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Association between 12α-hydroxylated bile acids and hepatic steatosis in rats fed a high-fat diet

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Running title

12α-hydroxylated BA and hepatic steatosis

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Abbreviations: Acta2, actin alpha 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AU, arbitrary unit; BA, bile acid; Cyp3a2, cytochrome P450 family 3 subfamily A member 2; Cyp7a1, cholesterol 7α-hydroxylase; Cyp27a1, sterol 27-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; CA, cholic acid; Col1α1, collagen type I alpha 1; CDCA, chenodeoxycholic acid; Cidea, cell death inducing DFFA like effector A; Cpt1, carnitine O-palmitoyltransferase 1; DCA,

deoxycholic acid; α-epoxy, 5α-epoxycholesterol; β-epoxy, 5β-epoxycholesterol; Fasn, fatty acid synthase; Fn1, fibronectin 1; Fxr, farnesoid X receptor; HF, high-fat; Hmgcr, hydroxymethylglutaryl-CoA reductase; IL-1β, interleukin-1 beta; IL-6, interleukin 6; 6keto, 6-ketocholestanol; 7keto, 7-ketocholesterol; LCA, lithocholic acid; Lxr, liver X receptor alpha; MCA, muricholic acid; NAFLD, nonalcoholic fatty liver disease; NDCA, nordeoxycholic acid; 7oDCA, 7-oxodeoxycholic acid; 12oLCA, 12-oxolithocholic acid; 4βOH, 4β-hydroxycholesterol; 7αOH, 7α-hydroxycholesterol; 7βOH, 7β-hydroxycholesterol; 12αOH BAs, 12α-hydroxylated BAs; 19OH, 19-hydroxycholesterol; 25OH, 25-hydroxycholesterol; 27OH, 27-hydroxycholesterol; Rplp0, ribosomal protein lateral stalk subunit P0; Shp, small heterodimer partner; Star, steroidogenic acute regulatory protein; Srebp, sterol regulatory element-binding protein; TCA, taurocholic acid; TG, triglyceride; TNFα, tumor necrosis factor alpha; βtriol, cholestan-3β,5α,6β-triol; UDCA, ursodeoxycholic acid.

Abstract (up to 250 words)

High-fat (HF) diet induces hepatic steatosis that is a risk factor for noncommunicable diseases such as obesity, type 2 diabetes, and cardiovascular disease. Previously, we found that HF feeding in rats increases the excretion of fecal bile acids (BAs), specifically 12α-hydroxylated (12αOH) BAs. Although the liver is the metabolic center in our body, the association between hepatic steatosis and 12αOH BAs in HF-fed rats is unclear. Thus, we investigated extensively BA composition in HF-fed rats and evaluated the association between hepatic steatosis and 12αOH BAs. Acclimated male inbred WKAH/HkmSlc rats were divided into two groups and fed either control or HF diet for eight weeks. Feeding HF diet increased hepatic triglyceride and total cholesterol concentrations, which correlated positively with 12αOH BAs concentrations but not with non-12αOH BAs in the feces, portal plasma, and liver. Accompanied by the increase in 12αOH BAs, the rats fed HF diet showed increased fat absorption and higher mRNA expression of liver *Cidea*. The enhancement of 12αOH BA secretion may contribute to hepatic steatosis by the promotion of dietary fat absorption and hepatic *Cidea*

mRNA expression. The increase in 12αOH BAs was associated with enhanced liver cholesterol 7α-hydroxylase (*Cyp7a1*) and sterol 12α-hydroxylase (*Cyp8b1*) mRNA expression. There was a significant increase in 7α-hydroxycholesterol, a precursor of BAs, in the liver of HF-fed rats. Altogether, these data suggest that the HF diet increases preferentially 12αOH BAs synthesis by utilizing the accumulated hepatic cholesterol and enhancing mRNA expression of *Cyp7a1* and *Cyp8b1* in the liver.

Keywords: Bile acids and salts/Metabolism, Cholesterol 7-alpha hydroxylase, Cholesterol/Metabolism, Diet and dietary lipids, Lipids, Obesity

1. Introduction

Epidemiological studies have shown that hepatic steatosis is a risk factor for the future development of noncommunicable diseases, including obesity, type 2 diabetes, and cardiovascular disease [1]. The development of hepatic steatosis is associated closely with excessive energy consumption [2]. Specifically, a diet high in saturated fat is metabolically more harmful to the liver than unsaturated fat or simple sugars [3]. In animal experiments, a lard-based high-fat (HF) diet, which includes a larger amount of saturated fat than a vegetable oil-based diet, causes fatty liver [4]. In contrast, the consumption of polyunsaturated fatty acids alleviates hepatic steatosis and improves insulin sensitivity in animals and humans [5,6]. However, the underlying factors involved in the above processes are not understood fully.

A significant change induced by the HF diet is alteration in bile acids (BAs) metabolism [7,8]. BAs are end products of cholesterol catabolism in the liver [9]. BAs have unique amphipathic structure, they can solutize lipids by forming mixed micelles [10] and contribute significantly to dietary lipid absorption because fatty acid uptake by the enterocyte is so rapid that diffusion becomes

rate limiting in the overall absorptive process [11]. After facilitating fat absorption in the small intestine, the secreted BAs is reabsorbed through the portal vein into the liver and reused. This process is called enterohepatic circulation [12]. The two critical enzymes in the BA synthesis pathway are cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1). The former is the rate-limiting enzyme of BA synthesis [13], and the latter controls the production of 12α-hydroxylated (12αOH) BAs [14]. The main products of the 12αOH BAs and non-12αOH BAs in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA), respectively. Additionally in rodents, CDCA is hydroxylated further at position-6 of the steroid ring immediately after its synthesis and converted to muricholic acids (MCAs). These primary BAs are conjugated with taurine or glycine in the liver before secretion. In the large intestine, gut microbiota deconjugates BAs and modifies further to produce secondary BAs by 7α-dehydroxylation [15]. Deoxycholic acid (DCA) is a major secondary 12αOH BAs that is generated from CA.

The importance of 12αOH BAs in metabolic disorders has been reported in a human study [16] and showed an association of low 12αOH BA levels in serum with lower body mass index, insulin sensitivity, and improvement of fatty liver. In rats, we previously found that HF diet ingestion results in a selective increase in fecal 12αOH BAs in rats [8]. In mice, consumption of a diet high in saturated fat but not polyunsaturated fat, enhances bile secretion of TCA in mice [17]. With regard to regulation of the BA metabolism, a specific reduction of 12αOH BAs by *Cyp8b1*$^{-/-}$ in mice protects Western diet-induced hepatic steatosis due to impaired fat absorption [18]. In addition, supplementation of taurocholic acid (TCA), a major 12αOH BA, to the *Cyp8b1*$^{-/-}$ mice restored fat absorption [18]. Therefore, we hypothesized that HF diet-induced increase of 12αOH BAs is associated with the development of hepatic steatosis by promoting fat absorption. To our knowledge, no studies have shown the association between 12αOH BAs and diet-induced hepatic steatosis. This study aims to show the increase in 12αOH BAs associated with a HF diet potentially contributes to the development of hepatic steatosis by promoting fat absorption.

2. Methods

2.1. Animal experiments and sample collection

The Institutional Animal Care and Use Committee of National Corporation Hokkaido University approved this study (approval number: 17-0050), and all animals were maintained following the guidelines of Hokkaido University Manual for Implementing Animal Experimentation. Male inbred WKAH/HkmSlc rats (3-weeks old; Japan SLC Inc., Shizuoka, Japan, NBRP Rat No: 0154) were housed individually in a controlled environment at 22 ± 2°C temperature and 55 ± 5% humidity. The light period was from 08:00 to 20:00. The rats had free access to food and water during the entire study period and were acclimated for two weeks with the AIN-93G-based control diet [19]. They were divided into two groups (n = 5-6 per group) and fed control diet (7% fat from soybean oil, wt/wt) or the HF diet (7% from soybean oil and 23% from lard, wt/wt) (Table 1) for 8-weeks. We measured cholesterol (Chol) level in casein and lard used in the present study and found to be 80 mg/kg and 860 mg/kg, respectively. Body weight and food intake were measured every two days. At the end of the 8-week experimental period, the rats were anesthetized by injecting sodium pentobarbital (50 mg/kg body weight). The aortic blood was collected into a syringe containing heparin (final concentration at 50 IU/mL) and aprotinin (final concentration at 500 KIU/mL) for separating plasma. All rats were euthanized by exsanguination. The liver and epididymal adipose tissue were removed from the animals and weighed, and all tissue samples collected within four hours. Feces were collected for 24 hours at the end of the experiment. The plasma was separated by centrifugation at 2,000 × g for 10 min at 4°C. The collected samples were stored at −80°C until analysis. Food deprivation was not conducted in the experiment. The same animal samples were used for evaluating the plasma lipid profile using untargeted lipidomics [20] in another part of the study.

2.2. Biochemical analysis

Liver and fecal lipids were analyzed as described earlier [21]. In brief, lipids were extracted from fresh liver and freeze-dried feces using chloroform: methanol = 2:1 (v/v) solution [22]. The extracts were evaporated and dissolved in 2-propanol for measurement. Chol, triglyceride (TG), and free fatty

acid (FFA) levels in the extracts were determined using a cholesterol E-test, triglyceride E-test, and NEFA C-test Wako kits (Fujifilm Wako Pure Chemical Corporation, Ltd., Osaka, Japan), respectively. The Chol level in lard and casein was analyzed for determining the concentration of Chol in each diet. Chol was extracted using 2-propanol from 1 g of lard or casein. Chol concentration in the extract was determined using a cholesterol E-test Wako kit (Fujifilm Wako). Hepatic free cholesterol levels were measured as described by Sperry and Webb [23]. The activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by the transaminase CII-test Wako kit (Fujifilm Wako).

2.3. Quantitative polymerase chain reaction (qPCR)

qPCR was performed using an Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) with SYBR Green method or TaqMan method as described earlier [21]. The SYBR Green method was performed with specific primer pairs for *ribosomal protein lateral stalk subunit P0* (*Rplp0*, forward: 5'-GGCAAGAACACCATGATGCG-3', reverse: 5'-GTGATGCCCAAAGCTTGGAA-3', 5 μM) and for *steroidogenic acute regulatory protein* (*Star*, forward: *5'*-GCCACACACTTTGGGGAGAT-3', reverse: 5'-GTGGTGGGCAGTCCTTAACA-3', 2.5 μM). Relative expression levels were calculated for each sample after normalization to *Rplp0* as a reference gene using the standard curve method. The TaqMan method was performed with TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) as follows: Rn03302271_gH for *Rplp0*, Rn00561420_m1 for *interleukin 6* (*IL-6*), Rn00580432_m1 for *interleukin-1 beta* (*IL-1β*), Rn99999017_m1 for *tumor necrosis factor alpha* (*TNFα*), Rn01759928_g1 for *actin alpha 2* (*Acta2*), Rn00569575_m1 for *fibronectin 1* (*Fn1*), Rn01463848_m1 for *collagen type I alpha 1* (*Col1α1*), Rn01495769_m1 for *sterol regulatory element-binding protein 1* (*Srebp1*), Rn01463550_m1 for *fatty acid synthase* (*Fasn*), Rn00580702_m1 for *Cpt1* (*Carnitine O-palmitoyltransferase 1*), Rn04181355_m1 for *cell death inducing DFFA like effector A* (*Cidea*), Rn01502638_m1 for *Srebp2*, Rn00565598_m1 for *hydroxymethylglutaryl-CoA reductase* (*Hmgcr*), Rn00756461_m1 for *cytochrome P450 family 3 subfamily A member 2* (*Cyp3a2*) which is a ortholog

of human *Cyp3a4*, Rn00572658_m1 for *farnesoid X receptor* (*Fxr*), Rn00589173_m1 for *small heterodimer partner* (*Shp*), Rn00581185_m1 for *liver X receptor alpha* (*Lxr*), Rn01445029_s1 for 12α-hydroxylase (*Cyp8b1*), Rn00564065_m1 for cholesterol 7α-hydroxylase (*Cyp7a1*), and Rn00710297_m1 for sterol 27-hydroxylase (*Cyp27a1*). Relative expression levels of these target mRNAs were calculated for each sample as shown in the SYBR Green method.

2.4. Oxysterol analysis

Liver oxysterol levels were analyzed using an earlier method [24] with minor modifications. Livers for oxysterol extraction were stored at −80°C until use, and lipids were extracted with chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene. After overnight saponification, unsaponified lipids were extracted with hexane. The extracted lipids were applied to a Sep-Pak Silica Vac cartridge (Nihon Waters K.K., Tokyo, Japan) for separating the oxysterols from cholesterol. The cartridge was equilibrated with hexane and eluted sequentially with a mixture of hexane and 2-propanol (1:200, v/v), and hexane and 2-propanol (3:7, v/v), thus enabling sequential elution of cholesterol and 19-hydroxycholesterol (5-cholesten-3β,19-diol, 19OH) plus oxysterols, respectively. After evaporating the oxysterol-containing solvent fraction under N_2, the dried residues were converted to trimethylsilyl ethers. Oxysterol was quantified by gas chromatography-mass spectrometry using a Shimadzu GC-17A version 3 instrument (Shimadzu Corporation, Kyoto, Japan) coupled with an SPB-1-fused silica capillary column (60 m × 0.25 mm i.d., 0.25 μm thickness, Supelco Inc., Bellefonte, PA, USA) connected to a QP5050A series mass-selective detector (Shimadzu). We used the following temperature program using helium (high purity, 99.9999%) as a carrier gas at a flow rate of 1.5 ml/min: 180°C for 1 min; from 180 to 250°C (20°C/min); from 250 to 290°C (5°C/min); and 290°C for 32.5 min. The total run time was 45 min. The injector was operated at a split ratio of 1:5, maintained at 300°C with the detector transfer line maintained at 300°C. The concentrations of individual oxysterols were measured using 19OH (Steraloids, Inc., Newport, RI, USA) as the internal standard. The oxysterols analyzed in this study were as follows: 4β-

hydroxycholesterol (5-cholesten-3β,4β-diol, 4βOH), 6-ketocholestanol (5α-cholestan-3β-ol-6-one, 6keto), 7-ketocholesterol (5-cholesten-3β-ol-7-one, 7keto), 7α-hydroxycholesterol (5-cholesten-3β,7α-diol, 7αOH), 7β-hydroxycholesterol (5-cholesten-3β,7β-diol, 7βOH), 5α-epoxycholesterol (cholestan-5α,6α-epoxy-3β-ol, α-epoxy), 5β-epoxycholesterol (cholestan-5β,6β-epoxy-3β-ol, β-epoxy), cholestan-3β,5α,6β-triol (β-triol), 25-hydroxycholesterol (5-cholesten-3β,25-diol, 25OH), 27-hydroxycholesterol (25*R*-cholest-5-en-3β,26-diol, 27OH), 22(*R*)-hydroxycholesterol (5-cholestene-3β,22(*R*)-diol), 7α,25-dihydroxycholesterol (5-cholesten-3β,7α,25-triol), and 24(*S*)-hydroxycholesterol (5-cholesten-3β,24(*S*)-diol).

2.5. BA analysis

BA extraction was performed as described earlier [25] with minor modifications. Briefly, an aliquot of the liver and fecal samples was freeze-dried and weighed for calculation of water content. Approximately 100 mg of ground dried samples were used for subsequent extraction steps. Plasma samples 100 µl each were freeze-dried for later use. 23-nor-5β-cholanic acid-3α,12α-diol (nordeoxycholic acid, NDCA) dissolved in ethanol was added as an internal standard for each sample (25 nmol for liver and plasma samples, and 250 nmol for feces). The samples were subjected to sonication and heating. Plasma samples with ethanol were homogenized before sonication and heating in boiled water. After centrifugation, the supernatant was collected and evaporated. The extraction step with ethanol was repeated twice. Next, methanol was added to the dried extracts and purified with an HLB cartridge (Waters, Milford, MA) [25]. The purified extracts were evaporated and stored at -30°C. The extracts were reconstituted with methanol and analyzed by an LC-MS system, as described earlier [26]. Individual BA concentrations were measured using NDCA as an internal standard. Primary 12αOH BAs were measured as follows: 5β-cholanic acid-3α,7α,12α-triol (CA); 5β-cholanic-3α,7α,12α-triol-*N*-(2-sulphoethyl)-amide (TCA); 5β-cholanic-3α,7α,12α-triol-*N*-(carboxymethyl)-amide (glycocholic acid, GCA). Secondary 12αOH BAs were measured as follows: 5β-cholanic acid-3α,12α-diol (DCA); 5β-cholanic-3α,12α-diol-*N*-(2-sulphoethyl)-amide (taurodeoxycholic acid, TDCA); 5β-cholanic-3α,12α-diol-*N*-(carboxymethyl)-amide

(glycodeoxycholic acid); 5β-cholanic acid-3α,12α-diol-7-one (7-oxodeoxycholic acid, 7oDCA); 5β-cholanic acid-3α-ol-12-one (12-oxolithocholic acid, 12oLCA); 5β-cholanic acid-12α-ol-3-one (3o12α); 5β-cholanic acid-3α,7β,12α-triol (ursocholic acid, UCA). Primary non-12αOH BAs were measured as follows: 5β-cholanic acid-3α,7α-diol (CDCA); 5β-cholanic-3α,7α-diol-*N*-(2-sulphoethyl)-amide (taurochenodeoxychlic acid, TCDCA); 5β-cholanic-3α,7α-di-ol-*N*-(carboxymethyl)-amide (glycochenodeoxycholic acid); 5β-cholanic acid-3α,6α,7α-triol (hyocholic acid); 5β-cholanic acid-3α,6β,7α-triol (αMCA); 5β-cholanic acid-3α,6β,7β-triol (βMCA); 5β-cholanic-3α,6β,7α-triol-*N*-(2-sulphoethyl)-amide (tauro-α-MCA, TαMCA); 5β-cholanic-3α,6β,7β-triol-*N*-(2-sulphoethyl)-amide (tauro-β-MCA, TβMCA). Secondary non-12αOH BAs were measured as follows: 5β-cholanic acid-3α-ol (lithocholic acid, LCA); 5β-cholanic-3α-ol-*N*-(2-sulphoethyl)-amide (tauro-LCA, TLCA); 5β-cholanic-3α-ol-*N*-(carboxymethyl)-amide (glyco-LCA); 5β-cholanic acid-3α,6α,7β-triol (ωMCA); 5β-cholanic-3α,6α,7β-triol-*N*-(2-sulphoethyl)-amide (tauro-ω-MCA, TωMCA); 5β-cholanicacid-3α,6α-diol (hyodeoxycholic acid); 5β-cholanic-3α,6α-diol-*N*-(2-sulphoethyl)-amide (taurohyodeoxycholic acid); 5β-cholanic-3α,6α-diol-*N*-(carboxymethyl)-amide (glycohyodeoxycholic acid); 5β-cholanic acid-3α,7β-diol (ursodeoxycholic acid, UDCA); 5β-cholanic-3α,7β-diol-*N*-(2-sulphoethyl)-amide (tauroursodeoxycholic acid); 5β-cholanic-3α,7β-diol-*N*-(carboxymethyl)-amide (glycoursodeoxycholic acid); and 5β-cholanic acid-3α-ol-7-one (7-oxolithocholic acid).

2.6. Statistical Analysis

All data are presented as the mean with SEM. A significant difference ($P < 0.05$) between the mean values were determined using the Student's *t*-test. Pearson's method was used to evaluate correlation. The statistical analysis was performed using JMP version 14.0 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Growth parameters in response to HF diet

Rats fed the HF diet consumed a lower quantity of food based on weight but consumed significantly higher calories compared to controls (Table 2). Consistently, we observed an increase in body and epididymal adipose tissue weights in the HF-fed rats compared to controls. The absolute liver weights in the HF-fed rats were significantly heavier (Control: 10.3 ± 0.4 g vs. HF: 11.7 ± 1.3 g, $P = 0.0164$). However, the relative liver weights were almost comparable between the groups (Table 2).

3.2. Impact of HF diet on fatty liver and related parameters

As expected, a significant increase in liver TG concentrations was observed in HF-fed rats compared to controls (Fig. 1A). There was a significant increase in concentration of hepatic total Chol but not in free Chol in HF-fed rats (Fig. 1A). A significantly increased plasma FFA level was shown in rats fed the HF diet (Fig. 1B). There was no significant change in plasma TG and Chol between the groups (Fig. 1B). We analyzed the lipid metabolic pathway gene expression in the liver (Fig. 1C) to evaluate lipid metabolism changes in the HF-fed rats. In the HF-fed rats, we observed a significant decrease in liver mRNA expression of *Srebp1* and *Srebp2*, the master regulators of fatty acid and Chol synthesis, respectively. Consistent with this, HF-fed rats showed significantly decreased mRNA expressions of *Fasn* and *Hmgcr*, the rate-limiting enzymes for fatty acid and Chol synthesis, respectively. In contrast, HF-fed rats exhibited a marked increase in mRNA expression of liver *Cidea* and *Cpt1*. We observed a significant increase in the BAs precursor 7αOH (Fig. 1D) in the liver accompanied by upregulation of *Cyp3a2* mRNA expression. Unexpectedly, a significant decrease was observed in liver *IL-1β* expression in the HF-fed rats (Fig. 1E), although the expression of other inflammatory cytokine genes, *IL-6* and *TNFα* (Fig. 1E) were not different. A higher expression of the fibrosis gene marker *Acta2* ($P = 0.0543$) compared to control was observed, but there was no difference in the expression of other fibrosis markers, *Fn1* and *Cola1* (Fig. 1E). No significant differences were observed in plasma ALT and AST between the groups (Fig. 1F).

3.3. BA metabolism on HF diet

To investigate the impact of dietary fat on BA metabolism, we analyzed thirty molecular species of BAs, including taurine- or glycine-conjugates in the liver, blood plasma and feces using an LC-MS system [27]. The total BA concentrations were higher in the fecal and portal plasma samples of HF-fed rats compared to controls (Fig. 2A). The total BA concentration in aortic plasma was considerably lower than that of the portal plasma BAs regardless of the diet (Fig. 2A). There was a significant increase in the concentration of total 12αOH BA but not total non-12αOH BA in the portal plasma and feces of HF-fed rats (Fig.2B-C). Accordingly, ratio of 12αOH BA to non-12αOH BA was higher in the liver, blood plasma, and feces of the HF-fed rats compared to controls (Fig. 2D). Also, a significant increase in the ratios of secondary to primary BAs and conjugated to unconjugated was observed in feces but not in the liver, portal plasma, and aortic plasma samples of the HF-fed rats (Fig. 2E-F).

CA and TCA were the dominant molecular species among 12αOH BAs in the liver, portal plasma, and aortic plasma in both groups (Fig. 3A-C). A trend towards higher CA and TCA was present in both the liver and portal plasma (Fig. 3A and 3B). We observed an increased DCA and its derivative 3o12α in feces (Fig. 3D), which was associated with enhanced portal plasma DCA (Fig. 3B) and liver TDCA (Fig. 3A) in rats fed the HF diet. Collectively, changes in 12αOH BAs were more significant than those of non-12αOH BAs on the HF diet.

In the hepatic BA synthetic pathway (Fig. 4A), Chol is hydroxylated at position-7 of the steroid ring by CYP7A1, the rate-limiting BA synthetic enzyme [13]. Subsequently, CYP8B1 controls the ratio of 12αOH BAs [14], and STAR transfers Chol to the inner mitochondrial membrane where hydroxylation by CYP27A1 takes place to produce non-12αOH BAs [28]. Chol stimulates *Cyp7a1* transcription via activation of LXR, whereas BAs repress that through FXR-mediated induction of SHP [29]. Both groups of rats expressed similar levels of *Fxr* and *Lxr* (Fig. 4B) mRNAs. Induction of SHP suppresses *Cyp7a1* mRNA expression. Although liver mRNA expression of *Shp* tended to

increase (Fig. 4B), there was a trend of increase in mRNA expression of liver *Cyp7a1* in the HF-fed rats (Fig. 4B) ($P = 0.062$). A significant increase in hepatic *Cyp8b1* mRNA expression was observed in the HF-fed rats (Fig. 4B). There was no significant difference in the expression of *Cyp27a1* and *Star* between the groups (Fig. 4B).

3.4. Correlation of 12αOH BAs with steatosis

The association of BAs and hepatic steatosis was examined by Pearson's correlation analysis of BAs and liver lipid concentrations, body weight, total energy intake, and total fat intake. The concentrations of liver TG and Chol correlated positively with the concentration of 12αOH BAs in the liver, portal plasma, and feces (Fig. 5A). In contrast, none of the non-12αOH BAs showed a correlation with the hepatic lipid parameters (Fig. 5A). Similarly, the fecal concentrations of each 12αOH BA correlated positively with liver TG (Fig. 5B). The total energy intake correlated closely with body weight (Fig. 5C, $r = 0.965$, $P < 0.001$). Although total fat intake correlated positively with liver TG concentration ($r = 0.9123$, $P < 0.0001$), we identified separate clusters in each dietary group (Fig. 5D). Total energy intake also correlated positively with liver TG concentration, but the association was more linear and stronger between fecal 12αOH BAs and liver TG concentration (Fig. 5D).

3.5. Fecal lipid excretion and apparent fat absorption.

Since the HF diet contains a higher proportion of fat than the control diet (7% for control; 30% for HF diet), lipid intake was considerably higher in the HF-fed rats (Fig. 6A). Both daily fecal TG and FFA excretion were significantly higher in the HF-fed rats (Fig. 6B). However, the amount of fecal lipid excretion was much lower than the lipid intake. Thus, the apparent lipid absorption was significantly increased in the HF-fed rats (Fig. 6C). The apparent lipid absorption rate was higher in the HF-fed rats (Control: 96.1 ± 1.4 % vs. HF: 97.5 ± 0.4 %, $P = 0.0144$) (Fig. 6D). The Chol level in control and HF diet we measured was 16 mg/kg diet and 214 mg/kg diet, respectively. Thus, Chol

intake increased significantly in the HF-fed rats (Fig. 6E). In contrast, the fecal Chol excretion showed no difference between the groups. We could not calculate the apparent Chol absorption since the fecal Chol excretion was higher than Chol intake, presumably due to the excretion of endogenous Chol in feces. In a separate experiment, we determined fecal lipid excretion and apparent fat absorption rate in rats fed a CA-supplemented diet (Supplementary Fig. 1A and B). The CA supplementation to diet reduced fecal lipid output and increased apparent fat absorption rate in the rats.

4. Discussion

A previous human study has shown that levels of serum 12αOH BAs are negatively associated with hepatic steatosis [16]. A recent study has demonstrated that *Cyp8b1*$^{-/-}$ mice that have a lower 12αOH BAs concentration are protected from Western diet-induced hepatic lipid accumulation due to impaired dietary lipid absorption and supplementation of TCA to the *Cyp8b1*$^{-/-}$ mice restores sufficiently the impaired fat absorption [18]. In the present study, we showed that HF feeding in rats specifically increased the levels of 12αOH BAs accompanied with enhanced *Cyp8b1* mRNA expression in the liver. Increased levels of hepatic total Chol and 7αOH are the likely sources for 12αOH BA synthesis. HF-fed rats had higher apparent fat absorption and the specific increase in 12αOH BA concentration was positively correlated with that of hepatic TG. Also, we observed reduced fecal lipid output and higher apparent fat absorption ratio in rats fed a CA-supplemented diet (Supplementary Fig. 1A and B). These results suggest that HF diet selectively increases the concentration of 12αOH BAs by enhancing *Cyp8b1* expression and HF-induced 12αOH BAs contribute to hepatic TG accumulation by enhancing fat absorption.

The HF feeding induced hepatic TG and Chol accumulation despite markedly reduced expression of lipid synthesis-related genes such as *Srepb1*, *Fasn*, *Srebp2*, and *Hmgcr* in the liver. Since lard samples naturally contain Chol, the increased lard consumption might exceed a range of the compensatory reduction of hepatic endogenous lipid synthesis in HF-fed rats. A previous study [34] in which ingredients in the AIN-93G-based control diet and HF diet are almost the same as those in the present study shows that the HF diet consumption for 4 weeks decreases serum HDL-Chol level and increased LDL-Chol in rats. Given that LDL takes up Chol from the liver whereas HDL carries

blood Chol to the liver, the changes in HDL and LDL would not contribute to the hepatic lipid accumulation in the present study.

The accumulated Chol can be excreted from the liver in the form of BAs partially [35]. HF diet consumption significantly increased the fecal BA concentration and excretion (Control: 7.6 ± 1.7 vs. HF: 11.3 ± 1.8, μmol/day, $P = 0.0026$), suggesting enhanced BA synthesis from accumulated Chol in the liver of HF-fed rats. Indeed, a significant increase in the concentration of liver 7αOH, a precursor of BAs, was observed (Fig. 1D). An increase in 7αOH was accompanied with an increase in hepatic *Cyp7a1* mRNA expression in the HF-fed rats, possibly because 7αOH activates LXR that enhances *Cyp7a1* mRNA expression [36]. On the other hand, 7αOH does not activate FXR [37] that is responsible for suppressing *Cyp7a1* expression through SHP induction. These observations suggest that hepatic free Chol in the HF-fed rats was rapidly converted to BAs. Indeed, the concentration of hepatic free Chol was similar between the groups although hepatic total Chol concentration was significantly increased in the HF-fed rats (Fig.1A). Notably, among BAs, fecal 12αOH concentration was increased specifically (Fig. 3D) in the HF-fed rats, suggesting that hepatic Chol is able to be converted selectively into BA, particularly as 12αOH BA. Interestingly, a strong correlation was observed between fecal 12αOH BAs and liver lipid concentrations (Fig. 5A and 5B). Since BAs are synthesized from Chol in a certain amount and excreted in the feces, fecal 12αOH BA concentration could be a predictor of hepatic lipid accumulation especially in diet rich in saturated fat condition.

CYP8B1 synthesizes 12αOH BAs [28], and thus, increased expression of CYP8B1 leads to a marked increase in CA biosynthesis in rats [14], whereas *Cyp8b1* knockdown results in decreased 12αOH BA concentration in mice [18]. In the present study, hepatic *Cyp8b1* mRNA expression was increased significantly (Fig. 4B) along with a preferential increase of 12αOH BA concentration in HF-fed rats, suggesting that HF diet increased 12αOH BA synthesis by enhancing the mRNA expression of *Cyp8b1* in the liver. Insulin signaling can suppress *Cyp8b1 [38]*. Considering that hepatic insulin resistance is developed after three days of HF diet intake [39], insulin resistance might involve an increase in *Cyp8b1*, which subsequently enhances 12αOH BA synthesis in HF-fed rats.

In contrast to CYP8B1, STAR transports Chol into inner mitochondria to synthesize non-12αOH BAs [28]. Qiu et al. [40] reported reduced mRNA and protein levels of STAR in the livers of

HF-fed mice, suggesting that reduced STAR could induce a preferential increase in 12αOH BA levels by suppressing the synthesis of non-12αOH BAs. However, in the present study the mRNA levels of *Star* and *Cyp27a1* were not significantly different in rats fed the HF diet. A previous study [40] reported that HF-fed mice had higher levels of liver inflammatory cytokines and serum ALT/AST activities; nonetheless, the HF-fed rats in the present study showed no evidence of hepatic inflammation and/or injury (Fig. 1E). These differences suggest the involvement of STAR expression in the late rather than in the early phase of fatty liver disease. Additionally, in this study, the liver *Star* mRNA expression was considerably lower than that of *Cyp8b1* (data not shown). Therefore, increased *Cyp8b1* mRNA expression might be responsible for the enhancement of fecal concentration of 12αOH BAs in HF-fed rats.

The increase in 12αOH BA levels in enterohepatic circulation would not cause FXR activation because CA and its conjugated form, TCA, are less potent FXR ligands [37,41]. Accordingly, in the present study, the induction of liver *SHP* mRNA expression was not significant. Increased expression of liver *Cyp7a1* in the HF-fed rats was observed, even though a substantial increase was noted in the total portal BA concentration (Fig. 2A), suggesting no feedback inhibition including fibroblast growth factor-15 in the intestine [42] to reduce the *Cyp7a1* mRNA expression. Thus, BA synthesis is ongoing to eliminate excess Chol while maintaining efficient lipid absorption through 12αOH BAs in HF-fed rats [18], which is physiologically sustainable where few instances offer such a high energy diet.

HF feeding for only three days significantly increased the hepatic TG concentration in rats [39]. In contrast, fecal 12αOH BA concentration was significantly increased after four weeks of HF diet feeding [8]. Hence, the increase in 12αOH BA concentration could be due to hepatic steatosis induced by the HF diet. Hepatic steatosis in the HF-fed rats might be due to the accumulation of dietary fats into the liver independent of the increase in 12αOH BAs. On the other hand, it is possible that increased 12αOH BA concentration paradoxically accelerates hepatic steatosis. Bertaggia et al. [18] showed that TCA treatment restored the impaired fat absorption in *Cyp8b1*$^{-/-}$ mice, suggesting that such a primary 12αOH BA at least in part contributes to hepatic steatosis development by promoting lipid absorption. Similarly, we found that the CA-supplemented diet significantly reduced fecal lipid

output and enhanced the apparent fat absorption rate in rats (Supplementary Fig. 1A and B). Importantly, unlike these primary 12αOH BAs, secondary 12αOH BAs such as DCA and TDCA may not contribute to dietary fat absorption. The critical micellar concentrations of DCA and TDCA are 2.3-2.5 M [43] and less than 0.7 M [44], respectively. As the concentration of TDCA in bile is less than 0.069 M in HF-fed rats [8], the biliary concentrations of DCA and TDCA should be out of the range. In the present study, the concentration of primary 12αOH BAs (sum of CA and TCA) was increased in the HF-fed rats (19.0±3.1 μM vs HF: 28.1±7.1 μM, $P = 0.0153$). These data suggest that primary 12αOH BAs but not secondary 12αOH BAs might contribute to hepatic steatosis by promoting fat absorption in HF-fed rats. Primary 12αOH BAs might be involved in the development of hepatic steatosis since the levels of 12αOH BAs are increased in subjects with NAFLD [45], obesity [46], insulin resistance [16], and in diabetic-animal models [47].

We further showed that HF feeding significantly increased the DCA in feces (Fig. 3D) along with hepatic TDCA (Fig. 3A). An increase in DCA has been reported in humans with fatty liver [16,48]. Obesity is associated with an increased abundance of Firmicutes [49], such as *Clostridium* cluster XI and XIVa, which are responsible for 7α-dehydroxylation to generate DCA in the large intestine [15]. In addition, DCA feeding promotes liver cancer in mice, even though the hepatic lipid concentration has not yet been determined [50]. Considering that the gut microbiota plays a significant role in NAFLD pathogenesis [51,52], increased fecal DCA might promote the development of hepatic steatosis. Liver *Cidea* is a candidate target for DCA, which promotes hepatic lipid storage in the liver [53]. Zhou and colleagues [53] reported the induction of *Cidea* mRNA expression by saturated fatty acids, which was reduced when *Srebp1* was knocked down in *ob*/*ob* mice hepatocytes. In contrast, we observed significantly increased liver *Cidea* mRNA expression in HF-fed rats despite a marked reduction in liver *Srebp1* mRNA expression (Fig. 1F). These results suggest a direct role for DCA in inducing liver *Cidea* mRNA expression, independent of *Srebp1*, to promote hepatic lipid storage. These data suggest that increased levels of primary 12αOH BAs promote lipid absorption, whereas those of secondary 12αOH BAs enhance liver *Cidea* mRNA expression. These changes subsequently contribute to the development of hepatic steatosis.

Consideration is necessary in two aspects to interpret results in the present study. At first, we can not separate the effects of fat and Chol on the association between 12αOH BAs and hepatic steatosis because the HF diet contains not only a high level of fat but also a high level of Chol than control. In literature, a high-Chol diet called western diet, usually supplemented with additional Chol, contains an extremely high level of Chol in the diet (1.5-12.5 g/kg diet) [18,30,31]. If humans consume such food at 1 kg/day, the amount of dietary Chol is nearly equivalent to 1.5 - 12.5 g/day. This value is extremely higher than human average intake of Chol (about 300 mg/day) [32]. Also, lard contains naturally Chol as we measured in the lard sample even without the addition of Chol to the diet. The casein sample used in the present study also contained Chol. The calculated Chol level in the HF diet (214 mg/kg diet) is considered to be comparable with the average level of humans Chol intake. In addition, if humans consume a saturated fat-rich diet, they would consume not only saturated fat but also Chol. Actually, it has been reported that nonalcoholic fatty liver disease (NAFLD) patients have higher intake both saturated fat and Chol compared to healthy subjects [33]. Thus, the HF diet which contains higher fat without the addition of Chol would be physiologically relevant to human conditions and thus we chose the HF diet to investigate the association between 12αOH BAs and hepatic steatosis. Secondly, it is difficult to separate the effects of fat and 12αOH BAs on fat absorption. Both the CA-supplemented and HF-fed showed a higher apparent fat absorption rate. These results imply the effect of 12αOH BAs on fat absorption. Unfortunately, however, it is unable to investigate the effect of the increase in fat intake itself on the apparent fat absorption rate *in vivo* because HF consumption increased not only fat intake but also 12αOH BAs level in the present study.

In conclusion, the levels of 12αOH BAs in enterohepatic circulation and feces were increased in hepatic steatosis through accumulated hepatic total Chol, 7αOH, and enhanced *Cyp8b1* mRNA expression in the liver. Fecal 12αOH BAs might predict ‘liver-specific’ lipid abundance especially in a diet rich in saturated fat condition. The increased levels of 12αOH BAs may accelerate hepatic steatosis development through several mechanisms depending on the BA species. This interdependence between hepatic steatosis and 12αOH BAs may result in their mutual promotion and accelerate NAFLD development.

Author contributions

All authors contributed to the development, analysis and drafting of this article.

Shota Hori: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original Draft, **Takayuki Abe**: Investigation, **Dong Geun Lee**: Investigation, **Satoru Fukiya**: Conceptualization, Supervision, Investigation, **Atsushi Yokota**: Conceptualization, Supervision, **Nao Aso**: Investigation, **Bungo Shirouchi**: Investigation, **Masao Sato**: Conceptualization, Investigation, **Satoshi Ishizuka**: Conceptualization, Investigation, Writing - Review & Editing, Supervision

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Conflict of interest

There is no conflict of interest

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Figure legends

Fig. 1. Hepatic and plasma parameters in HF diet-induced steatosis. (A) Concentration of liver TG, total Chol, and free Chol. (B) Concentration of plasma free FFA, TG, total Chol. (C) mRNA expression of genes involved in hepatic lipid metabolism. (D) Hepatic oxysterol concentration. (E) mRNA expression of genes involved in hepatic inflammation and fibrosis. (F) Plasma aminotransferase activities. Open bars, n = 6 for control; filled bars, n = 7 for HF. In C and E, data were normalized to *Rplp0* mRNA expression. Values are shown as the mean with the SEM (n = 6-7). Asterisks indicate a significant difference compared to control ($P < 0.05$).

Fig. 2. BA profiles in various sites in rats fed control and HF diet. (A) Total BA concentration, (B) total 12αOH BA concentration, (C) total non-12αOH BA concentration, (D) ratio of 12αOH BAs to non-12αOH BAs, (E) ratio of secondary to primary BA, and (F) ratio of BA conjugates to

unconjugated BAs in the liver, portal plasma, aortic plasma, and feces of rats fed control or HF diet for eight weeks. Open bars, n = 6 for control; filled bars, n = 7 for HF. Values are shown as the mean with the SEM (n = 6-7). Asterisks indicate a significant difference compared to control ($P < 0.05$).

Fig. 3. BA compositions in rats fed control and HF diet. BAs concentration in (A) liver, (B) portal plasma, (C) aortic plasma, and (D) feces in the rats fed control or HF diet for eight weeks. Open bars, n = 6 for control; filled bars, n = 7 for HF. Values are shown as the mean with the SEM (n = 6-7). Asterisks indicate significant differences compared to control ($P < 0.05$).

Fig. 4. Hepatic gene expression regulates BA metabolism. (A) BA metabolism scheme in the liver. (B) mRNA level of hepatic BA synthesis genes expression in rats fed control or HF diet for eight weeks. In B, data were normalized to *Rplp0* mRNA expression. Open bars, n = 6 for control; filled bars, n = 7 for HF. Values are shown as the mean with the SEM. Asterisks indicate a significant difference compared to control ($P < 0.05$).

Fig. 5. Correlation among the concentrations of 12αOH BAs, hepatic steatosis, body weight, total energy intake, and total fat intake. (A) The heatmap shows Pearson's correlation of total 12αOH BAs in each site and liver lipids. Red and black cells indicate positive and negative correlations, respectively. The values of correlations were shown on the cells. Asterisks indicate significant correlations ($P < 0.05$). Pearson's correlation of (B) fecal BA species and liver TG, (C) the total energy intake and body weight, (D) liver TG concentration and total fat intake, total energy intake, and fecal 12αOH BAs. In B, red and gray bars indicate 12αOHs BA and non-12αOH BAs, respectively. Total energy intake was calculated from diet consumption throughout the experimental period with the caloric densities of each diet (4.04 kcal/g for control; 5.19 kcal/g for the HF diet). Body weight was shown in Table 1. Concentrations of fecal 12αOH BAs were calculated from Fig. 3D. Liver TG concentration was shown in Fig. 1A. Total fat intake was calculated from diet

consumption throughout the experimental period and fat content in each diet (7% for control; 30% for the HF diet). In C and D, individual data are shown as circles (open circles, n = 6 for control; filled circles, n = 7 for HF).

Fig. 6. Lipid intake, fecal lipid excretion, and apparent fat absorption. (A) Daily fat intake was calculated from diet consumption at the end of this experiment and fat content in each diet (7% for control; 30% for HF diet). (B) Fecal excretion of FFA and TG per day were calculated using fecal weight collected over 24 hours at the end of the experiment and the concentrations in the feces. (C) Apparent fat absorption was calculated by subtracting the fecal lipid excretion shown in B from the fat intake shown in A. (D) Apparent fat absorption rate was calculated from the fat intake and fat absorption shown in A and C, respectively. (E) Daily Chol intake was calculated from diet consumed at the end of this experiment and Chol content in each diet (16 mg/kg diet for control; 214 mg/kg diet for HF diet). (F) Fecal excretion of Chol per day was calculated using fecal weight collected for 24 hours at the end of the experiment and the concentrations in the feces. Values are shown as the mean with the SEM (n = 6-7). Asterisks indicate a significant difference compared to control ($P < 0.05$).

Table

Table 1. Diet compositions

	Control	HF
	g/kg diet	
Casein[1]	200	200
Dextrin[2]	529.5	299.5
Sucrose[3]	100	100
Soybean oil[4]	70	70
Lard	-	230
Cellulose[5]	50	50
Mineral mixture[6]	35	35
Vitamin mixture[7]	10	10
L-Cystine[8]	3	3
Choline hydrogen tartrate[8]	2.5	2.5

[1] NZMP Acid Casein (Fonterra Co-Operative Group Limited, Auckland, New Zealand),

[2] TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

[3] Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan

[4] J-Oil Mills, Inc., Tokyo, Japan

[5] Crystalline cellulose (Ceolus PH-102, Asahi Kasei Chemicals Corp., Tokyo, Japan)

[6] AIN-93G Mineral mixture [19]

[7] AIN-93 Vitamin mixture [19]

[8] Fujifilm Wako Pure Chemical Corporation, Osaka, Japan

Table 2. Food intake, growth, and organ weights

	Control	HF
Cumulative food intake (per entire study)		
(g)	848 ± 14	722 ± 57*
(kcal)	3426 ± 58	3745 ± 297*
Final body weight (g)	316.7 ± 7.0	348.4 ± 28.3*
Organ weight (g/100g body weight)		
Liver	3.25 ± 0.12	3.34 ± 0.16
Epididymal adipose tissue	2.23 ± 0.24	2.63 ± 0.20*

Means values with SEM. * Significant difference from the values of control (Student's *t*-test, $P < 0.05$, n = 6-7)

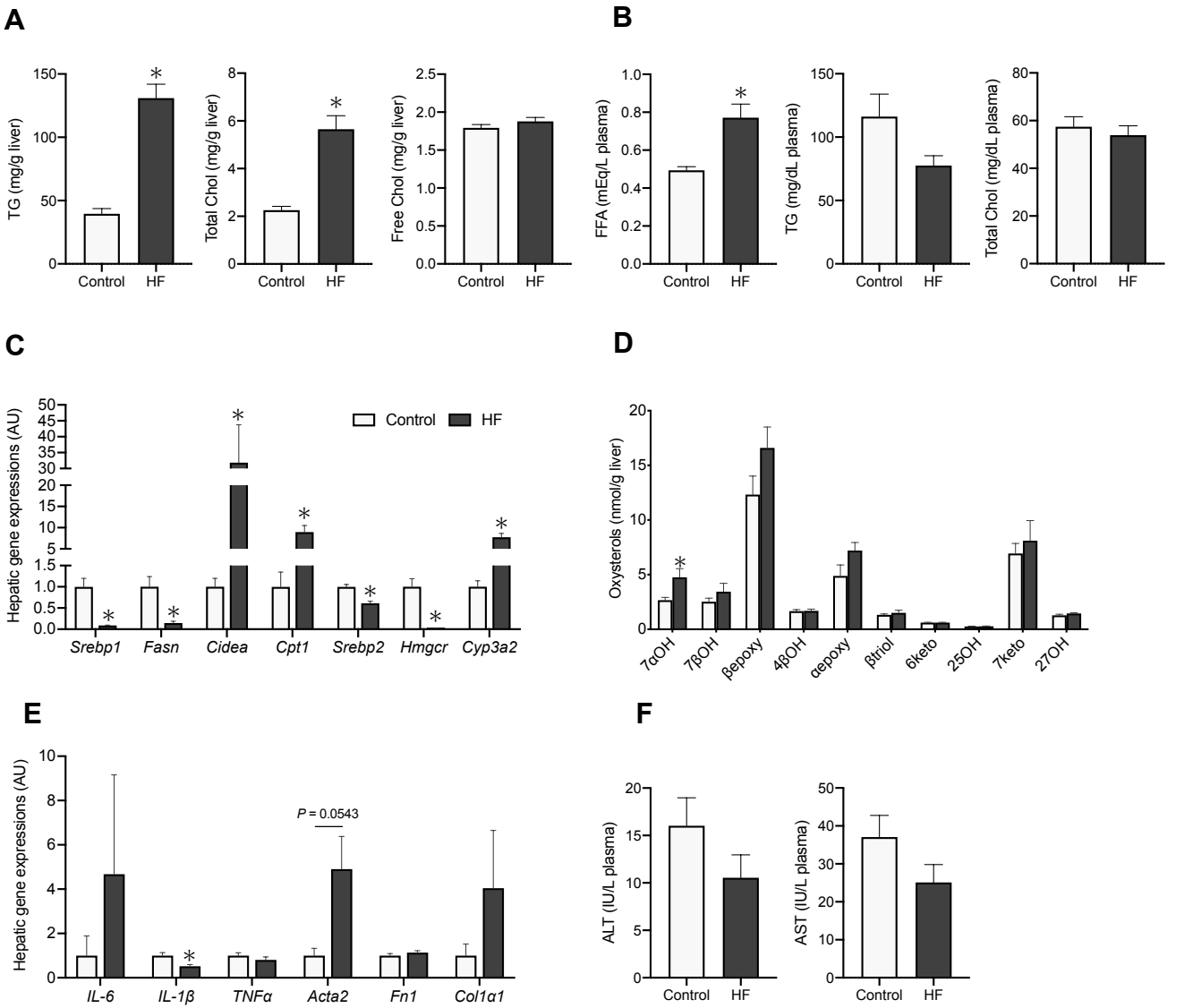

A
TG (mg/g liver)
Total Chol (mg/g liver)
Free Chol (mg/g liver)
Control
HF
B
FFA (mEq/L plasma)
TG (mg/dL plasma)
Total Chol (mg/dL plasma)
C
Hepatic gene expressions (AU)
Srebp1
Fasn
Cidea
Cpt1
Srebp2
Hmgcr
Cyp3a2
D
Oxysterols (nmol/g liver)
7αOH
7βOH
βepoxy
4βOH
αepoxy
βtriol
6keto
25OH
7keto
27OH
E
Hepatic gene expressions (AU)
P = 0.0543
IL-6
IL-1β
TNFα
Acta2
Fn1
Col1α1
F
ALT (IU/L plasma)
AST (IU/L plasma)

Figure 1

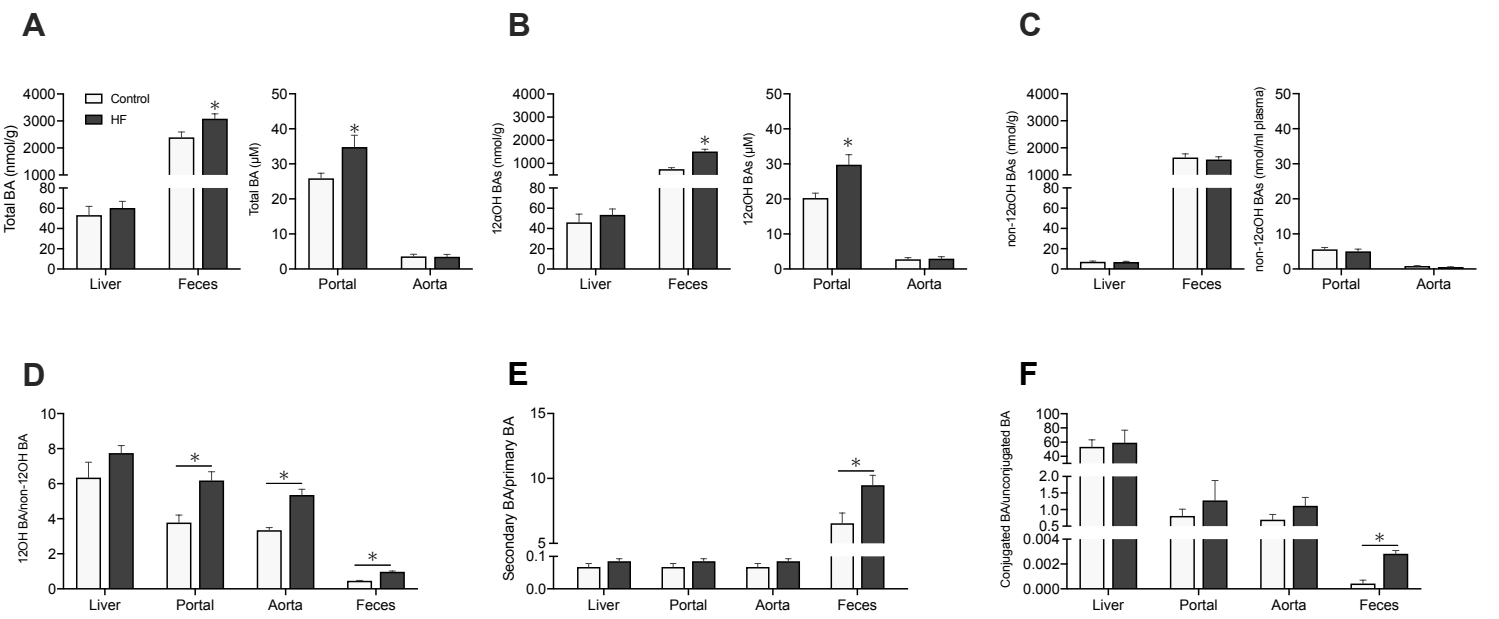

A
B
C
D
E
F
Control
HF
Total BA (nmol/g)
Total BA (μM)
12αOH BAs (nmol/g)
12αOH BAs (μM)
non-12αOH BAs (nmol/g)
non-12αOH BAs (nmol/ml plasma)
12OH BA/non-12OH BA
Secondary BA/primary BA
Conjugated BA/unconjugated BA
Liver
Feces
Portal
Aorta

Figure 2

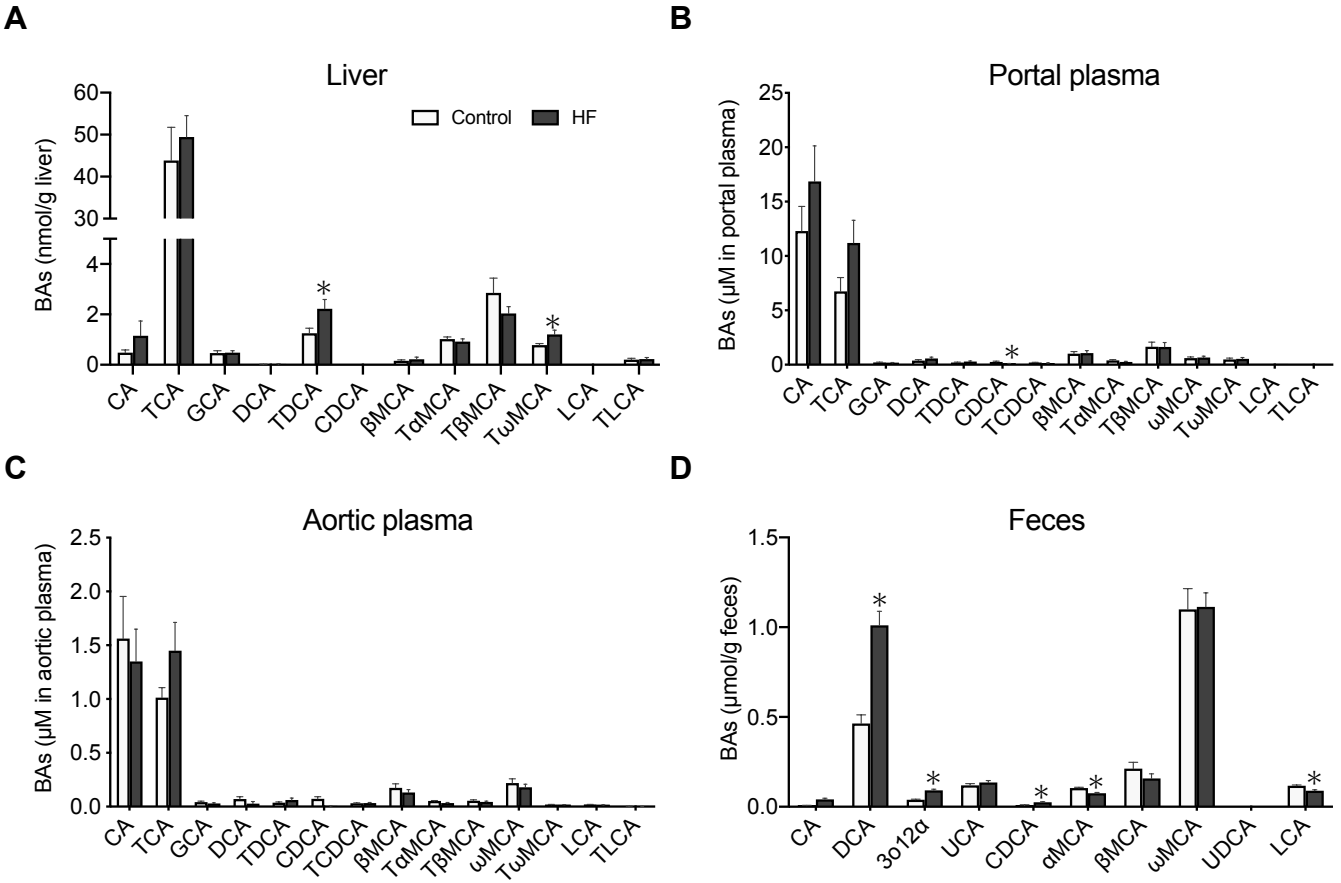
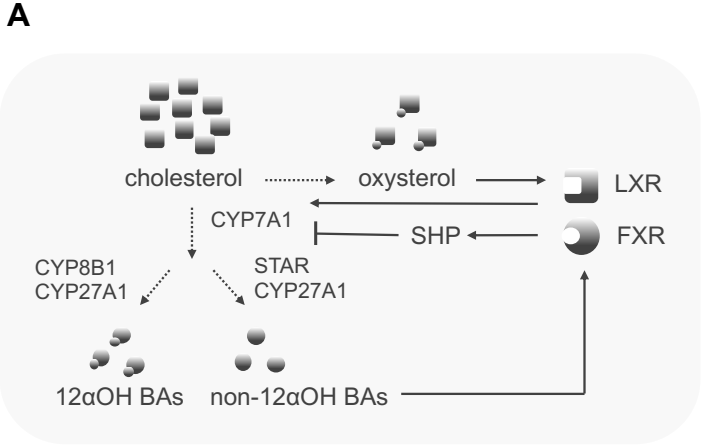

A
Liver
Control
HF
BAs (nmol/g liver)
60
50
40
30
4
2
0
CA
TCA
GCA
DCA
TDCA
CDCA
βMCA
TαMCA
TβMCA
TωMCA
LCA
TLCA
B
Portal plasma
BAs (μM in portal plasma)
25
20
15
10
5
0
CA
TCA
GCA
DCA
TDCA
CDCA
TCDCA
βMCA
TαMCA
TβMCA
ωMCA
TωMCA
LCA
TLCA
C
Aortic plasma
BAs (μM in aortic plasma)
2.5
2.0
1.5
1.0
0.5
0.0
CA
TCA
GCA
DCA
TDCA
CDCA
TCDCA
βMCA
TαMCA
TβMCA
ωMCA
TωMCA
LCA
TLCA
D
Feces
BAs (μmol/g feces)
1.5
1.0
0.5
0.0
CA
DCA
3o12α
UCA
CDCA
αMCA
βMCA
ωMCA
UDCA
LCA

Figure 3


A
cholesterol
oxysterol
LXR
CYP7A1
SHP
FXR
CYP8B1
CYP27A1
STAR
CYP27A1
12αOH BAs
non-12αOH BAs

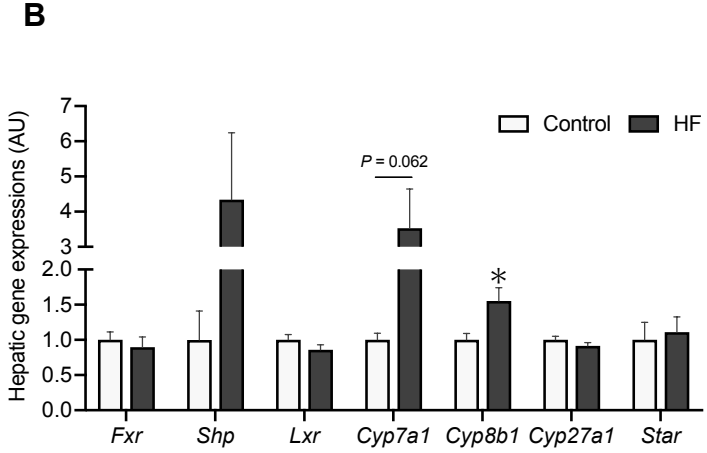

B
Hepatic gene expressions (AU)
Control
HF
P = 0.062
*
Fxr
Shp
Lxr
Cyp7a1
Cyp8b1
Cyp27a1
Star

Figure 4

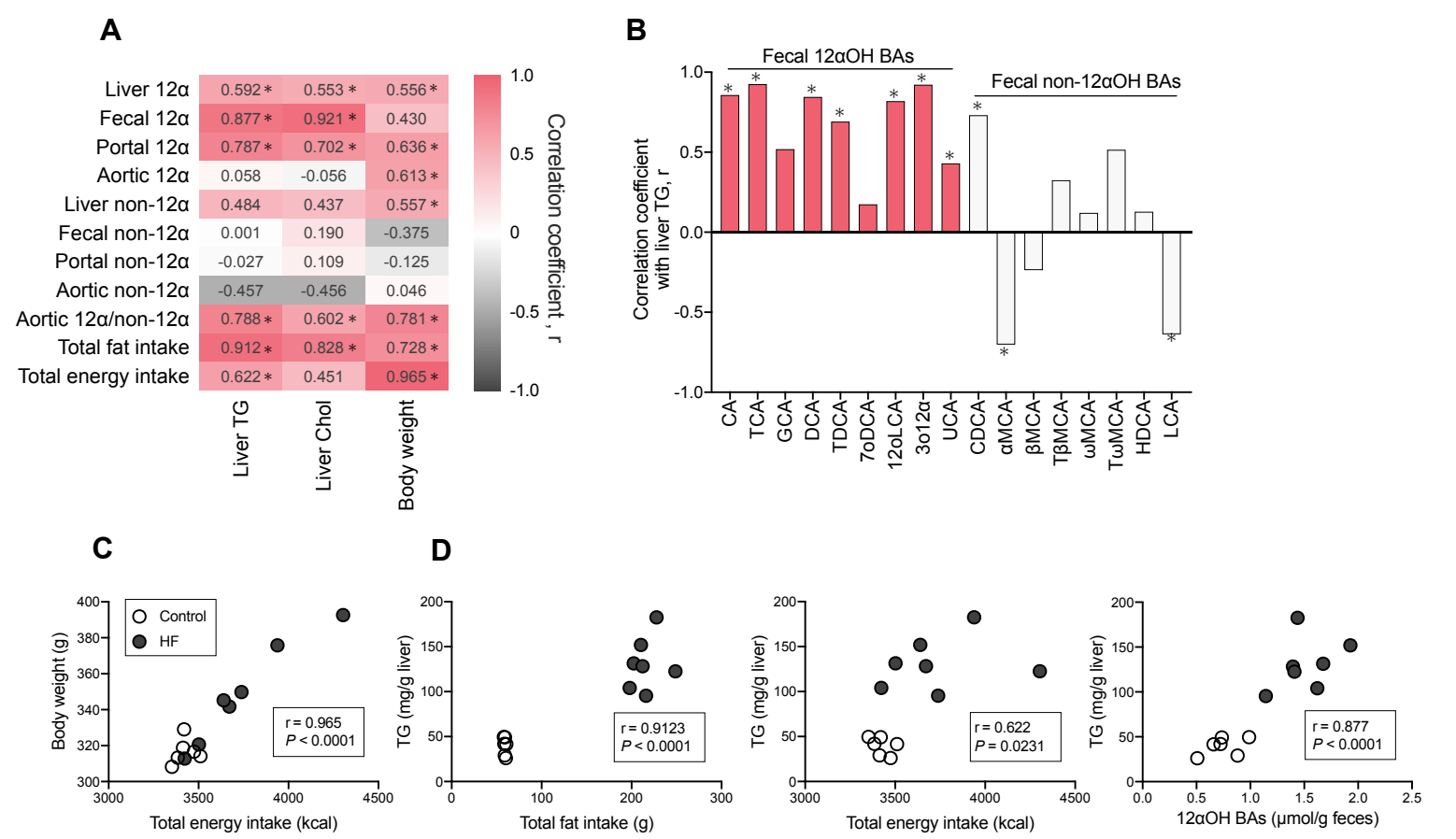

A
Liver 12α 0.592* 0.553* 0.556*
Fecal 12α 0.877* 0.921* 0.430
Portal 12α 0.787* 0.702* 0.636*
Aortic 12α 0.058 -0.056 0.613*
Liver non-12α 0.484 0.437 0.557*
Fecal non-12α 0.001 0.190 -0.375
Portal non-12α -0.027 0.109 -0.125
Aortic non-12α -0.457 -0.456 0.046
Aortic 12α/non-12α 0.788* 0.602* 0.781*
Total fat intake 0.912* 0.828* 0.728*
Total energy intake 0.622* 0.451 0.965*
Liver TG
Liver Chol
Body weight
Correlation coefficient, r
1.0
0.5
0
-0.5
-1.0
B
Fecal 12αOH BAs
Fecal non-12αOH BAs
Correlation coefficient with liver TG, r
CA
TCA
GCA
DCA
TDCA
7oDCA
12oLCA
3o12α
UCA
CDCA
αMCA
βMCA
TβMCA
ωMCA
TωMCA
HDCA
LCA
C
Control
HF
Body weight (g)
r = 0.965
P < 0.0001
Total energy intake (kcal)
D
TG (mg/g liver)
r = 0.9123
P < 0.0001
Total fat intake (g)
r = 0.622
P = 0.0231
Total energy intake (kcal)
r = 0.877
P < 0.0001
12αOH BAs (μmol/g feces)

Figure 5

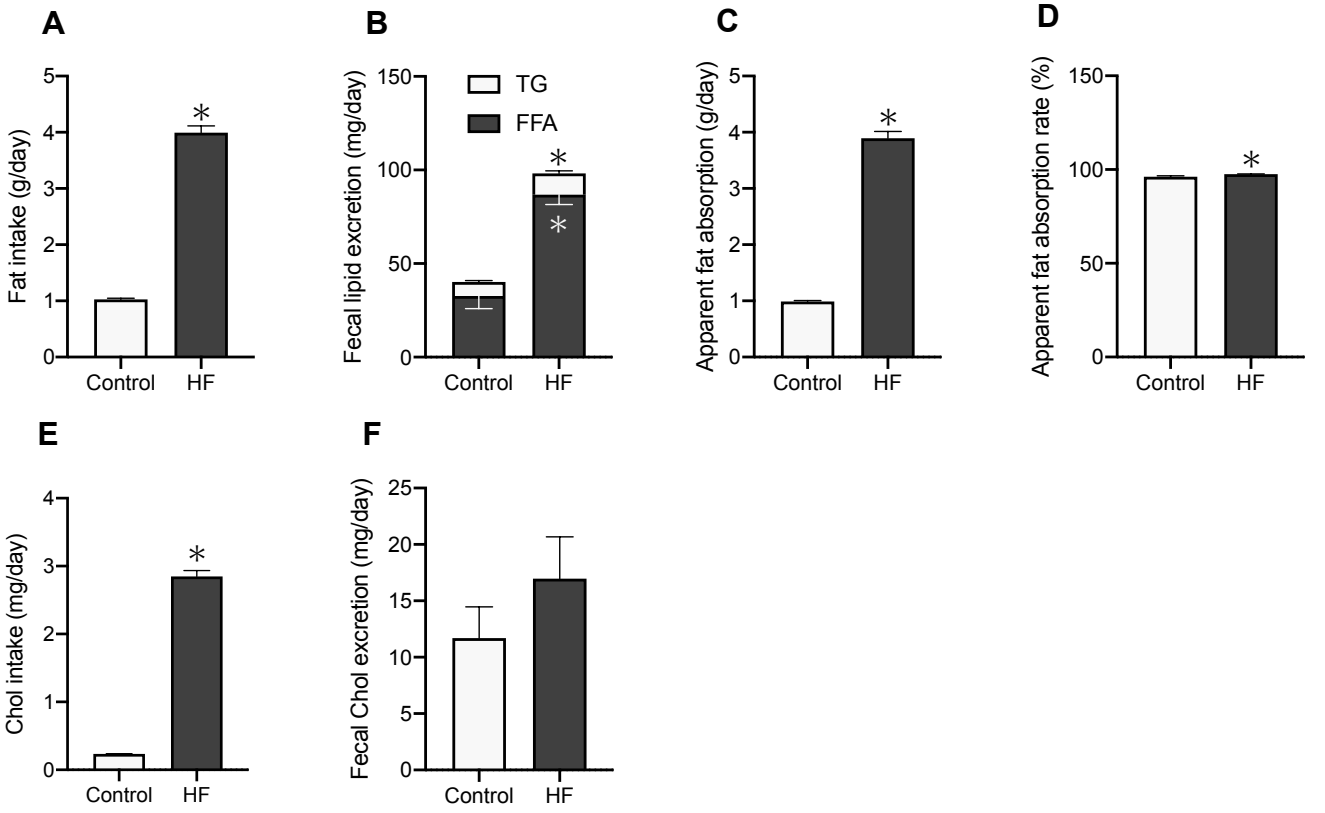

A
Fat intake (g/day)
5
4
3
2
1
0
Control
HF
*
B
Fecal lipid excretion (mg/day)
150
100
50
0
TG
FFA
Control
HF
*
*
C
Apparent fat absorption (g/day)
5
4
3
2
1
0
Control
HF
*
D
Apparent fat absorption rate (%)
150
100
50
0
Control
HF
*
E
Chol intake (mg/day)
4
3
2
1
0
Control
HF
*
F
Fecal Chol excretion (mg/day)
25
20
15
10
5
0
Control
HF

Figure 6