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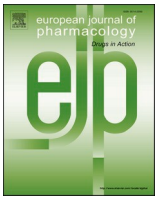

Glucocorticoid receptor modulator CORT125385 alleviates diet-induced hepatosteatosis in male and female mice

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a common condition that can progress to the more severe conditions like non-alcoholic steatohepatitis (NASH) for which limited effective therapeutic options are available. In this study, we set out to evaluate the novel glucocorticoid receptor modulator CORT125385, an analogue of the previously studied miricorilant but without mineralocorticoid receptor binding activity. Male and female mice that received high-fat diet and fructose water were treated with either vehicle, CORT125385 or mifepristone. We found that CORT125385 significantly lowered hepatic triglyceride levels in male mice, and hepatic triglyceride and cholesterol levels in female mice. Mifepristone treatment had no effect in male mice, but significantly lowered hepatic triglyceride and cholesterol levels in female mice. In reporter assays *in vitro*, CORT125385 showed weak partial agonism on the progesterone receptor (PR) at high doses, as well as PR antagonism at a potency 1000-fold lower than mifepristone. *In vivo*, CORT125385 treatment did not influence PR-responsive gene expression in the oviduct, while mifepristone treatment strongly influenced these genes in the oviduct, thus excluding *in vivo* PR cross-reactivity of CORT125385 at a therapeutically active dose. We conclude that CORT125385 is a promising glucocorticoid receptor modulator that effectively reduces liver steatosis in male and female mice without affecting other steroid receptors at doses that lower hepatic lipid content.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common condition with an estimated worldwide prevalence of over 30%. It can progress to the more severe conditions like non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma (Riazi et al., 2022; Teng et al., 2022). Given the limited number of effective treatment options for NAFLD, there is an urgent need for novel therapeutics (Friedman et al., 2018). Endogenous glucocorticoids have been implicated to play a dominant role in the development of NAFLD and NASH (Woods et al., 2015). Glucocorticoids bind and activate the glucocorticoid receptor (GR), which is a transcription factor known to regulate the expression of thousands of genes in the liver including genes involved in lipid metabolism. As such, GR signaling is known to influence hepatic fatty acid uptake, β-oxidation, de novo lipogenesis and VLDL-production. GR signaling in the liver may therefore represent a relevant therapeutic target for the treatment of NAFLD and NASH.

Miricorilant (CORT118335) is a selective GR modulator (SGRM) with mineralocorticoid receptor (MR) antagonistic properties. We previously showed that miricorilant strongly lowered hepatic lipid levels in male mice that were fed a high-fat diet (Koorneef et al., 2018). In contrast, pure GR agonism with dexamethasone provided no benefit, which is possibly in part explained by the differential regulation of fatty acid transporter CD36 (Du et al., 2015; Koorneef et al., 2018; Chen et al., 2022). Based on these preclinical findings, miricorilant was evaluated in patients with presumed NASH, and initial analyses of this clinical trial showed a rapid and robust reduction of hepatic lipid content after miricorilant administration (Kowdley et al., 2021). Although the GR is the presumed therapeutic target of miricorilant, its MR antagonism precludes this as a formal conclusion from our previous work. We therefore developed CORT125385, a newly developed GR modulator and analogue of miricorilant (Nguyen et al., 2019; Hunt et al., 2021) that lacks MR activity. We hypothesized that treatment with CORT125385 would mimic the therapeutic lipid-lowering effects of CORT118335 in

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Table 1
Primer sequences for real-time quantitative PCR.

Gene	Primer Fwd	Primer Rev
18S	AGGACCTGGAGAGGCTGAAG	CAGTGGTCTTGGTGTGCTGA
Acc1	AACGTGCAATCCGATTTGTT	GAGCAGTTCTGGGAGTTTCG
Acc2	AGATGGCCGATCAGTACGTC	GGGGACCTAGGAAAGCAATC
Actg2	GAGCTTCGAGTAGCACCAGA	GAACGATGCCTGTGGTACGG
Adamts1	TTGAATGGTGTGAGTGGCGA	TCAAACATTCCCCGTGTCCAT
Agtr2	GAGCTTCGAGTAGCACCAGA	GAACGATGCCTGTGGTACGG
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
B2M	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG
Cd36	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Des	GAGGCCAATGGCTATCAGGAC	GGATAGGAAGGTTGATCCTGCTC
Edn3	TTGTACTTGTATGGGGGCGG	GACATCAACCTTTGACGTGGG
Fasn	GCGCTCCTCGCTTGTCGTCT	TAGAGCCCAGCCTTCCATCTCCTG
Gilz	TGGCCCTAGACAACAAGATTGAGC	CCACCTCCTCTCTCACAGCAT
Got1	GGAGCTGACTTCTTAGGGCG	GGGGCATTCTCCAGATCATTCA
Got2	ATGGTGAAGGATGCCTGG	TTCATCCGCATCTTTGCAGACC
Gpt1	CACCTATCATTTCCGGATGACC	ATAGTGAGGGCTCCCAAGGA
Gpt2	TGAACCCGCAGGTGAAGG	CTCGGATTACCTCAGTGAATGG
Fabp1	GAGGAGTGCGAACTGGAGAC	GTAGACAATGTCGCCCAATG
Fkbp5	GCCGACTGTGTGTGTAATGC	CACAATACGCACTTGGGAGA
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Myocd	GAGCAGCTGGCTAACCAAGG	GGAATGGACCTTTCTGCCGT
Nr3c2	TGGTCCTTGGAGGTCGTAAGT	AGAGCAACACCGTCAAGGG
Prlr	TGAGTGGGAGATCCACTTCAC	GAACGGCCACAATGATCCAC
Ptgfr	ATGCCGAGATGTCTGCACTC	TTCTCCGTCTGGCAGGTTGT
Ptgs2	AGAACCGCATTGCCTCTGAA	CTTCCCCCAGCAACCCG

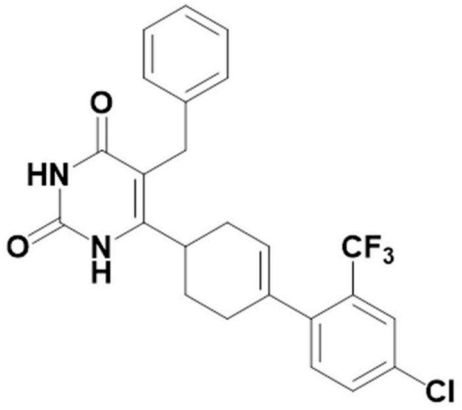

Fig. 1. The chemical structure of CORT125385. The chemical structure of CORT125385 (5-benzyl-6-(4′-chloro-2'-(trifluoromethyl)-2,3,4,5-tetrahydro-[1,1′-biphenyl]-4-yl)pyrimidine-2,4(1*H*,3*H*)-dione).

the liver of mice.

In this study, we show that CORT125385 treatment readily prevents high-fat diet-induced liver steatosis in male and female mice, without relevant cross-reactivity for the MR and the progesterone receptor (PR).

2. Methods

2.1. Glucocorticoid, progesterone, mineralocorticoid and androgen receptor signaling assays in human HEK293 cells

Human HEK293 cells were seeded 80,000 cells per well in a 24-wells plate using DMEM-Glutamax medium supplemented with 10% charcoal-stripped fetal bovine serum, 100 I.U/mL penicillin and 100 μg/mL streptomycin. Cells were transfected using Fugene HD (Promega) with 25 ng tyrosine aminotransferase (TAT) 1-GRE-firefly-luciferase or TAT3-GRE-firefly-luciferase, 10 ng human GR, PR, MR or androgen receptor (AR) expression vector, 1 ng CMV-renilla-luciferase control plasmid and 100 ng pcDNA-3.0 (Kroon et al., 2018). In GR agonist mode, cells were treated with 1–1000 nM dexamethasone, CORT118335 or CORT125385 for 24 h. In parallel, we performed a dexamethasone dose-response study on GR signaling, in which cells were pretreated with vehicle (DMSO), 1.0 μM CORT118335, 1.0 μM CORT125385 or 1.0 μM mifepristone for 1 h before dexamethasone exposure for 24 h. In GR antagonism mode, cells were pretreated with 0.1–1000 nM CORT125385 or mifepristone for 1 h before exposure to 100 nM cortisol for 24 h. In PR agonist mode, cells were treated with 10 nM progesterone or 10–1000 nM CORT125385 for 24 h. In PR antagonist mode, cells were pretreated with 0.1–1000 nM CORT125385 or mifepristone for 1 h before exposure to 10 nM progesterone for 24 h. In MR and AR antagonist mode, cells were pretreated with 10–1000 nM CORT125385 for 1 h before treatment with 10 nM cortisol (MR) or 100 nM dihydrotestosterone (DHT) (AR) for 24 h. At the end of the experiment, cells were lysed and firefly- and renilla-luciferase were measured using a dual-luciferase assay (Promega). EC_{50} and IC_{50} values were calculated using a non-linear fit model.

2.2. Microarray assay for coregulator-nuclear receptor interaction

In coregulator-nuclear receptor interaction assay, 154 leucine-rich binding motifs were attached to the PamChip array, and these motifs were incubated with HEK293 cell lysates containing human GR. Interactions of the human GR with coregulators were investigated in the presence of 1 μM cortisol, 1 μM CORT125385 or 1 μM mifepristone as compared to vehicle (DMSO), as previously described (Desmet et al., 2014; Viho et al., 2023). The modulation index was calculated for each ligand as compared to DMSO and are depicted as $\log_{10}$-transformed values and are available in Suppl. Tables 1A–B.

2.3. Animal studies

All mouse experiments were reviewed by the animal welfare body of Leiden University Medical Center (IvD Leiden) and executed under a license granted by the Central Authority for Scientific Procedures on Animals (CCD) under the license number AVD1160020186605, in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU. Mice were group-housed with 4 mice per cage at ambient temperature with a 12 h light-12 h dark cycle (lights on at clock time 07.00 and lights off at 19.00). Mice had *ad libitum* access to food

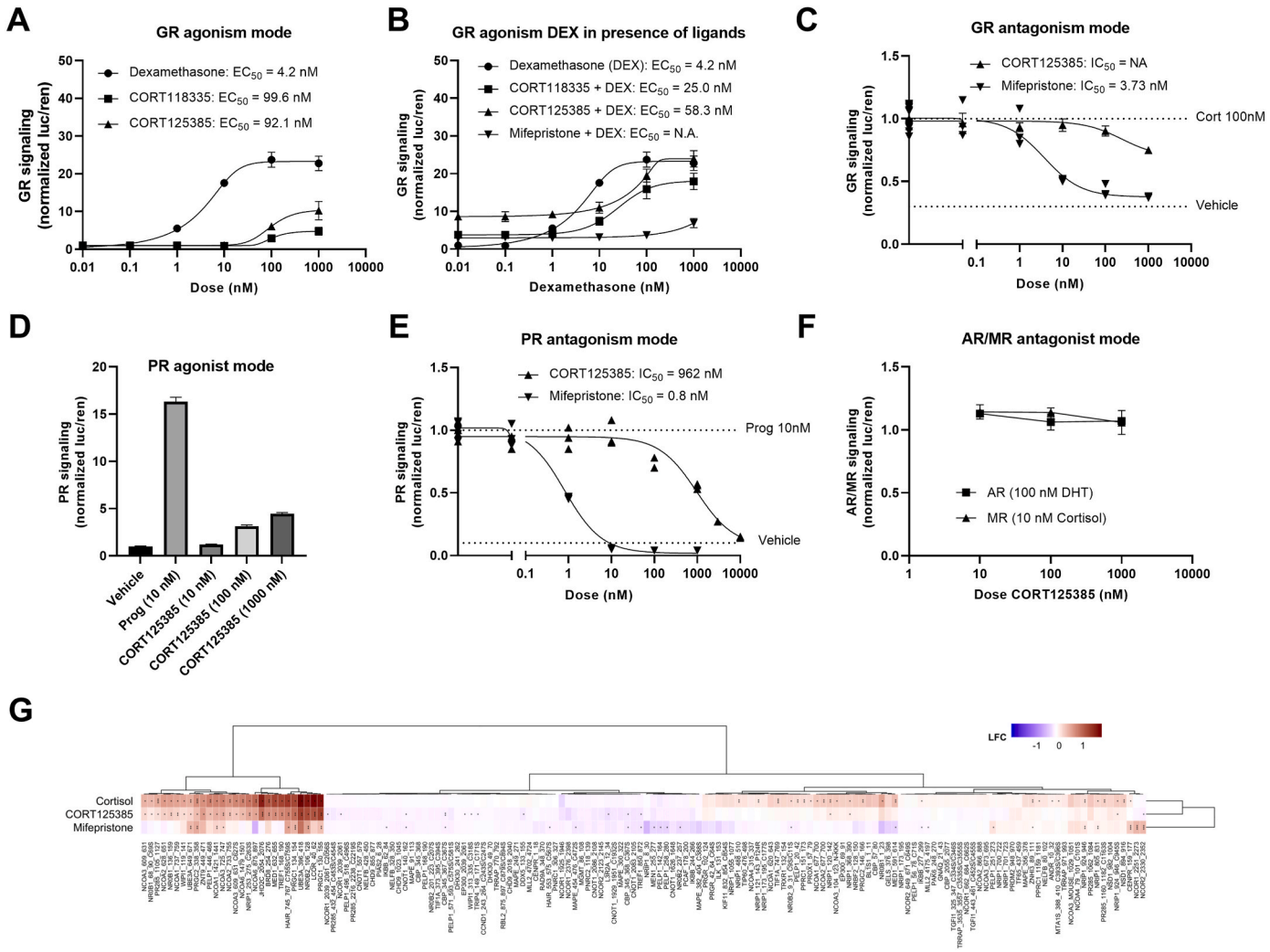

Fig. 2. Characterization of CORT125385 on steroid hormone receptor signaling. (A) Dose-dependent responses to dexamethasone, CORT118335 and CORT125385 on glucocorticoid receptor (GR) signaling in agonism mode in human HEK293 cells. (B) The effect of 1 μM CORT118335, CORT125385 or mifepristone pretreatment on dexamethasone (DEX)-induced GR signaling. The same dexamethasone dose-response curve (without additional ligand) is plotted in the graphs in Fig. 2A and B. (C) The effect of CORT125385 or mifepristone on cortisol-induced GR signaling. (D) The effect of progesterone or CORT125385 progesterone receptor (PR) signaling in agonism mode in human HEK293 cells. (E) The effect of CORT125385 or mifepristone pretreatment on progesterone-induced PR signaling. (F) The effect of CORT125385 pretreatment on cortisol-induced mineralocorticoid receptor (MR) signaling and dihydrotestosterone (DHT)-induced androgen receptor (AR) signaling in human HEK293 cells. EC_{50} and IC_{50} values were calculated using a non-linear fit model. (G) GR-coregulator interactions in the presence of 1 μM cortisol, CORT125385 or mifepristone.

and water, with the exception during fasting.

Male (N = 24) and female (N = 24) 8-week old C57BL/6J mice received high-fat diet (HFD, 60% fat, ResearchDiets D12492) in combination with 10% D-fructose water for a period of 3 weeks. Mice were assigned to one of the following groups: 1) HFD control (N = 8), 2) HFD + mifepristone treatment (N = 8, via diet supplementation, 500 mg compound per kg diet, resulting in an estimated exposure of 60 mg/kg/day), or 3) HFD + CORT125385 treatment (N = 8, via diet supplementation, 500 mg compound per kg diet, resulting in an estimated exposure of 60 mg/kg/day). The dose of 60 mg/kg/day CORT125385 was selected based on pharmacokinetic studies in mice that showed that administration of CORT125385 at this dose resulted in plasma levels expected to have a pharmacological effect.

Body weight and composition were determined every week (echo-MRI). At day 10, blood was collected via a nick in the tail vein within 2 min of removal of the mice from the home-cage, in order to determine circulating levels of corticosterone. At day 20, mice were fasted for 4 h and blood was collected via a nick in the tail vein, in order to determine white blood cell count (WBC, Sysmex XT-2000iV) and plasma levels of total cholesterol (TC), triglycerides (TG), aspartate transaminase (AST) and alanine transaminase (ALT). At day 21, mice were sacrificed between 10.00 h and 13.00 h via CO_2 asphyxiation, perfused for 5 min with ice-cold PBS, and tissues of interest were collected for further molecular and biochemical measurements.

To evaluate different treatment regimens, we performed a follow-up experiment in male (N = 35) 8-week old C57BL/6J mice that received 60% HFD in combination with 10% D-fructose water for a run-in period of 20 days. Mice were assigned to one of the following groups: 1) HFD + vehicle treatment ('vehicle'; 10% DMSO 0.1% Tween-80, 0.5% hydroxypropyl methylcellulose in H_2O; total volume of 100 μl administered via oral gavage; N = 7), 2) HFD + 60 mg/kg daily CORT125385 treatment for 14 days ('daily 14 days'; dissolved in 10% DMSO 0.1% Tween-80, 0.5% hydroxypropyl methylcellulose in H_2O; N = 7), 3) HFD + 60 mg/kg CORT125385 treatment every 2 days ('intermittent 2'; treatment at day 0, 2, 4, 6, 8, 10, 12 and 14; N = 7), 4) HFD + 60 mg/kg CORT125385 treatment every 4 days ('intermittent 4'; treatment at day 2, 6, 10 and 14; N = 7), 5) HFD + 60 mg/kg daily CORT125385 for 7 days followed by 7 days of treatment discontinuation ('daily 7 days'; N

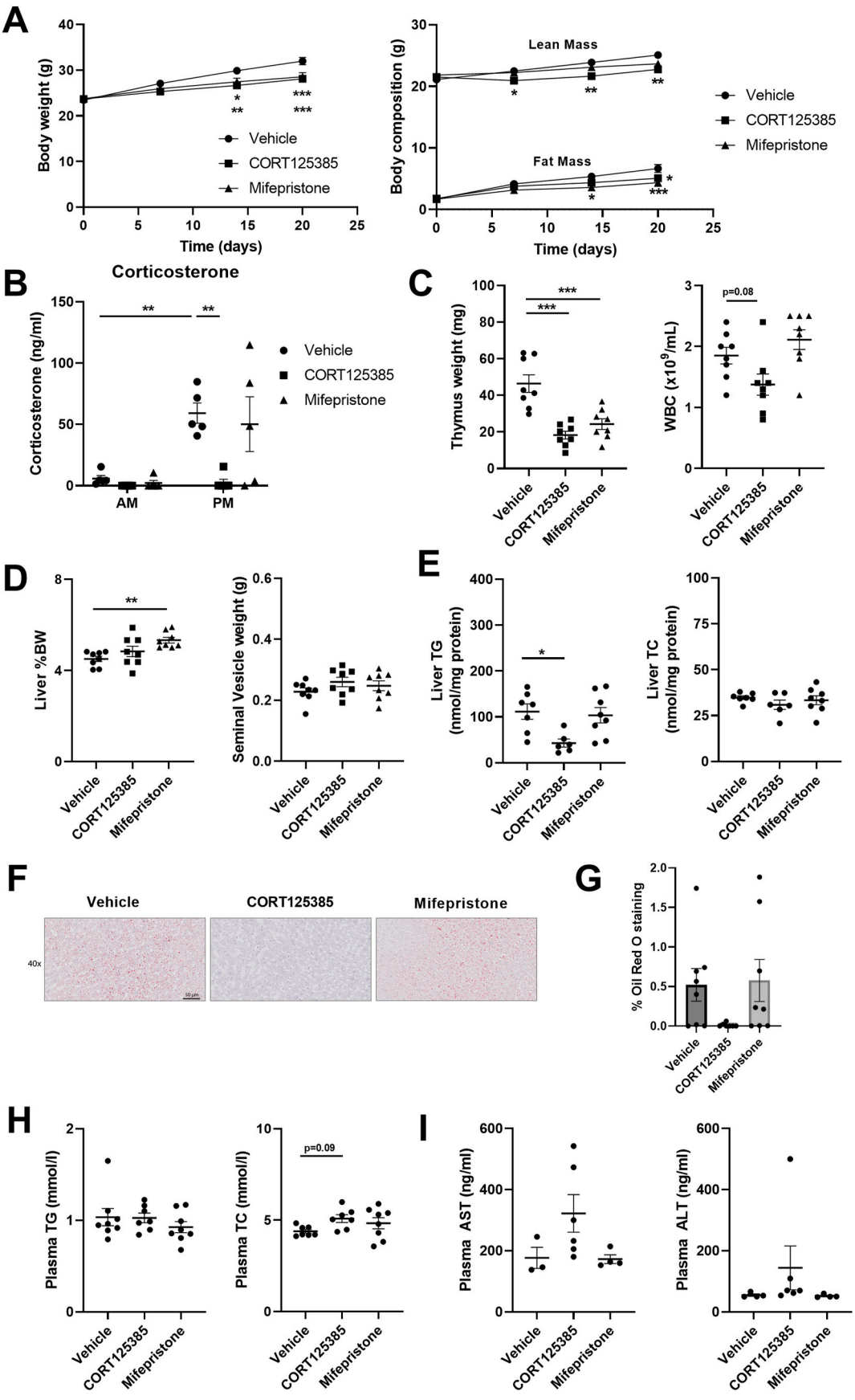

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Fig. 3. CORT125385 treatment alleviates hepatic steatosis in high-fat diet-fed male mice. (A) The effect of continuous CORT125385 or mifepristone treatment on total body weight (left panel) or fat and lean mass (right panel) of male C57BL/6J mice. Statistical differences were calculated using a two-way ANOVA * $p < 0.05$ vs Vehicle, ** $p < 0.01$ vs Vehicle, *** $p < 0.001$ vs Vehicle. Main effect body weight: treatment $p < 0.0001$, time $p < 0.0001$; fat mass: treatment $p = 0.0002$, time $p < 0.0001$; lean mass: treatment $p < 0.0001$, time $p < 0.0001$. The effect of continuous CORT125385 or mifepristone treatment on (B) corticosterone levels at AM (08h00) and PM (18h00), on (C) thymus weight and white blood cell count (WBC). The effect of continuous CORT125385 or mifepristone treatment on (D) liver and seminal vesicle weight, (E) liver triglyceride (TG) and total cholesterol (TC) content. (F) Representative images of the histological analysis and (G) quantification of hepatic content using Oil Red O staining. (H) Plasma levels of TG and TC, and (I) plasma levels of AST and ALT. Statistical differences were calculated using a one-way ANOVA (B–G). N = 8/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

= 7). Body weight and composition were determined every week (echo-MRI). On day 0, 4, 8 and 12, mice were fasted for 4 h and blood was collected via a nick in the tail vein, in order to determine plasma levels of TC, TG and AST. At day 21, mice were sacrificed between 09.00 h and 13.00 h via CO_2 asphyxiation, blood was collected by heart puncture (for WBC and corticosterone), mice were perfused for 5 min with ice-cold PBS, and tissues of interest were collected for further molecular and biochemical measurements.

2.4. Hepatic lipid determination

Lipids were extracted and measured as previously described (Koorneef et al., 2018). In short, HPLC-grade isopropanol was added to liver samples and samples were homogenized, vortexed and incubated at 70 °C for 25 min. The lipid extract was analyzed for TC and TG concentration (ref 03039773190 and 20767107322; Cobas C111 clinical analyzer, Roche). For 3 samples in the experiment with male mice, measurements of hepatic lipid content failed (2 CORT125385 and 1 vehicle).

2.5. Liver histology, Oil Red O staining and quantification

Snap-frozen liver fragments were sectioned at 8 μm per slice on silane-coated glass slides (631–1166, VWR) and air-dried at room temperature for 30 min. Sections were fixed in 3.7% formaldehyde (Sigma-Aldrich) in PBS for 30 min and washed with 60% isopropanol (Merck). After staining with filtered Oil Red O working solution (3 g/L; O0625, Sigma-Aldrich) for 15 min, samples were counterstained with Mayer's Hematoxylin Solution (51275, Sigma-Aldrich) diluted in H_2O (1:4) for 30 s. Finally, sections were mounted with Kaiser's glycerine gelatine (Merck). Quantification of Oil Red O staining was performed using ImageJ Software (National Institutes of Health). For each sample, 2 to 7 pictures of different tissue areas per sample were analyzed by quantifying the amount of stained area and the amount of total picture area. Per sample, the sum of the amount of stained area of each picture was expressed as a percentage of the sum of the amount of total area of each picture. Due to the poor quality, two samples were excluded from the female mice (one in the CORT125385 group and one in the mifepristone group).

2.6. Plasma biochemistry

Plasma samples were analyzed to determine TC (Roche), TG (Roche), AST (ab263882, abcam), ALT (ab282882, abcam) and corticosterone (EIA AC-15F1, Immunodiagnosticsystems). Due to space limitations on the ELISA plates, for the AST, ALT and corticosterone measurements only a subset of samples were analyzed. For several plasma samples, we did not have sufficient volume to perform all measurements (i.e. one plasma TC/TG in the CORT125385-treated male mice and one plasma TG in the vehicle group female mice).

2.7. Real-time quantitative PCR

Total RNA was isolated from frozen liver and oviduct tissue using TriPure RNA isolation reagent (Roche). RNA was reserved transcribed to cDNA using M-MLV reverse transcriptase (Promega), and real-time quantitative PCR was performed with IQ SYBR-Green Supermix on a MyIQ Thermal Cycler (Bio-Rad CFX96). Primer sequences are shown in Table 1. For the expression of *Nr3c3* (PR), CT values above 35 were considered as not detected (ND).

2.8. Western blot

To detect protein expression, we used the WES automated Western blot apparatus (ProteinSimple). Liver lysate was loaded on 66–440 kDA separation modules (BioTechne) and these were incubated with the following antibodies: primary rabbit anti-FABP1 (0.02 μg/ml protein, antibody dilution 1:50; Cell Signaling 13368S), primary rabbit anti-GAPDH (0.8 μg/ml protein, antibody dilution 1:20; Santa Cruz Biotechnology sc-25778), primary mouse anti-actin (0.8 μg/ml protein antibody dilution 1:50, Sigma A2228), secondary HRP anti-rabbit (1:100 dilution; ProteinSimple DM001). Only a subset of samples were analyzed using Western blot. Images of whole blots are shown in Suppl. Figs. 1A–C. For the detection of all proteins, antibody dilution and protein input were previously optimized.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism Software Version 9.3.1. All data are expressed as mean ± SEM. Data were analyzed using a one-way or two-way ANOVA.

3. Results

3.1. Chemical structure and characterization of CORT125385 on steroid hormone receptor signaling

CORT125385 (5-benzyl-6-(4′-chloro-2'-(trifluoromethyl)-2,3,4,5-tetrahydro-[1,1′-biphenyl]-4-yl)pyrimidine-2,4(1*H*,3*H*)-dione; molecular weight 460.88; Fig. 1) was initially selected on the basis of its ability to inhibit dexamethasone-induced tyrosine aminotransferase activity in human HepG2 cells (Ali et al., 2004). In this assay, we determined a maximum inhibition of 98% and a K_i of 38 nM for CORT125385. To evaluate receptor specificity of CORT125385, human HEK293 cells were transfected with a luciferase-reporter that can be activated by the GR, PR, MR or AR. This revealed GR agonism of CORT125385 at 100–1000 nM, albeit at a much lesser extent as compared to dexamethasone but more pronounced than analogue CORT118335 (Fig. 2A). Pretreatment with the high 1 μM dose of CORT125385 caused a right-shift of the dexamethasone dose-response curve and resulted in a higher EC_{50} value of dexamethasone (58.3 nM with CORT125385 pretreatment as compared to 4.2 nM with vehicle pretreatment), similar as CORT118335 (EC_{50} of dexamethasone of 25.0 nM). Mifepristone pretreatment almost completely abolished dexamethasone-induced GR signaling (Fig. 2B). At a constant agonist dose (100 nM cortisol), pretreatment with high concentrations of CORT125385 only showed a modest inhibition of GR signaling, while mifepristone pretreatment potently antagonized cortisol-induced GR signaling with an IC_{50} of 3.7 nM (Fig. 2C). On PR signaling, CORT125385 exhibited both agonistic and antagonistic properties at doses above 100 nM, and with a potency over 1000-fold lower than mifepristone (Fig. 2D and E). We did not observe antagonistic activity of CORT125385 on cortisol-induced MR signaling and DHT-induced AR signaling (Fig. 2F). We evaluated the coregulator interactions of human GR in the presence of cortisol, CORT125385 or

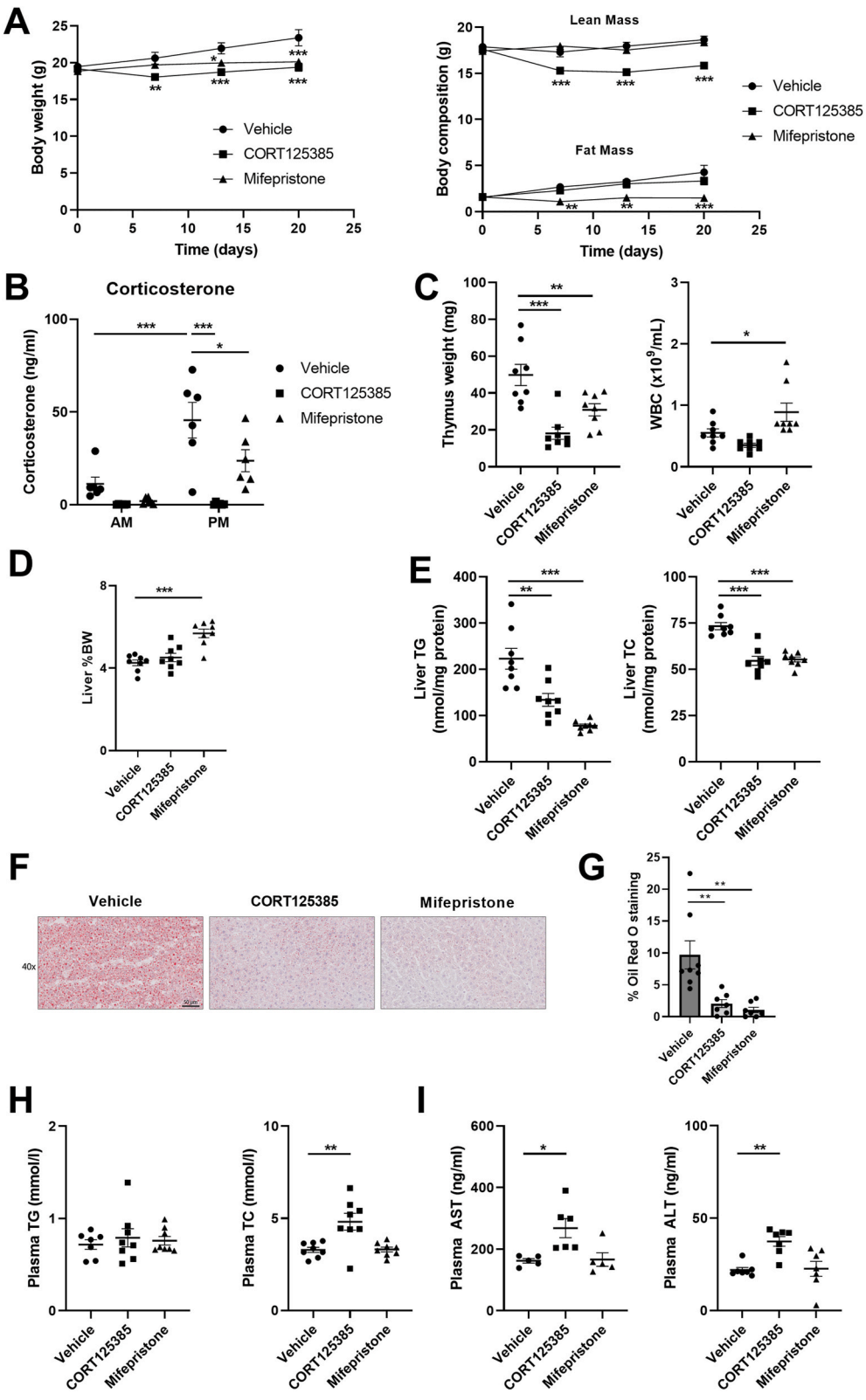

Fig. 4. CORT125385 treatment alleviates hepatic steatosis in high-fat diet-fed female mice. (A) The effect of continuous CORT125385 or mifepristone treatment on total body weight (left panel) or fat and lean mass (right panel) of female C57BL/6J mice. Statistical differences were calculated using a two-way ANOVA; *p < 0.05 vs Vehicle, **p < 0.01 vs Vehicle, ***p < 0.001 vs Vehicle. Main effect body weight: treatment p < 0.0001, time p = 0.0013; fat mass: treatment p < 0.0001, time p < 0.0001; lean mass: treatment p < 0.0001, time p = 0.0029.The effect of continuous CORT125385 or mifepristone treatment on (B) corticosterone levels at AM (08h00) and PM (18h00), on (C) thymus weight and white blood cell count (WBC). The effect of continuous CORT125385 or mifepristone treatment on (D) liver weight, (E) liver triglyceride (TG) and total cholesterol (TC) content. (F) Representative images of the histological analysis and (G) quantification of hepatic content using Oil Red O staining. (H) Plasma levels of TG and TC, and (I) plasma levels of AST and ALT. Statistical differences were calculated using a two-way ANOVA (A) or a one-way ANOVA (B–G). N = 8/group. *p < 0.05, **p < 0.01, ***p < 0.001.

mifepristone, and this revealed that GR-interactome profile of CORT125385 is similar to that of cortisol (but with an overall lower intensity; e.g. for NCOA1, NCOA2, NCOA3, Suppl. Table 1A) and distinct from that of mifepristone (Fig. 2G). Examples of coregulators that are included by CORT125385 include NCOA1-3, NCOR1-2 and NROB1-2 (Suppl. Table 1B).

3.2. CORT125385 treatment alleviates hepatic steatosis in high-fat diet-fed male mice

We next analyzed the effect of CORT125385 treatment in a model of HFD feeding in male mice, in direct comparison with the non-specific GR antagonist mifepristone. CORT125385 and mifepristone treatments

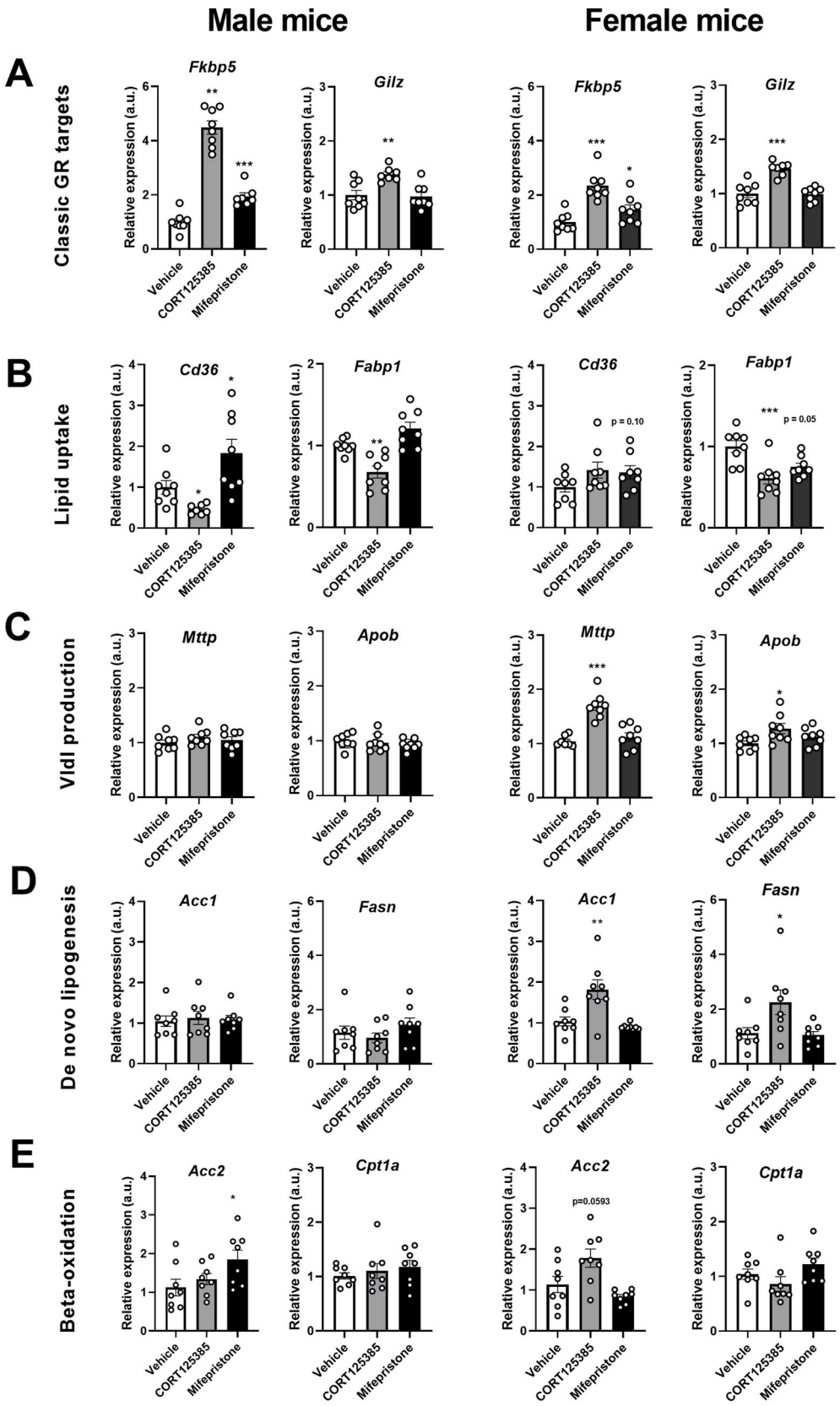

Fig. 5. The effect of CORT125385 and mifepristone on hepatic gene expression. The effect of continuous CORT125385 or mifepristone treatment of male and female C57BL/6J mice on the hepatic expression of (A) GR-responsive genes, (B) genes involved in lipid uptake, (C) genes involved in VLDL-production or (D) liver enzymes. Statistical differences were calculated using a one-way ANOVA. N = 8/group. *p < 0.05 vs Vehicle, **p < 0.01 vs Vehicle, ***p < 0.001 vs Vehicle.

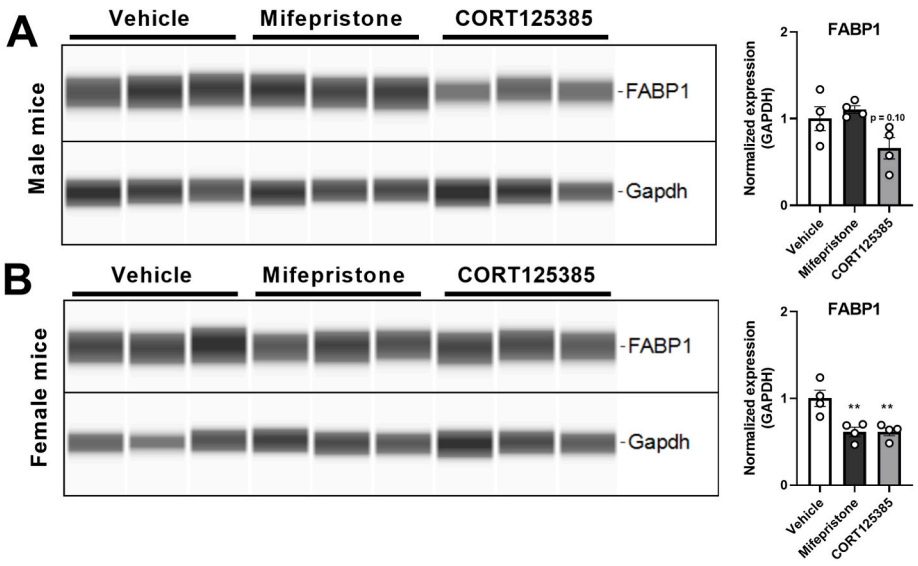

Fig. 6. The effect of CORT125385 and mifepristone on hepatic FAPB1 protein expression.
The effect of CORT125385 and mifepristone on the hepatic protein expression of FABP1 I (A) male and (B) female mice. For each replicate, protein expression of FABP1 was normalized to GAPDH protein expression. Statistical differences were calculated using a one-way ANOVA. N = 4/group. **p < 0.01 vs Vehicle.

both decreased total body weight, which for mifepristone-treated animals was attributed to a significant decrease in fat mass ($p < 0.05$ at day 14, $p < 0.001$ at day 21; Fig. 3A). CORT125385 treatment lowered lean mass ($p < 0.05$ at day 7, $p < 0.01$ at 14 and 21; Fig. 3A). CORT125385 exhibited agonistic GR properties *in vivo*, as indicated by strongly decreased endogenous corticosterone levels at PM (main effect time: $p < 0.0001$; main effect treatment: $p = 0.0044$; Fig. 3B), decreased thymus weight ($p < 0.0001$) and a trend towards lower WBC ($p = 0.08$; Fig. 3C). CORT125385 did not influence total liver weight, nor the weight of the androgen-sensitive seminal vesicles (Fig. 3D). CORT125385 treatment strongly reduced triglyceride levels in the liver ($p < 0.05$), while mifepristone did not influence hepatic lipid content (Fig. 3E). Oil Red O staining of the liver confirmed the strong lipid lowering activities of CORT125385 in the liver (Fig. 3F and G). Plasma triglyceride levels were unaltered by CORT125385 and mifepristone treatment, while a trend for increased plasma total cholesterol levels was observed upon CORT125385 treatment ($p = 0.09$; Fig. 3H). CORT125385 and mifepristone treatment did not significantly influence plasma AST and ALT levels, although AST levels did show more variation after CORT125385 treatment (Fig. 3I).

3.3. CORT125385 treatment alleviates hepatic steatosis in high-fat diet-fed female mice

We next evaluated the therapeutic utility of CORT125385 in female mice fed with HFD for 3 weeks, in an identical experimental setup to that performed in male mice. Both CORT125385 and mifepristone treatment lowered total body weight (Fig. 4A). CORT125385 treatment significantly lowered lean mass ($p < 0.001$ at day 7–21) while mifepristone treatment lowered fat mass ($p < 0.01$ at day 7 and 14, $p < 0.001$ at day 21; Fig. 4A). CORT125385 significantly decreased endogenous corticosterone levels at PM (main effect time: $p < 0.0001$; main effect treatment: $p < 0.0001$; Fig. 4B) and thymus weight ($p < 0.001$; Fig. 4C). Mifepristone treatment also significantly lowered endogenous corticosterone levels and thymus weight, but to a lesser extent ($p < 0.05$ and $p < 0.01$; Fig. 4B and C), but increased WBC ($p < 0.05$; Fig. 4C). CORT125385 did not influence liver weight, while mifepristone significantly increased liver weight (Fig. 4D). We measured lipid accumulation in the liver as a result of HFD feeding, and found that treatment with both CORT125385 and mifepristone strongly reduced hepatic triglyceride and cholesterol content (Fig. 4E). In line with this, Oil Red O staining showed a strong decrease in lipids in livers after both CORT125385 and mifepristone treatment (Fig. 4F and G). CORT125385 treatment elevated plasma cholesterol levels (Fig. 4H), and significantly enhanced plasma AST and ALT levels in female mice (Fig. 4I).

3.4. The effect of CORT125385 on hepatic gene and protein expression

We next performed gene and protein expression analyses on livers obtained from male and female mice treated with CORT125385 or mifepristone. We observed a significant upregulation of GR-responsive genes *Fkbp5* and *Gilz* (*Tsc22d3*) in both male and female mice after CORT125385 treatment ($p < 0.01$ in male mice; $p < 0.001$ in female mice; Fig. 5A), suggesting (partial) GR agonism on these genes. CORT125385 treatment downregulated lipid uptake gene *Cd36* in male, but not female mice, while lipid chaperone gene *Fabp1* was downregulated in both male and female mice (Fig. 5B). *Mttp* and *Apob*, involved in hepatic VLDL-production, were significantly increased upon CORT125385 treatment in female mice, but not in male mice (Fig. 5C). CORT125385 treatment significantly increased the expression of *Acc1* and *Fasn*, genes involved in de novo lipogenesis, in female but not male mice (Fig. 5D). In both male and female mice, genes involved in beta-oxidation were not significantly regulated after CORT125385 or mifepristone treatment (Fig. 5E). In male mice, we found that CORT125385 did not influence the expression of the genes encoding AST and ALT, namely *Got1*, *Got2*, *Gpt1* and *Gpt2* (Suppl. Fig. 2). In female mice, CORT125385 and mifepristone significantly increased expression of *Got1*, *Got2* and *Gpt2* (Suppl. Fig. 2). We did not find a significant effect of CORT125385 treatment on hepatic FABP1 protein in male mice ($p = 0.10$; Fig. 6A), while both CORT125385 and mifepristone treatment readily lowered FABP1 protein expression in female mice (both $p < 0.01$; Fig. 6B).

3.5. CORT125385 treatment does not influence progesterone receptor activity in the oviduct of female mice

In our *in vitro* investigations, we observed some agonistic and antagonistic properties of high doses of CORT125385 on PR signaling. We therefore studied the potential effect of CORT125385 treatment on PR activity *in vivo* by analyzing expression of PR-responsive transcripts in the oviduct of female mice (Akison et al., 2014). We first confirmed PR (*Nr3c3*) expression in the oviduct of female mice (Fig. 7A), and

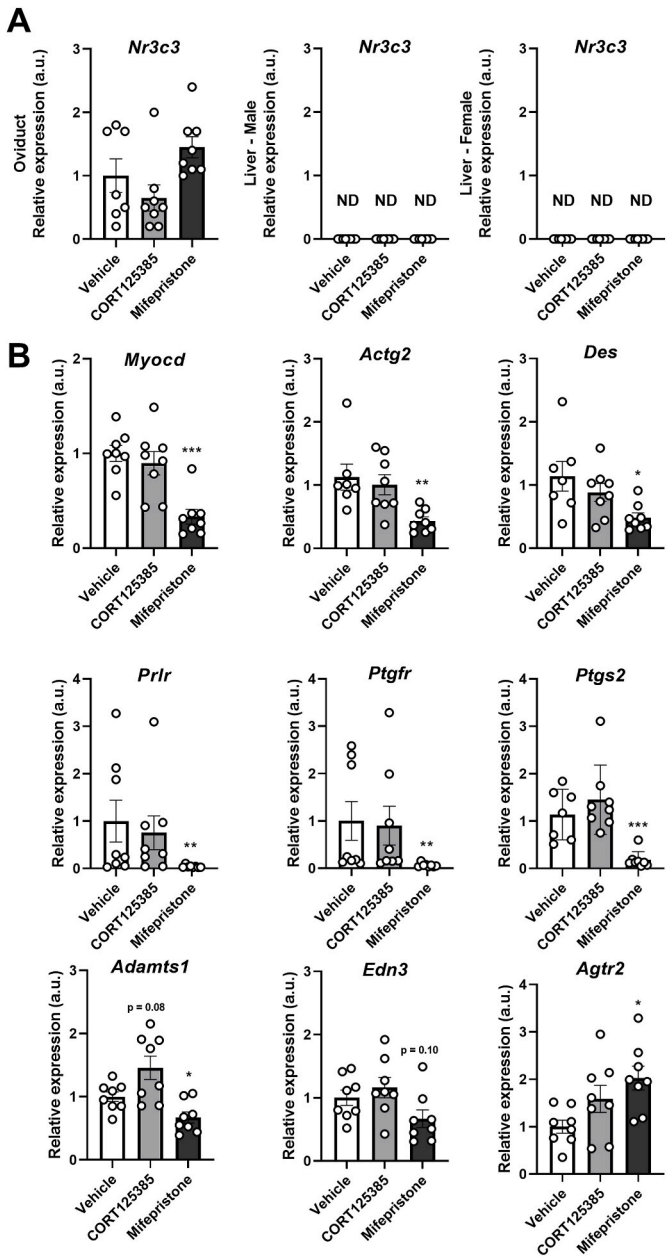

Fig. 7. CORT125385 treatment does not influence progesterone receptor activity in the oviduct of female mice. The effect of continuous CORT125385 or mifepristone treatment on the (A) oviduct and liver expression of the progesterone receptor (*Nr3c3*). (B) Progesterone receptor-responsive genes *Myocd, Actg2, Des, Prlr, Ptgfr, Ptgs2, Adamts1, Edn3* and *Agtr2* in the oviduct. Statistical differences were calculated using a one-way ANOVA. N = 8/group. *p < 0.05 vs Vehicle, **p < 0.01 vs Vehicle, ***p < 0.001 vs Vehicle.

excluded PR expression in both male and female liver (Fig. 7A). As expected, mifepristone treatment had a dominant effect on oviduct PR signaling, as it downregulated PR-responsive genes *Myocd*, *Actg2*, *Adamts1*, *Des*, *Prlr*, *Ptgfr* and *Ptgs2*, while there was a trend for decreased *Edn3* expression and increased *Agtr2* expression (Fig. 7B). In strong contrast with mifepristone, CORT125385 treatment did not influence expression of any of the PR-responsive genes in this panel, suggesting no PR cross-reactivity in the oviduct at the evaluated dose (Fig. 7B).

3.6. Exploring different CORT125385 treatment regimens and the effect of hepatic lipid accumulation

We next evaluated different treatment regimens with CORT125385, in which we compared daily treatment by oral gavage with intermittent treatment (CORT125385 administration every 2 or 4 days) and transient treatment (for 7 consecutive days before treatment discontinuation), all in mice on HFD. Throughout the course of the 2 week treatment period, we monitored total body weight and body composition. Daily treatment for 14 days every day or every 2 days significantly reduced body weight gain after 7 and 14 days (Fig. 8A). Treatment discontinuation after 7 days of daily treatment showed a recovery of body weight comparable to control mice (Fig. 8A). Daily CORT125385 treatment significantly reduced fat mass gain after 14 days. Lean mass was decreased after CORT125385 every day or every 2 days, which recovered after treatment discontinuation (Fig. 8A). As with continuous administration, oral gavage administration of CORT125385 every two or four days diminished endogenous corticosterone levels, an effect that was not observed one week after treatment discontinuation (Fig. 8B). When focusing on the liver, we did not observe an effect of any CORT125385 treatment regimen on total liver weight (Fig. 8C). Liver triglyceride levels were readily decreased by daily CORT125385 treatment for 14 days ($p < 0.05$), while a trend was observed for CORT125385 treatment every 2 days ($p = 0.06$), and no effects were observed upon CORT125385 treatment every 4 days or after treatment discontinuation (Fig. 8D). Consistent with our previous study in male mice, we did not observe any changes on hepatic total cholesterol levels by CORT125385 treatment (Fig. 8D). As a readout for systemic glucocorticoid agonism, we analyzed thymus weight and white blood cell count (WBC). We observed significant decreases in both readouts after treatment with CORT125385 daily, every 2 and every 4 days (Fig. 8E). Transient treatment for the first 7 days and treatment discontinuation thereafter revealed no change in thymus weight relative to vehicle treatment, suggesting that the effect of repeated CORT125385 treatment on thymus weight is reversible (Fig. 8E). Plasma biochemistry measurements showed an increase in 6 h-fasted plasma total cholesterol levels after 4 and 8 days of daily treatment with CORT125385, and no significant changes on plasma TG (Fig. 8F). Finally, we did not observe any robust changes in plasma AST levels (Fig. 8G). The GR-responsive gene *Fkbp5* was upregulated after daily treatment for 14 days and intermittent treatment every 2 and 4 days, but not after treatment discontinuation (daily 7 group; Fig. 9A). Daily and intermittent treatment every 2 days with CORT125385 seemed to downregulate expression of *Cd36* and *Fabp1*, although this was not significant, while a significant downregulation of *Fabp1* was observed after treatment every 4 days (Fig. 9A). On protein level, we observed an apparent modest and not statistically significant reduction of FABP1 protein after daily treatment with CORT125385 for 14 days (Fig. 9B).

4. Discussion

In this study, we report on the novel GR modulator CORT125385, an analogue of the previously reported miricorilant (CORT118335) (Hunt et al., 2012, 2021; Koorneef et al., 2018). As expected, we found highly similar effects of CORT125385 and miricorilant, with strong lipid lowering effects in the livers of high-fat diet-fed mice. These effects are likely GR-mediated, given that CORT125385 and miricorilant share the GR as a target, while the respective cross-reactivities for the PR and MR are compound-specific.

We observed sex-specific effects in response to treatment with GR ligands mifepristone and CORT125385. Most strikingly, we found that treatment with the GR antagonist mifepristone exhibited strong sexually dimorphic effects as the hepatic lipid-lowering effect of mifepristone is female-specific. The sex-differences of CORT125385 effects are more subtle with female-specific cholesterol-lowering effects but triglyceride-lowering effects in both sexes. Sex-specific effects in the response to

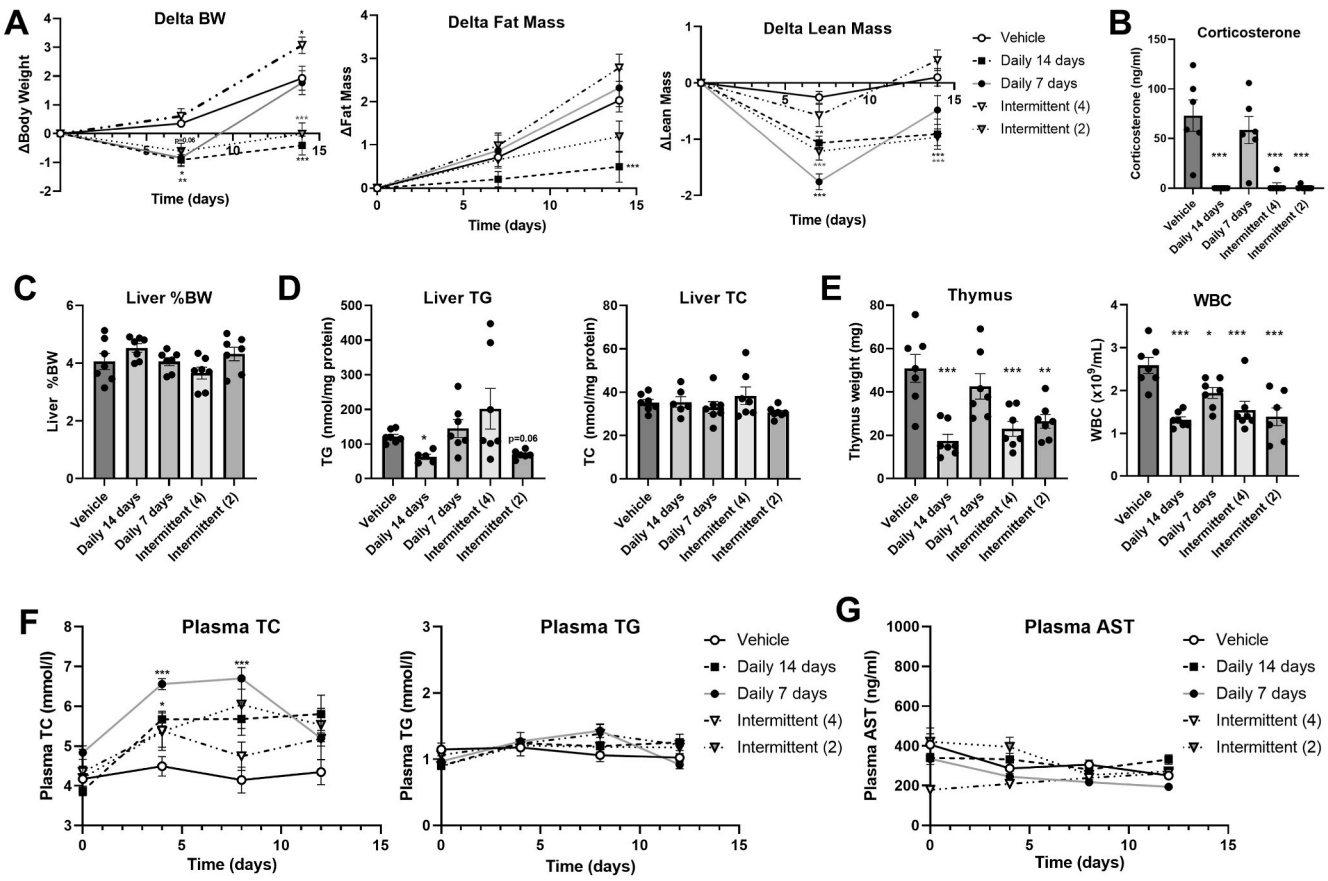

Fig. 8. Exploring different CORT125385 treatment regimens and the effect on hepatic lipid accumulation. The effect of daily, intermittent or transient oral gavage treatment of male C57Bl/6 mice with CORT125385 on (A) delta body weight, fat mass and lean mass (normalized to day 0; all in grams), (B) plasma corticosterone levels at endpoint, (C) liver weight (% of body weight), (D) liver lipid content, (E) thymus weight and white blood cell count (WBC), (F) plasma levels of total cholesterol (TC) and triglycerides (TG) after a 4 h fast and (G) plasma AST levels. Statistical differences were calculated using a two-way ANOVA with repeated measures (A, F, G) or a one-way ANOVA (B–E). N = 6–7/group. *p < 0.05 vs. Vehicle, **p < 0.01 vs Vehicle, ***p < 0.001 vs Vehicle.

glucocorticoid agonists (corticosterone) were shown previously for glucose metabolism (Kaikaew et al., 2019), fat accumulation (Gasparini et al., 2019) and muscle function (Li et al., 2022), while GR antagonist treatment with CORT125329 showed sex-specific gene regulation in brown adipose tissue (Kroon et al., 2021). Another noteworthy sex difference is consistent and significant increase in plasma AST and ALT in female mice after CORT125385 treatment, while these liver enzymes were variable in male mice with no consistent increase upon CORT125385 treatment. We do not understand the reason for this sex-specific transaminitis. Transaminases link to gluconeogenesis, and it was shown that there are quite notable sex differences in activation of gluconeogenesis in response to fasting (Della Torre et al., 2018). The removal of triglycerides from the liver may likewise trigger sex-dependent responses in hepatocytes. We did not observe increased hepatic inflammation (in male and female mice) based on histological examination. Sex-specific functional responses to CORT125385 and mifepristone can be related to differential GR signaling in male and female subjects. PR is not expressed in the liver of male and female mice, and we therefore rule out that hepatic PR is involved in the sexual dimorphic effects. We cannot exclude that the ligands influence PR signaling in other tissues, which via endocrine mechanisms could influence lipid accumulation in the liver. It seems unlikely that this plays a role in the beneficial effects of CORT125385, as we observed no effect on the expression of PR-responsive genes in the oviduct at therapeutically active doses in female mice.

We observed that CORT125385 regulated gene expression of several targets that are involved in hepatic lipid metabolism. Also for gene regulation, we found sex-dependent effects of CORT125385 treatment as *Cd36* expression was downregulated in the livers of male mice with no obvious effect in female mice. *Fabp1* mRNA is of particular interest as the regulation of this readout most closely mirrored the therapeutic lowering of hepatic lipid content by glucocorticoid ligands. More specifically, CORT125385 downregulated the expression of this gene in both male and female mice, both also presenting with lower lipid content upon CORT125385 treatment. On the other hand, mifepristone treatment lowered *Fabp1* expression only in female mice, in which a decrease in hepatic triglycerides and cholesterol was also observed, while in male mice *Fabp1* expression was not influenced by mifepristone treatment and hepatic lipid content was not changed. Analysis of FABP1 protein was in line with the patterns observed at the RNA level. FABP1 is involved in intracellular lipid transport and is predominantly found in the cytoplasm of hepatocytes (Wang et al., 2015), where it is capable of binding long-chain fatty acids, in addition to many other intracellular ligands. However, a previous study in rats showed a downregulation of hepatic FABP1 expression upon dexamethasone treatment (Foucaud et al., 1998), a GR ligand that does not lower hepatic lipid content (Koorneef et al., 2018). It is thus unclear from our study if regulation of FABP1 expression is causally involved in the therapeutic effect of CORT125385, or if the decreased FABP1 expression reflects its response to changes in local lipid levels. Given that the GR has many target genes in any context, we conclude that FABP1 expression may be part of one of the ensembles of regulated proteins that is able to reduce liver triglyceride content.

We investigated different treatment regimens with CORT125385 and found that intermittent treatment every 2 days is sufficient to lower hepatic lipid content while treatment every 4 days is not. We did observe

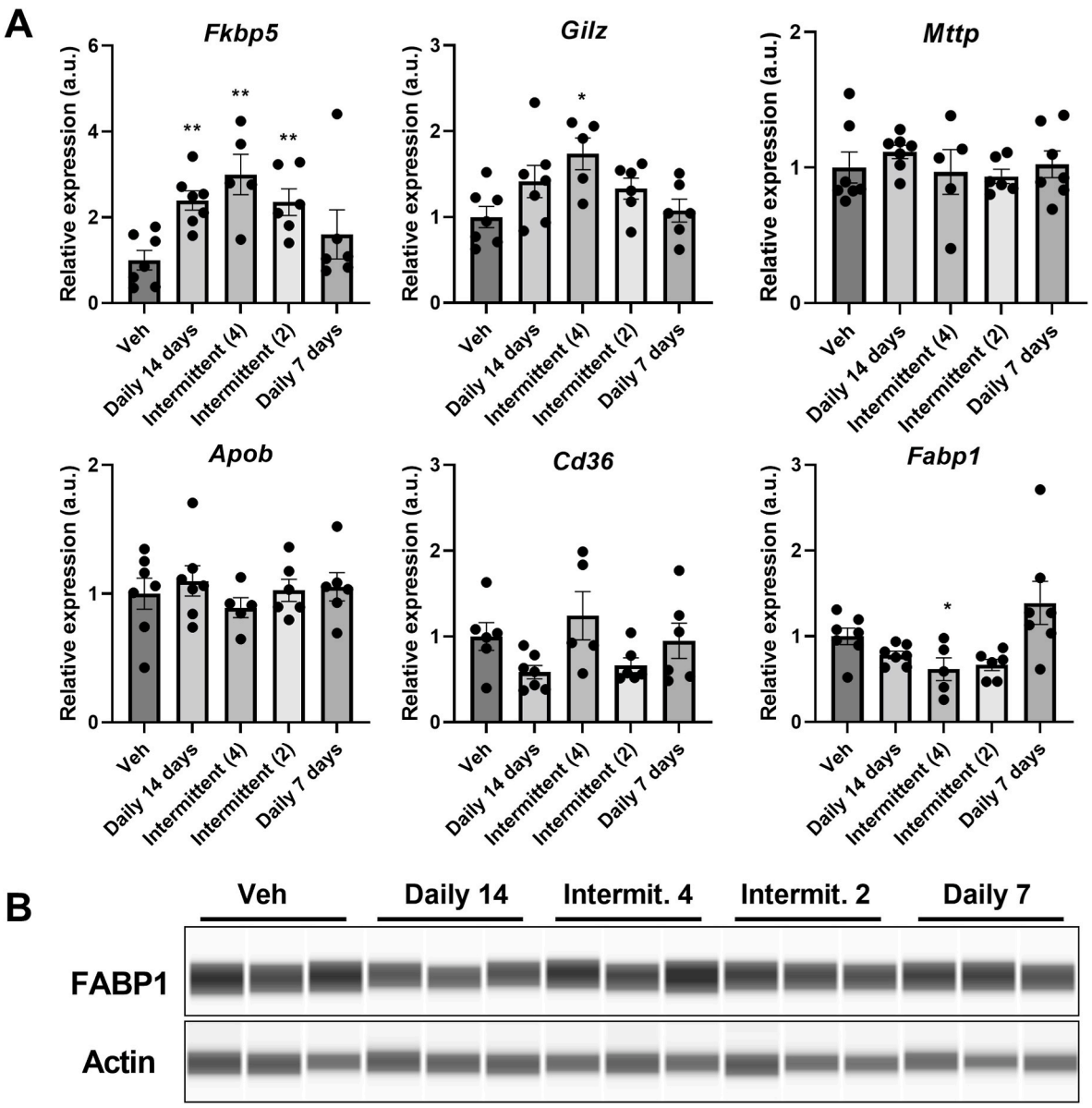

Fig. 9. The effect of CORT125385 on hepatic gene and protein expression. (A) The effect of continuous, intermittent and transient CORT125385 treatment of male C57BL/6J mice on the hepatic expression of GR-responsive genes, genes involved in lipid uptake and genes involved in VLDL-production. Statistical differences were calculated using a one-way ANOVA. N = 6–7/group. *p < 0.05 vs Vehicle, **p < 0.01 vs Vehicle. (B) FABP1 protein expression after vehicle or CORT125385 treatment. N = 4–5/group.

transcriptional effects after treatment every 4 days with significant effects on *Fkbp5*, *Gilz* and *Fabp1* expression, but this is likely related to the experimental design in which the last CORT125385 dose was administered 24 h before sample collection. The therapeutic (lipid-lowering) effects of CORT125385 were transient, and discontinuation of dosing for 7 days results in hepatic lipid levels comparable to 5 weeks of HFD feeding. Also other effects by CORT125385, i.e. on plasma corticosterone, thymus weight, WBC and lean mass, were transient and recovered within a week of treatment discontinuation.

Our study has several limitations. Firstly, we did not directly compare CORT125385 to miricorilant in a head-to-head comparison *in vivo*, but rather chose mifepristone as a benchmark drug and a positive control for PR cross-reactivity. Secondly, we used PR-responsive gene expression in the oviduct as a proxy for PR cross-reactivity (Akison et al., 2014), but we did not exclude the possibility that PR signaling is affected by CORT125385 in other tissues in the female reproductive tract. It is thus still possible that CORT125385 exerts (parts of) its activities via non-GR-mediated mechanisms. With regards to other nuclear steroid receptors, we did not observe cross-reactivity for the AR and MR in this study, and previous unpublished data also exclude binding of CORT125385 to the structurally distinct estrogen receptor (Thornton, 2001). Finally, the method of administration differed between experiments. In the first two experiments in male and female mice we aimed to provide a proof-of-principle that CORT125385 lowers hepatic lipid content and achieved this by diet-administration of the compound. In the last experiment we wanted to study the effects of the compound in a more clinically-relevant setting which we performed by daily oral administration.

Based on our studies in male and female mice, we propose that CORT125385 is a promising novel treatment option for NAFLD via modulating GR activity, and that it provides a suitable alternative for miricorilant.

CRediT authorship contribution statement

Jan Kroon: Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Validation, Writing – original draft, Writing – review & editing. **Max Gentenaar:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Tijmen J.A. Moll:** Formal analysis, Investigation, Validation, Writing – review & editing. **Hazel Hunt:** Conceptualization, Resources, Writing – review & editing. **Onno C. Meijer:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

MG and TJAM have nothing to declare. HH is employed by Corcept Therapeutics and JK works on a secondment basis for Corcept Therapeutics. OCM receives funding from Corcept Therapeutics, which is used to fund this study.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2023.176012.

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