an opposite effect on the regulation of cholesterol biosynthesis from CMPF. Further studies to elucidate the underlying mechanism responsible for CMPF-increased cholesterol production are warranted.

SREBP-1c is the key lipogenic transcription factor responsible for transcription of the genes involved in fatty acid synthesis.[37,38] The hepatic insulin resistance that is associated with diet-induced NAFLD is probably the major driving force behind the increased expression and activity of SREBP-1c, as suggested by many studies.[39,40] It is intriguing to see that the expression/activity of SREBP1c is maintained at the same level as that observed in CHOW-fed mice, even after 5 weeks of an HFD following cessation of CMPF treatment (Figure 5). Within CMPF-treated livers, both shortly after and 6wks after CMPF treatment, insulin signalling, as measured through phosphorylation of AKT, was improved.[9] Therefore, maintenance of functional insulin signalling within the hepatocyte could possibly explain CMPF's ability to prevent the deregulation of SREBP-1c protein expression and activity as observed in the livers of Control-HFD mice.

Importantly, we present here a novel concept that inhibition of ACC, either by phosphorylation via upstream kinases or by direct allosteric inhibition, can induce hepatic FGF21 expression. FGF21 is regulated primarily via the ligand-activated transcription factor PPARα; however, CMPF failed to directly stimulate PPARα (Figure S2B, S2C), confirming that CMPF itself is not an activating ligand. It is more likely that inhibition of ACC results in the accumulation of intracellular metabolic intermediates that may have the ability to activate PPARα and, thus, to subsequently enhance FGF21 gene transcription.[41] In contrast to the liver, neither CMPF accumulation nor OAT expression was detected in any fat depots. This suggests that the observed

FIGURE 5 CMPF directly increases hepatic fatty acid oxidation and lipid production in vitro. A, Oral lipid tolerance test and respective AUC on 7-day injected mice ($n = 6$). B, Fatty acid uptake ($n = 6$), C, fatty acid oxidation ($n = 5$), D, lipid production ($n = 3$), E, intracellular cholesterol ($n = 3$–4) and F, secreted cholesterol ($n = 3$–4) in isolated hepatocytes treated for 24 hours with CMPF (200 μM), TOFA (100 μM) or insulin (100 nM). G) Representative western blot and quantification of AKT phosphorylation following 100 nM insulin stimulation for 15 minutes in isolated hepatocytes following 48-hour FAT (300 μM oleic acid and 300 μM palmitic acid) supplementation with CMPF (200 μM) treatment in the final 24 hours ($n = 4$). H, Glucose uptake ($n = 4$) and I, Glucose production ($n = 3$). J, p-AMPK/AMPK and p-ACC/ACC western blot in isolated hepatocytes treated with CMPF (200 μM) and glucagon (100 nM). K) mRNA FGF21 expression calculated as $2^{-\Delta\Delta CT}$. I, FGF21 secretion into hepatic media following treatment with CMPF, TOFA (100 μM), S-2E (10 μM) and PF-05175157 (10 μM) and AICAR in isolated hepatocytes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. All error bars, SEM

preventive effect of CMPF on diet-induced enlargement of adipocytes probably occurs as a prolonged metabolic consequence of CMPF's maintenance of hepatic insulin sensitivity and elevation in circulating FGF21.

Despite the observed beneficial effects of CMPF on regulating hepatic lipid homeostasis and insulin sensitivity, it cannot be ignored that they occur in combination with the negative effects of CMPF on beta cells.[10,11]

In conclusion, the results of our study provide further insight into the hepatic mechanism behind CMPF's beneficial effect in preventing diet-induced fatty liver. CMPF increases hepatic fatty acid oxidation in parallel with an increase in hepatic FGF21 production and secretion via its inhibitory effect on ACC. In return, FGF21 can further enhance hepatic fatty acid oxidation via its autocrine or endocrine action. This feed-back loop that is established among fatty acid oxidation, ACC inhibition, and FGF21 production during CMPF exposure, primes the liver with a prolonged effect that prevents hepatic lipid synthesis and storage via negative regulation of the Insig2/SREBP-1c/FAS axis. Simultaneously, it promotes lipid utilization to fight against diet-induced hepatic lipid accumulation and to maintain the insulin sensitivity that remains even 5 weeks after CMPF treatment.

ACKNOWLEDGMENTS

This study was funded by a CIHR operating grant to MBW (FDN-143219). H.M. and S.L.B. were supported by a BBDC postdoctoral Fellowship and BBDC Graduate Studentship respectively. A CIHR doctoral research award supported KJP. Some of the equipment used in this study was supported by The 3D (Diet, Digestive Tract and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442 and 30961.

Conflict of interest

All authors have no conflicts of interest to declare.

Author contributions

Y. L. and M. B. W. designed the study and edited the manuscript. S. L. B. and H. M. conducted experiments, acquired data, analysed data, and wrote and edited the manuscript. J. H. K., M. L., L. M., B. B., K. J. P. D. A. R, A. B. and E. B. conducted experiments and reviewed the manuscript. F. W. and B. C. performed statistical analysis on data collected from gene microarray procedures and reviewed the manuscript. C. L. C. and D. D. B. reviewed the manuscript.

ORCID

Carolyn L. Cummins https://orcid.org/0000-0001-7603-6577
Ying Liu https://orcid.org/0000-0001-7710-0809
Michael B. Wheeler https://orcid.org/0000-0002-7480-7267

REFERENCES

1. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology*. 2010;51:679-689.
2. Smith BW, Adams LA. Non-alcoholic fatty liver disease. *Crit Rev Clin Lab Sci*. 2011;48:97-113.
3. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. *Annu Rev Pathol*. 2010;5:145-171.
4. Ahmed M. Non-alcoholic fatty liver disease in 2015. *World J Hepatol*. 2015;7:1450-1459.
5. Birkenfeld AL, Shulman GI. Nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes. *Hepatology*. 2014;59:713-723.
6. Adams LA, Angulo P. Treatment of non-alcoholic fatty liver disease. *Postgrad Med J*. 2006;82:315-322.
7. Ratziu V, Goodman Z, Sanyal A. Current efforts and trends in the treatment of NASH. *J Hepatol*. 2015;62(suppl 1):S65-S75.
8. Eslick GD, Howe PRC, Smith C, Priest R, Bensoussan A. Benefits of fish oil supplementation in hyperlipidemia: a systematic review and meta-analysis. *Int J Cardiol*. 2009;136:4-16.
9. Prentice KJ, Wendell SG, Liu Y, et al. CMPF, a metabolite formed upon prescription Omega-3-Acid ethyl ester supplementation, prevents and reverses steatosis. *EBioMedicine*. 2018;27:200-213.
10. Liu Y, Prentice KJ, Eversley JA, et al. Rapid elevation in CMPF may act as a tipping point in diabetes development. *Cell Rep*. 2016;14:2889-2900.
11. Prentice KJ, Luu L, Allister EM, et al. The furan fatty acid metabolite CMPF is elevated in diabetes and induces beta cell dysfunction. *Cell Metab*. 2014;19:653-666.
12. Davidson MH, Johnson J, Rooney MW, Kyle ML, Kling DF. A novel omega-3 free fatty acid formulation has dramatically improved bioavailability during a low-fat diet compared with omega-3-acid ethyl esters: the ECLIPSE (Epanova([R]) compared to Lovaza([R]) in a pharmacokinetic single-dose evaluation) study. *J Clin Lipidol*. 2012;6:573-584.
13. Glueck CJ, Khan N, Riaz M, Padda J, Khan Z, Wang P. Titrating lovaza from 4 to 8 to 12 grams/day in patients with primary hypertriglyceridemia who had triglyceride levels >500 mg/dl despite conventional triglyceride lowering therapy. *Lipids Health Dis*. 2012;11:143.
14. Zhang S, Chen P, Jin H, et al. Circulating 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) levels are associated with hyperglycemia and beta cell dysfunction in a Chinese population. *Sci Rep*. 2017;7:3114.
15. Zheng JS, Lin M, Imamura F, et al. Serum metabolomics profiles in response to n-3 fatty acids in Chinese patients with type 2 diabetes: a double-blind randomised controlled trial. *Sci Rep*. 2016;6:29522.
16. Tovar J, de Mello VD, Nilsson A, et al. Reduction in cardiometabolic risk factors by a multifunctional diet is mediated via several branches of metabolism as evidenced by nontargeted metabolite profiling approach. *Mol Nutr Food Res*. 2017;61(2).
17. Carvalho BS, Irizarry RA. A framework for oligonucleotide microarray preprocessing. *Bioinformatics*. 2010;26:2363-2367.
18. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43:e47.
19. Merico D, Isserlin R, Stueker O, Emili A, Bader GD. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One*. 2010;5:e13984.
20. Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. *Nat Rev Immunol*. 2015;15:511-523.
21. Yabe D, Komuro R, Liang G, Goldstein JL, Brown MS. Liver-specific mRNA for Insig-2 down-regulated by insulin: implications for fatty acid synthesis. *Proc Natl Acad Sci U S A*. 2003;100:3155-3160.
22. Galman C, Lundåsen T, Kharitonenkov A, et al. The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARalpha activation in man. *Cell Metab*. 2008;8:169-174.
23. Badman M, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab*. 2007;5:426-437.
24. Rusli F, Deelen J, Andriyani E, et al. Fibroblast growth factor 21 reflects liver fat accumulation and dysregulation of signalling pathways in the liver of C57BL/6J mice. *Sci Rep*. 2016;6:30484.
25. Parker RA, Kariya T, Grisar JM, Petrow V. 5-(Tetradecyloxy)-2--furancarboxylic acid and related hypolipidemic fatty acid-like alkyloxyarylcarboxylic acids. *J Med Chem*. 1977;20:781-791.

26. Ohmori K, Yamada H, Yasuda A, Yamamoto A, Matsuura N, Kiniwa M. Anti-hyperlipidemic action of a newly synthesized benzoic acid derivative, S-2E. *Eur J Pharmacol*. 2003;471:69-76.
27. Griffith DA, Kung DW, Esler WP, et al. Decreasing the rate of metabolic ketone reduction in the discovery of a clinical acetyl-CoA carboxylase inhibitor for the treatment of diabetes. *J Med Chem*. 2014; 57:10512-10526.
28. Gluchowski NL, Becuwe M, Walther TC, Farese RV. Lipid droplets and liver disease: from basic biology to clinical implications. *Nat Rev Gastroenterol Hepatol*. 2017;14:343-355.
29. Herms A, Bosch M, Ariotti N, et al. Cell-to-cell heterogeneity in lipid droplets suggests a mechanism to reduce lipotoxicity. *Curr Biol*. 2013; 23:1489-1496.
30. Wilfling F, Wang H, Haas JT, et al. Triacylglycerol synthesis enzymes mediate lipid droplet growth by relocalizing from the ER to lipid droplets. *Dev Cell*. 2013;24:384-399.
31. Mofrad P, Contos MJ, Haque M, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. *Hepatology*. 2003;37:1286-1292.
32. Matsumoto M, Hada N, Sakamaki Y, et al. An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis. *Int J Exp Pathol*. 2013;94:93-103.
33. Henjakovic M, Hagos Y, Krick W, Burckhardt G, Burckhardt BC. Human organic anion transporter 2 is distinct from organic anion transporters 1 and 3 with respect to transport function. *Am J Physiol Renal Physiol*. 2015;309:F843-F851.
34. Kobayashi Y, Ohshiro N, Sakai R, Ohbayashi M, Kohyama N, Yamamoto T. Transport mechanism and substrate specificity of human organic anion transporter 2 (hOat2 [SLC22A7]). *J Pharm Pharmacol*. 2005;57:573-578.
35. Prentice KJ, Wendell SG, Liu Y, et al. CMPF, a metabolite formed upon prescription Omega-3-acid ethyl Ester supplementation, prevents and reverses steatosis. *EBioMedicine*. 2018;27:200-213.
36. Feingold KR, Grunfeld C. Introduction to lipids and lipoproteins. In: De Groot LJ, Chrousos G, Dungan K, et al., eds. *Endotext*. South Dartmouth, MA: MDText.com, Inc; 2000.
37. Eberle D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie*. 2004; 86:839-848.
38. Espenshade PJ. SREBPs: sterol-regulated transcription factors. *J Cell Sci*. 2006;119:973-976.
39. Kohjima M, Higuchi N, Kato M, et al. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. *Int J Mol Med*. 2008;21:507-511.
40. Taniguchi CM, Ueki K, Kahn CR. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest*. 2016;126: 4387.
41. Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta*. 1812;2011:1007-1022.

SUPPORTING INFORMATION

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

How to cite this article: Mohan H, Brandt SL, Kim JH, et al. 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) prevents high fat diet-induced insulin resistance via maintenance of hepatic lipid homeostasis. *Diabetes Obes Metab*. 2019;21:61–72. https://doi.org/10.1111/dom.13483