

High-Fat Diet Alters Serum Fatty Acid Profiles in Obesity Prone Rats: Implications for *In Vitro* Studies

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Abstract High-fat diets (HFD) are commonly used in rodents to induce obesity, increase serum fatty acids and induce lipotoxicity in various organs. *In vitro* studies commonly utilize individual free fatty acids (FFA) to study lipid exposure in an effort to model what is occurring *in vivo*; however, these approaches are not physiological as tissues are exposed to multiple fatty acids *in vivo*. Here we characterize circulating lipids in obesity-prone rats fed an HFD in both fasted and fed states with the goal of developing physiologically relevant fatty acid mixtures for subsequent *in vitro* studies. Rats were fed an HFD (60 % kcal fat) or a control diet (10 % kcal fat) for 3 weeks; liver tissue and both portal and systemic blood were collected. Fatty acid profiles and absolute concentrations of triglycerides (TAG) and FFA in the serum and TAG, diacylglycerol (DAG) and phospholipids in the liver were measured. Surprisingly, both systemic and portal serum TAG were ~40 % lower in HFD-fed compared to controls. Overall, compared to the

control diet, HFD feeding consistently induced an increase in the proportion of circulating polyunsaturated fatty acids (PUFA) with a concomitant decline in monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) in both serum TAG and FFA. The elevations of PUFA were mostly attributed to increases in n-6 PUFA, linoleic acid and arachidonic acid. In conclusion, fatty acid mixtures enriched with linoleic and arachidonic acid in addition to SFA and MUFA should be utilized for *in vitro* studies attempting to model lipid exposures that occur during *in vivo* HFD conditions.

Keywords Fatty acids · HFD · Development of obesity · PUFA

Abbreviations

DAG Diacylglycerol(s)
FFA Non-esterified fatty acids

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MAG	Monoacylglycerol(s)
MUFA	Monounsaturated fatty acid(s)
PL	Phospholipids
PUFA	Polyunsaturated fatty acid(s)
SFA	Saturated fatty acid(s)
TAG	Triacylglycerol(s)

Introduction

Increased ectopic lipid storage is a hallmark of high-fat diet (HFD)-induced obesity and metabolic disease [1–3]. FFA exposure has frequently been used to increase lipid storage and insulin resistance in various cell culture models of tissues known to participate in the development of obesity-associated metabolic diseases (e.g., muscle, liver, adipose, endothelium, pancreas). The liver plays a central role in handling dietary lipids; it utilizes lipids as an energy source, stores them as TAG, or repackages them into lipoproteins for export to the periphery. Excessive lipid accumulation in the liver, or hepatic steatosis, can impair factors that suppress VLDL secretion and eventually lead to chronic dyslipidemia [4, 5]. Liver receives exposure to fatty acids from the blood stream of both systemic circulation and the portal vein. However, the differences of fatty acid profiles between the systemic circulation and portal blood in relation to an HFD or obesity are still unclear. Obesity has been casually linked with elevated circulating lipids including both FFA [6] and TAG [7–9], which correlates strongly with insulin resistance [10]. These elevated lipids travel through the circulation and, after being hydrolyzed by lipoprotein lipase, can putatively result in tissue lipotoxicity, which is associated with impaired insulin action and inflammation [11]. Due to the important regulatory roles of liver, primary hepatocytes and immortalized hepatocyte cell lines have been widely used to study hepatic lipid metabolism and insulin resistance following FFA exposures [12, 13].

Many different fatty acid species or fatty acid mixtures have been used to study lipotoxicity *in vitro*. Palmitic acid (16:0) and oleic acid (18:1n-9) are the two most commonly used FFAs [14, 15] as they are the dominant saturated fatty acid (SFA) and mono-unsaturated fatty acids (MUFA) in circulation and in most tissues, respectively [16]. The SFA palmitic acid has been most commonly used to induce increased lipid accumulation and insulin resistance [17–19] despite potential problems with cell toxicity if concentrations are too high. Oleic acid is sometimes added with palmitic acid to increase physiological relevancy [20]. However, tissues are exposed to more than one or two fatty acids *in vivo* [16]; therefore, the most commonly used approaches to induce lipid accumulation *in vitro* ignore the importance of fatty acid combinations in cellular lipid

metabolism. Different fatty acid species cause various toxicity and/or inflammatory reactions to cells that may eventually alter cell functions [21]; thus, it would be most logical to recapitulate *in vivo* events that occur during HFD feeding conditions during the development of obesity. Here, we quantified the *in vivo* circulating systemic and portal fatty acid profiles of obesity-prone rats fed a commonly used lard-based HFD (60 % kcal fat) for 3 weeks to capture the initial stage of obesity. We made these measurements in rats who were fasted overnight or who were allowed *ad libitum* access to food up until sample analysis to determine whether the fasting or fed state impacted results. We also made measurements to compare the fatty acid profile in the portal blood vs. systemic circulation to elucidate the fatty acids that liver is exposed to when fed an HFD. The results demonstrate the unique fatty acid profiles develop in the circulation following a short-term HFD feeding and strongly suggest that *in vitro* work should utilize PUFA in addition to SFA and MUFA if the goal is to model *in vivo* conditions. The proportion of certain fatty acid species dramatically differs between portal serum and systemic serum, suggesting that both fatty acid pools should be considered when treating hepatocytes, whereas systemic fatty acid profiles may be used in cell culture studies involving other cell types such as adipocytes, myocytes, endothelial cells, etc.

Materials and Methods

Animals

The protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia and the Subcommittee for Animal Safety for the Research Service at the Harry S. Truman Memorial VA Hospital. Thirty-four 5-week-old obesity-prone Sprague-Dawley male rats were purchased from Taconic (Hudson, NY, USA) and housed in a room controlled for temperature (75 °F), humidity (50 %) and light cycle (12 h light/dark). Obesity-prone rats were chosen so that there would be a consistent development of obesity upon feeding an HFD. Rats had access to *ad libitum* standard rodent chow (Purina Formulab 5008, St. Louis, MO, USA) and water. At the age of 7 weeks, rats were switched to a low-fat control diet with 10 % kcal of total energy from fat [(50 % of the fat from lard, 50 % from soybean oil); 20 % kcal protein, 70 % kcal carbohydrate, D12450B, Research Diet, Inc., New Brunswick, NJ, USA] for 3 weeks. At 10 weeks of age, half of the animals were switched to an HFD with 60 % kcal fat [(92 % of the fat from lard, 8 % from soybean oil), 20 % kcal protein, 20 % kcal carbohydrate, D12492, Research Diets Inc., New Brunswick, NJ, USA] for 3 weeks while the other half were maintained on the

control diet. This specific HFD was chosen because it is one of the most commonly reported diets in the literature to induce obesity and ectopic lipid storage. Diet composition and fatty acid profiles obtained from the manufacturer are shown in Supplemental Tables S1 and S2. We chose to perform a 3-week HFD feeding to access the changes of fatty acid profiles while animals were developing obesity, in which the changes of fatty acid profiles most likely reflect more the dietary intervention instead of causing by diseases. Fourteen rats ($n = 7/\text{group}$) were euthanized at 9 a.m. after a 12-h food withdraw (fasted state), whereas the other 20 rats ($n = 10/\text{group}$) were fed *ad libitum* until euthanasia at 7 a.m. in the morning (fed state). More rats were used in the fed state group because more variation was expected because of *ad libitum* feeding prior to killing. Blood was collected at both times to determine whether fatty acid profiles differed during fed and fasted conditions. Both systemic circulation blood (cardiac puncture under anesthesia) and portal blood samples (portal vein catheterization) were collected and centrifuged (7000g, 10 min, 4 °C) to separate serum after allowing clotting at room temperature for 20 min. Serum samples were aliquoted and stored at -80 °C until analysis.

Lipid Extraction and Separation

Lipids were extracted using the method described by Folch *et al.* [22] with slight modifications as described previously [23]. Serum and liver samples from each animal were mixed or homogenized in ice-cold Trizma/EDTA buffer (50 mmol l^{-1} Tris and 1 mmol l^{-1} EDTA; pH 7.4). Twenty micrograms of 17:0 NEFA (Sigma, St. Louis, MO, USA) in chloroform and 25 μg of 17:0 TG (Nu-chek, Elysian, MN, USA) were added to each serum sample as internal standards. Similarly, 100 μg of 17:0 TG, 25 μg of 17:0 DG (Nu-chek, Elysian, MN, USA) and 100 μg of 17:0 phospholipids (PL; Avanti, Alabaster, AL, USA) were added to each liver sample as internal standards. Samples were mixed with 3 ml of 0.5 % of acetic acid in methanol and with 6 ml of chloroform. The mixtures were then centrifuged (190g, 10 min), and the organic phase was transferred into another glass conical tube through an IPS filter (Whatman, Piscataway, NJ, USA) and evaporated under a nitrogen stream before being reconstituted with 100 μl of 2:1 chloroform: methanol. Extracted lipids were run on a silica gel 60 Å analytical thin-layer chromatography (TLC) plate (Sigma, St. Louis, MO, USA) in a solvent system containing hexane/diethyl ether/acetic acid (70:30:1, by vol). Lipid class standards were also spotted for detecting target bands. Then 2, 7-dichlorofluorescein was used to visualize individual lipid classes on the plate under a UV lamp. TAG and FFA bands in the serum samples and the TAG, DAG and PL bands in the liver samples were scraped and collected.

Fatty Acid Analyses

Collected lipid fractions were reconstituted in 500 μl toluene. For TAG, DAG and PL fractions, 1 ml of 0.5 M sodium methoxide in anhydrous methanol was mixed with each sample for 1 min and then mixed with 750 μl distilled deionized water and 2 ml iso-octane. The samples were vortexed thoroughly and centrifuged under 190g for 10 min. After centrifugation, the upper layer was transferred into another glass tube through a sodium sulfate-filled glass pipette before blowing down under a nitrogen stream. For the FFA fractions, 1.2 ml of methanol and 100 μl of acetyl chloride were mixed with the scraped FFA fractions; the mixture was heated in a 100 °C water bath for 60 min. Two milliliters hexane and 750 μl distilled deionized water were mixed thoroughly with the FFA mixture. The upper phase was transferred into another conical glass tube through a sodium sulfate-filled glass pipette before blowing down under a nitrogen stream. Fatty acid methyl esters were reconstituted with 100 μl heptane and transferred into auto-injector vials for analysis by gas chromatography (Agilent Technologies, Wilmington, DL, USA).

Statistical Analysis

One-way ANOVA was performed using SPSS software (IBM SPSS Statistics, V.19.0, IBM Corp., Armonk, NY, USA) to determine differences in body weight, body weight gain, fat pad weight, food intake and each fatty acid in the liver between groups. The differences between SFA, MUFA and PUFA in each diet group were also determined by one-way ANOVA. Two-way ANOVA was performed to compare serum fatty acids in control diet vs. HFD and portal vs. systemic blood samples within the fasted state or within the fed state. Post hoc analysis was performed by LSD comparisons where significant differences were found by ANOVA. Data are shown as mean \pm SEM. Statistical significance was set at $p < 0.05$.

Results

Body Weight, Fat Pad Weight and Food Intake

As expected, both final mean body weight and weight gain over the 3-week period were higher in the HFD group compared to the control group (Table 1). HFD-fed rats had significantly heavier fat pad mass (sum of epididymal and retroperitoneal fat pads) compared to the control group. Food intake by weight in the HFD group was markedly lower ($p < 0.05$) than in the control diet group, but caloric intake was higher ($p < 0.05$) because of the greater energy density of the HFD.

Table 1 Animal characteristics

	Control diet	HFD
Final body weight (g)	440.1 ± 8.9	479.0 ± 8.7*
Body weight change (g)	126.8 ± 10.6	141.9 ± 14.1*
Fat pad weight (g)	18.4 ± 0.7	25.6 ± 1.0*
Average daily food intake (g/day)	25.4 ± 0.6	21.4 ± 0.7*
Average daily energy intake (kcal/day)	97.7 ± 2.1	111.9 ± 3.7*

Data are shown as mean ± SEM, $n = 17$. Fat pad weight was the sum of epididymal and retroperitoneal fat pads

* Indicated statistically significant difference ($p < 0.05$) in the HFD group compared to the control diet group

Total Serum TAG and FFA Concentrations

Total serum concentrations of TAG and FFA are shown in Fig. 1. Total serum TAG level was significantly lower ($p < 0.05$) in the HFD-fed rats compared to the control under both fasted and fed states (Fig. 1a). Total circulating TAG level was significantly higher ($p < 0.05$) in the portal serum compared to systemic serum in the fed state, but no difference was found in the fasted state. In the fasted state, no significant differences were found either between diets or between the serum sources of total FFA (Fig. 1b). However, the HFD group had a markedly higher total FFA concentration compared to the control diet in the fed state. Furthermore, the total FFA level in the portal serum was significantly higher than systemic serum in the HFD-fed rats.

Fatty Acid Profile in Systemic Serum TAG

The complete serum fatty acid profile of serum TAG is shown in Supplemental Table S3. Total SFA, MUFA and PUFA were calculated to evaluate global changes in fatty acid composition. Control diet-fed animals killed under the fasted state had 54 % MUFA, 23 % PUFA and 23 % SFA of total fatty acids in their systemic serum TAG samples, whereas the systemic serum of HFD-fed rats had 32 % MUFA, 48 % PUFA and 21 % SFA in total fatty acids of serum TAG (Fig. 2a). Thus, HFD drastically shifted the dominant fatty acids from MUFA to PUFA in the systemic TAG after 3 weeks of HFD feeding. The PUFA in HFD serum was comprised of 72 % linoleic acid and 15 % arachidonic acid. In the fasted state, the majority of the TAG in serum comes from liver, suggesting that livers of HFD-fed rats were releasing more n-6 PUFA than MUFA or SFA in TAG.

In the fed state, systemic serum of control rats had 51 % MUFA, 19 % PUFA and 30 % SFA of the total fatty acids in TAG, whereas the HFD group had 42 % MUFA, 36 %

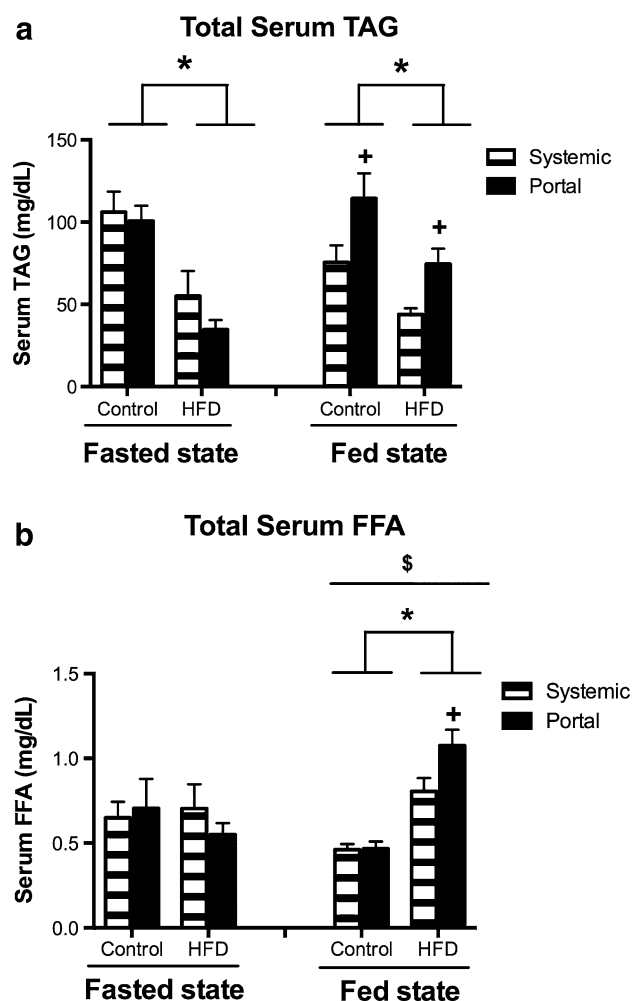


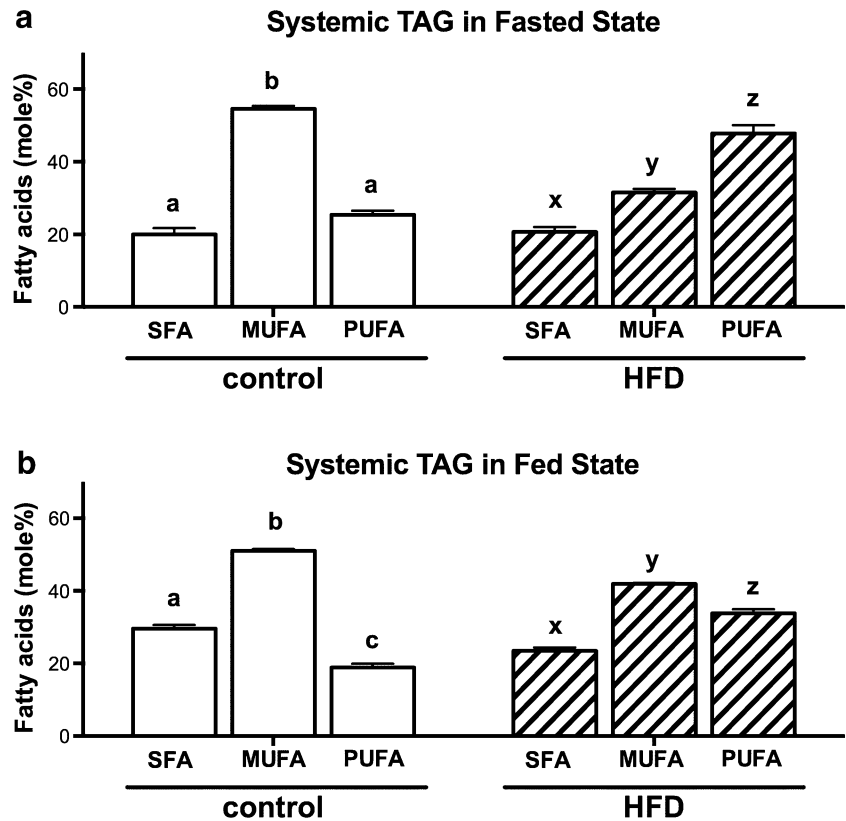
Fig. 1 Total serum **a** TAG and **b** FFA concentrations. Data are shown as mean ± SEM, $n = 5$ –10 per group. Asterisk denotes diet effect (control vs. HFD); plus denotes serum source effect (systemic vs. portal) within the diet group; dollar denotes interaction, $p < 0.05$

PUFA and 22 % SFA in the TAG fraction of their systemic serum (Fig. 2b). It appeared that a similar shift from MUFA in the control group to more PUFA in the HFD group also occurred in the fed state; however, the shifting was not as dramatic as seen in the fasted state. This is likely due to the serum TAG fraction measured in the *ad libitum* fed state reflecting the fatty acid composition of the diet, which is characterized by a MUFA-dominant profile.

Fatty Acid Profile of Portal Serum TAG

Similar to what we observed in the systemic serum TAG, control diet-fed animals displayed a fatty acid profile with 53 % MUFA, 18 % PUFA and 29 % SFA in the portal serum under fasted state (Fig. 3a). HFD also shifted the predominant fatty acids to PUFA in the portal serum TAG during the fasted state. In this condition, the fatty acid profile

Fig. 2 Fatty acid composition of systemic serum in TAG fraction under **a** fasted and **b** fed state in rats fed control or HFD. Data are shown as mean \pm SEM, $n = 5-10$ per group. *a, b, c* Means with different subscripts differ by $p < 0.05$ within control diet. *x, y, z* Means with different subscripts differ by $p < 0.05$ within HFD



of fasted portal serum TAG was composed of 32 % MUFA, 39 % PUFA and 29 % SFA in the total fatty acids. In the fed state, the majority of fatty acids in the portal serum TAG of the control diet-fed animals were MUFA (42 % of total fatty acid), while PUFA and SFA accounted for 22 and 36 %, respectively. HFD-fed animals had 42 % MUFA of total fatty acid, 34 % PUFA and 24 % SFA in their portal serum TAG (Fig. 3b).

Overall, the profile in the portal serum was identical with systemic serum, and the HFD group had a similar degree of shifting from MUFA to PUFA in both portal serum and systemic serum TAG. The proportion of SFA under the fasted state did not change between the control diet and HFD in both serum sources. In the fed state, however, HFD-fed rats had a lower proportion of SFA and higher proportion of PUFA compared to the control diet.

Fatty Acid Profile of Systemic Serum FFA

The complete serum fatty acid profile in percent fatty acid of the FFA fraction is shown in Supplemental Table S4. In the fasted state, the dominant fatty acids were SFA (45 % of total fatty acids), followed by MUFA (32 %) and then PUFA (23 %) in the systemic serum FFA of control diet-fed animals (Fig. 4a). The majority of the fatty acids in the SFA were stearic acid (50 %) and palmitic acid (40 %). In the systemic serum FFA of HFD-fed animals, SFA was still

the dominant fatty acid, which represented 47 % of the total fatty acids in the fasted state. However, there were more PUFAs (31 % of total fatty acids) in the systemic serum FFA of the HFD group compared to the control group. MUFAs only accounted for 22 % of the total FFAs in the systemic serum of the animals fed with HFD under fasted state (Fig. 4a). This finding suggested that a similar shift from MUFA to PUFA with HFD feeding still existed in the FFA fraction of systemic serum samples.

In the fed state, 61 % of the total fatty acids were SFA in the FFA fraction of systemic serum; 21 % were MUFAs; 18 % were PUFAs in the control diet-fed animals (Fig. 4b), whereas in the HFD-fed rats, 45 % of the total fatty acids in the FFA fraction of systemic serum were SFA, 25 % were MUFA, and 30 % were PUFA (Fig. 4b). The proportion of PUFA in systemic FFA increased significantly with HFD feeding in both fasted and fed states.

Fatty Acid Profile of Portal Serum FFA

The pattern of fatty acid composition in the FFA fraction of portal serum, as shown in Fig. 5, was similar to the systemic serum results. In the portal serum FFA of the control diet-fed rats, the predominant fatty acids were still SFAs, representing 53 % of the total fatty acids in the fasted state; 32 % of the total fatty acids were MUFAs; 15 % were PUFAs under fasted state. In the HFD group, 50 % of the

Fig. 3 Fatty acid composition of portal serum in the TAG fraction under **a** fasted and **b** fed state in rats fed a control or HFD. Data are shown as mean \pm SEM, $n = 5$ –10 per group. *a, b, c* Means with different subscripts differ by $p < 0.05$ within control diet. *x, y, z* Means with different subscripts differ by $p < 0.05$ within HFD

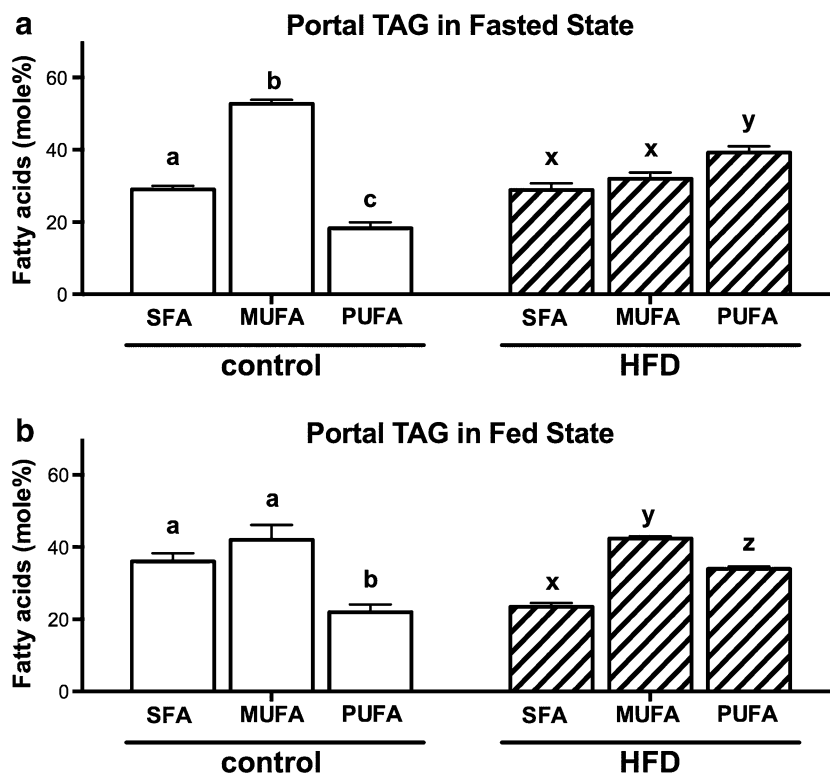


Fig. 4 Fatty acid composition of systemic serum in the FFA fraction under both **a** fasted and **b** fed state in rats fed a control or HFD. Data are shown as mean \pm SEM, $n = 5$ –10 per group. *a, b, c* Means with different subscripts differ by $p < 0.05$ within control diet. *x, y, z* Means with different subscripts differ by $p < 0.05$ within HFD

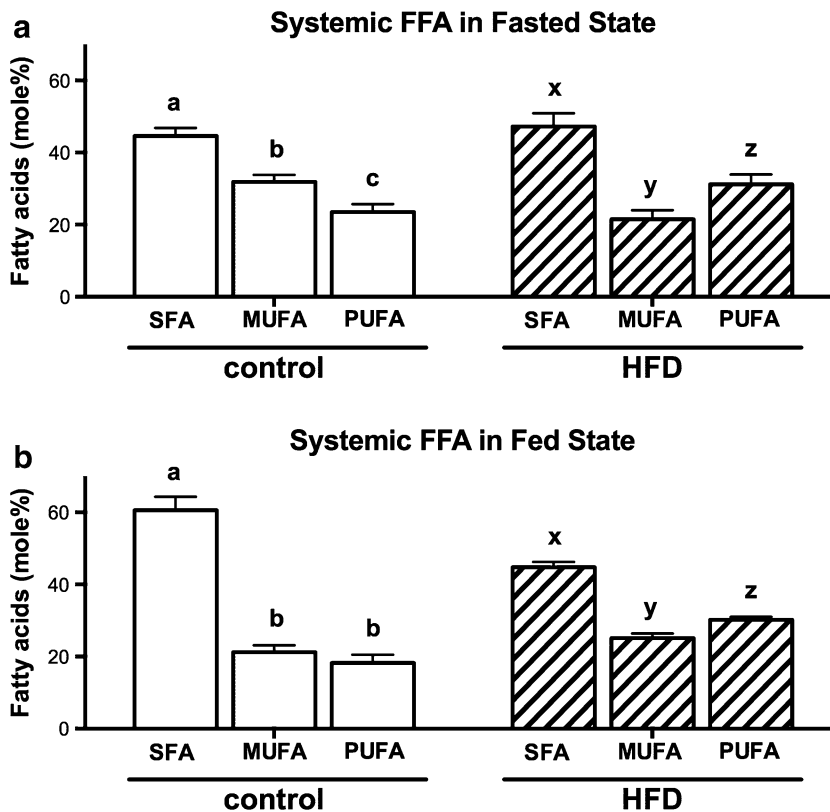
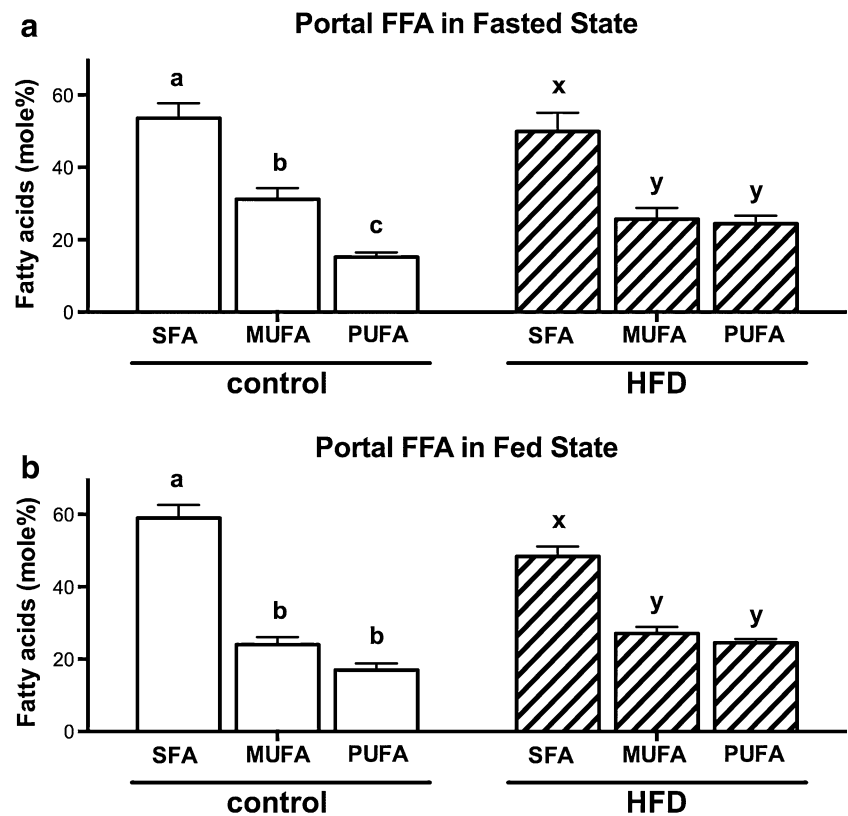


Fig. 5 Fatty acid composition of portal serum in the FFA fraction under **a** the fasted and **b** fed state in rats fed a control or HFD. Data are shown as mean \pm SEM, $n = 5\text{--}10$ per group. *a, b, c* Means with different subscripts differ by $p < 0.05$ within the control diet. *x, y, z* Means with different subscripts differ by $p < 0.05$ within HFD



total fatty acids in the portal serum FFA were SFA, 26 % were MUFA, and 24 % were PUFA under the fasted state (Fig. 5a). Overall, the percentage of MUFA decreased in the HFD group and shifted to PUFA compared to the control diet group. Fed-state fatty acid profiles nearly mimicked those found with fasting. In the fed state, 59 % of the total fatty acids in the FFA fraction of portal serum were SFA, 24 % were MUFA, and the remaining 17 % were PUFA in the control diet group (Fig. 5b). In the HFD-fed rats, 48 % of the total fatty acids were SFA, 27 % were MUFA, and 25 % were PUFA in the FFA fraction of their portal serum under the fed state.

Palmitic Acid (16:0) in the Serum

Percent palmitic acid in the TAG and FFA fraction is shown in Table 2. HFD-fed animals had significantly lower ($p < 0.05$) palmitic acid in the TAG fraction under both fasted and fed states. Additionally, the HFD-fed portal serum TAG palmitic acid was lower vs. systemic TAG palmitic acid ($p < 0.05$) under fasted state. However, HFD-fed portal serum TAG palmitic acid was not lower under the fed state when compared to systemic TAG palmitic acid. A significant interaction between diet interventions and serum sources was observed in the fed state ($p < 0.05$). The increased palmitic acid in the control diet-fed animals might indicate an increase in *de novo* lipogenesis, which is

logical given their high carbohydrate intake. Similarly with the TAG fraction, the HFD group had significantly lower ($p < 0.05$) palmitic acid in the FFA fraction in both the fasted and fed state. The portal serum had higher ($p < 0.05$) palmitic acid under fasted state; no difference was found between serum sources in the fed state.

Stearic Acid (18:0) in the Serum

Percent stearic acid in the TAG and FFA fraction is shown in Table 2. The HFD group had significantly higher ($p < 0.05$) stearic acid in the TAG fraction in both the fasted and fed state; however, the percent stearic acid in the portal vs. systemic serum TAG did not differ. The proportion of stearic acid in the portal serum FFA was significantly lower compared to the systemic serum FFA in both diet conditions in the fasted state. Neither diet nor serum source effects were found in the FFA fraction under the fed state.

Oleic Acid (18:1n-9) in the Serum

Percent oleic acid in the TAG and FFA fraction is shown in Table 2. The HFD group had significantly lower ($p < 0.05$) oleic acid in the TAG fraction in the fasted state, but markedly higher in the fed state. As for percent oleic acid in the FFA, the fed state, HFD group had higher ($p < 0.05$) levels in the FFA fraction compared

Table 2 Percent fatty acid in TAG and FFA fractions of systemic and portal serum under the fasted and fed state

Fatty acid	Lipid fraction	Fasted state				Fed state			
		Control		HFD		Control		HFD	
		Systemic	Portal	Systemic	Portal	Systemic	Portal	Systemic	Portal
16:0	TAG (%)	17.4 ± 1.6	27.0 ± 0.9 ^a	12.5 ± 1.5*	21.6 ± 1.4 ^{a,*}	26.3 ± 1.0	31.6 ± 1.9 ^a	15.5 ± 0.9*	15.6 ± 1.1*
	FFA (%)	18.7 ± 3.9	34.0 ± 1.8 ^a	15.1 ± 1.5*	25.8 ± 2.8 ^{a,*}	25.8 ± 2.6	19.8 ± 2.2	14.4 ± 1.4*	16.1 ± 1.2*
18:0	TAG (%)	2.6 ± 0.2	2.0 ± 0.2	8.1 ± 0.9*	7.3 ± 2.5*	3.3 ± 0.2	4.4 ± 0.5	7.9 ± 0.3*	7.9 ± 0.3*
	FFA (%)	22.4 ± 2.5	17.2 ± 2.6 ^a	27.6 ± 1.9	22.0 ± 2.3 ^a	33.9 ± 2.9	36.4 ± 3.5	29.5 ± 1.4	30.8 ± 2.5
18:1n-9	TAG (%)	39.7 ± 1.4	34.1 ± 1.0	28.4 ± 1.0*	28.5 ± 1.9*	35.0 ± 0.6	28.0 ± 3.2	38.4 ± 0.3*	39.2 ± 0.4*
	FFA (%)	24.4 ± 1.8	19.8 ± 1.8	20.3 ± 2.1	22.4 ± 2.4	16.7 ± 1.4	17.6 ± 1.5	23.4 ± 1.4*	26.2 ± 1.6*
18:2n-6	TAG (%)	15.9 ± 1.2	14.3 ± 1.4	29.6 ± 0.8*	28.3 ± 0.9*	15.7 ± 0.9	19.4 ± 1.6 ^a	26.5 ± 0.7*	27.9 ± 0.2*
	FFA (%)	10.5 ± 0.8	9.5 ± 1.0	15.8 ± 1.8*	17.5 ± 2.1*	8.3 ± 1.4	10.8 ± 1.2	17.9 ± 1.0*	19.5 ± 1.2*
20:4n-6	TAG (%)	3.3 ± 0.6	2.0 ± 0.3 ^a	7.9 ± 0.8*	5.9 ± 0.9 ^{a,*}	0.7 ± 0.1	1.0 ± 0.1	3.3 ± 0.3*	3.9 ± 0.4*
	FFA (%)	8.1 ± 1.4	3.8 ± 0.4 ^a	8.8 ± 1.0	5.0 ± 0.5 ^a	6.3 ± 0.6	5.1 ± 1.2 ^a	8.4 ± 0.8	5.0 ± 0.8 ^a

Data are shown as mean ± SEM, $n = 5$ –10 per group

* Main diet effect (control vs. HFD), $p < 0.05$

^a Serum source effect (systemic vs. portal serum) within the diet group

to the control diet group; neither diet nor serum source effects were found in the FFA fraction under the fasted state.

Linoleic Acid (18:2n-6) in the Serum

Percent linoleic acid in the TAG and FFA fraction is shown in Table 2. The percentages of linoleic acid in the serum TAG of HFD-fed animals were significantly higher ($p < 0.05$) compared to the control diet group in both the fasted and fed state. Portal serum TAG had higher ($p < 0.05$) linoleic acid than systemic serum in the fed state. The percent linoleic acid in the FFA fraction was also significantly higher ($p < 0.05$) in the HFD group in both fasted and fed states compared to control diet-fed animals, but no difference were found between serum sources.

Arachidonic Acid (20:4n-6) in the Serum

A significantly higher ($p < 0.05$) percent of arachidonic acid in the serum TAG was found in the HFD-fed compared to the control diet group in both the fasted and fed states (Table 2). Portal serum had a significantly lower ($p < 0.05$) percentage of arachidonic acid compared to systemic serum TAG in the fasted state. The percent of arachidonic acid in the portal serum was lower ($p < 0.05$) than systemic serum in the FFA fraction under both the fasted and fed states. No diet effect was found in the percent of arachidonic acid in the FFA fraction.

Liver TAG, DAG and PL

In the fasted state, the total liver TAG level was higher in the HFD group compared to the control diet-fed animals (Fig. 6a). In contrast, the total liver TAG level did not differ between diet groups under the fed state. However, liver TAG was higher in the fed compared to the fasted state. Diet interventions did not change the level of total DAG in the liver, but the total liver DAG in the fed state was higher than in the fasted state (Fig. 6b). The total PL concentration in the liver did not differ between the diet groups or fasted/fed status (Fig. 6c). The fatty acid profile in the liver TAG, DAG and PL fractions is shown in the supplemental materials (Supplemental Table S5, S6, S7). Fasted liver TAG data in Supplemental Table S5 have been previously published by our group [24].

SCD1 Indices in the Serum and Liver

SCD-1, also called delta-9 desaturase, is an enzyme that converts SFA (16:0 and 18:0) to MUFA (16:1 and 18:1), and thus modulates the percentages of SFA and MUFA. Previous studies have reported that liver SCD1 mRNA expression is positively correlated with SCD1 desaturase indices in the liver and the plasma triglyceride fractions, and the elevated SCD-18 index is associated with increased plasma TAG levels [25, 26]. SCD1 desaturase indices are good biomarkers for actual enzyme activity and have also been proposed as a good clinical indicator of SCD1 gene expression. Hence, to assess SCD1 activity we calculated two different SCD1 indices by dividing

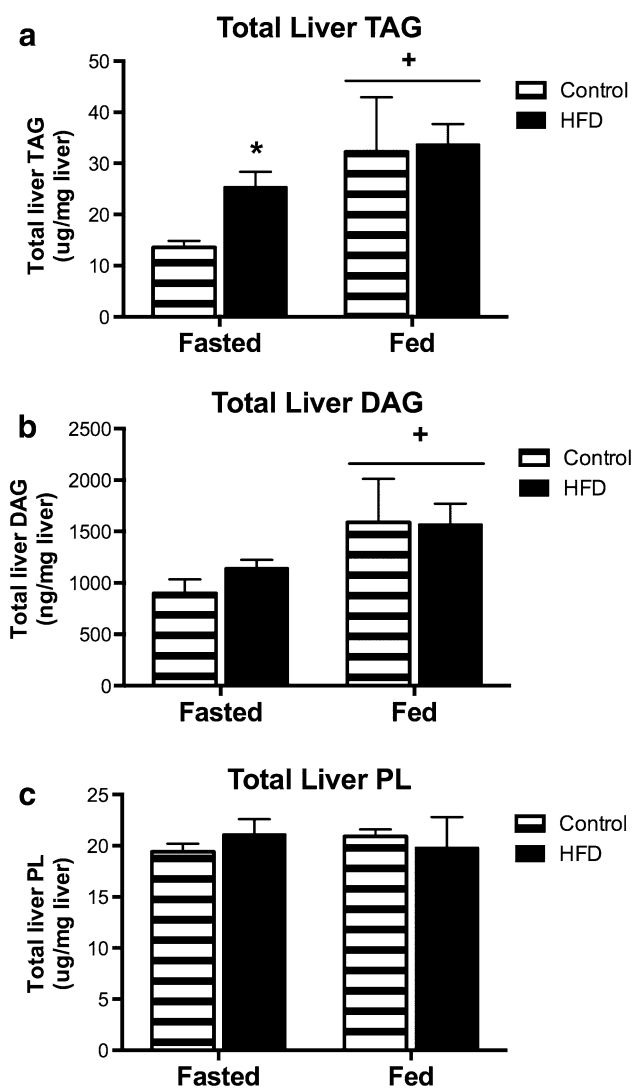


Fig. 6 Total liver **a** TAG, **b** DAG and **c** PL concentrations. Data are shown as mean \pm SEM, $n = 4$ per group. Asterisk denotes diet effect (control vs. HFD); plus denotes feeding status (fasted vs. fed), $p < 0.05$

the total concentration of 16:1 with 16:0 (SCD-16) and 18:1 with 18:0 (SCD-18) in each lipid fraction from serum and liver extracts [25, 27]. In the HFD group, the SCD-16 and SCD-18 indices were decreased in the liver TAG, DAG and PL fractions as well as fasting systemic serum TAG (Fig. 7). SCD-16 in the systemic serum FFA fraction was not calculable because 16:1 was not detectable in most of the samples. Fasting systemic serum of HFD-fed rats had significantly lower ($p < 0.05$) SCD-16 and SCD-18 indices compared to the control (Fig. 7a, b). SCD-18 in the fasting systemic serum FFA fraction did not change with HFD feeding (Fig. 7b). SCD-16 and -18 in the liver TAG, DAG and PL were all significantly

lower ($p < 0.05$) with HFD feeding under the fasted state (Fig. 7c, d).

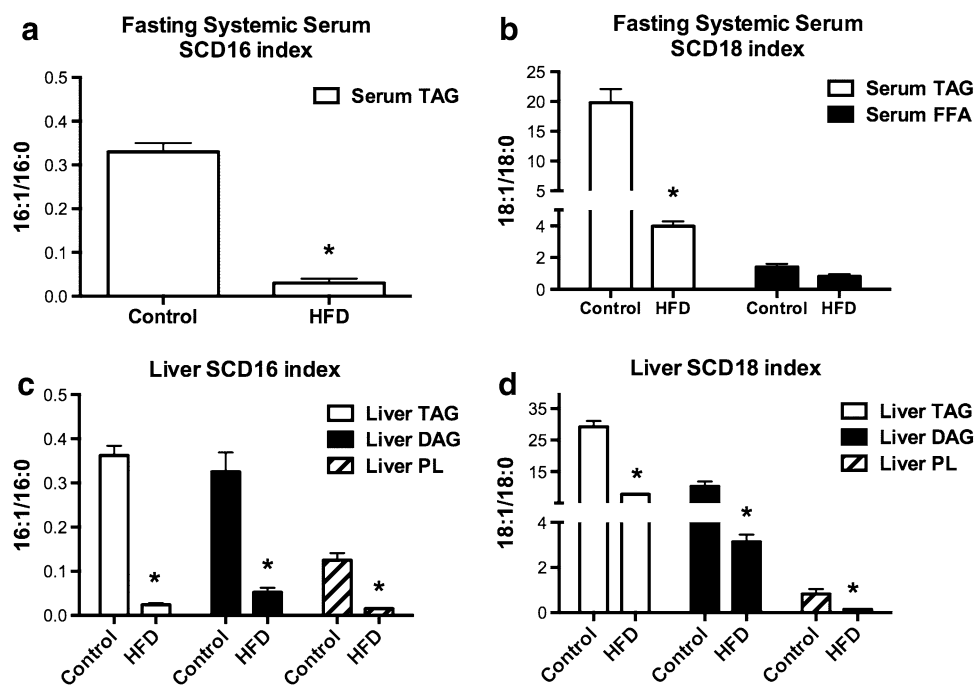
Discussion

Overconsumption of dietary fat is widely used to induce obesity and insulin resistance in *in vivo* rodent models, whereas excessive fatty acids are commonly used in cell culture to mimic the *in vivo* environment of HFD-induced lipotoxicity. The impact of an HFD on fatty acids in the circulation is unclear; therefore, the fatty acid species currently used for creating lipotoxicity in cell culture models potentially fail to imitate what organs are exposed to *in vivo*. Here, we report that 3 weeks of HFD feeding in obesity-prone Sprague-Dawley rats led to a greater proportion of circulating PUFA compared to MUFA in both TAG and FFA fractions compared to the control diet-fed animals. The increased proportion of PUFA was mainly attributed to increases in linoleic and arachidonic acids. Overall, these data suggest that *in vitro* experiments solely using palmitic or oleic acids do not adequately mimic circulating lipids in HFD-fed rodents.

The short 3-week HFD used in the current study was chosen to examine the effect of the HFD and to avoid the abnormal metabolism that may be induced by a more chronic HFD. Obesity-prone rats have a thrifty phenotype marked by a consistent, elevated storage of fat into adipose tissue and reduced oxidation in skeletal muscle [28]. The larger fat pad masses and twofold higher liver TAG content in the HFD group support this theory. Under fasted conditions, the total serum FFA level was no different between the control diet and HFD, conflicting with the elevated FFA levels previously reported in the serum of obese human subjects [6]. However, the animals in the current study received HFD over a relatively short duration, and the resulting obesity might not have been severe enough to display abnormal FFA levels under a fasted state. Moreover, some data suggest that obesity is not always linked with elevated serum FFA [29]. In contrast, the HFD led to greater circulating serum FFA levels in the fed state, likely a result of both increased dietary intake of lipids and an impaired ability of postprandial insulin to effectively inhibit lipolysis.

In the current study, 3 weeks of HFD feeding altered both serum TAG and FFA composition from predominantly MUFA to predominantly PUFA. Linoleic acid and arachidonic acid largely accounted for the increased percentage of PUFA in the serum TAG (72 and 15 % of the PUFA were linoleic acid and arachidonic acid, respectively) and serum FFA (83 and 4 %, respectively). Although we can only speculate, it is possible that the increased percentage

Fig. 7 SCD16 and SCD18 indices in (a, b) fasting systemic serum and (c, d) liver. Data are shown as mean \pm SEM, $n = 4$ per group. Asterisk indicated a significant difference in the HFD vs. control diet, $p < 0.05$



of PUFA in circulating FFA pools result from an increased flux of SFA and MUFA to storage or oxidative pathways. But this is purely speculation. The mechanisms by which PUFA increased in circulating FFA and TAG following an HFD are beyond the scope of this investigation but clearly deserve further study.

Linoleic acid (18:2n-6) is an essential dietary fatty acid that serves as a precursor for *in vivo* arachidonic acids synthesis. The HFD used in the current study had more linoleic acid compared to the control diet, and linoleic acid is present in relatively high amounts in the portal and systemic serum of the HFD-fed rats. This has particular implications for *in vitro* FFA incubation studies. Serum utilized to culture cells typically has linoleic acid an important component given that it is an essential fatty acid. However, *in vitro* fatty acid exposure studies are usually performed using serum-free media and thus media free of linoleic acid. This is a major concern and could negatively impact a number of outcome measures and further highlights that current methods for *in vitro* fatty acid exposure studies largely ignore physiological relevance.

n-6 PUFAs have long been suggested to be proinflammatory because of their downstream pathways that produce proinflammatory eicosanoids; however, this view has been challenged by a human randomized clinical trial showing that serum inflammatory markers were not affected by increased n-6 PUFA consumption [30]. At the same time, there are numerous reports showing that linoleic acid and arachidonic derived metabolites have potent and substantial effects on the nuclear receptors LXR and PPARs, which subsequently can alter the lipid metabolism, insulin

signaling and adipogenesis [31–33]. Overall, our data show that the percentage of fatty acids comprised of n-6 PUFA increases with HFD feeding and subsequently that *in vitro* studies should not ignore the important role that these fatty acids may have on outcome measures.

The changes observed in the percentage of SFA and MUFA due to HFD could also be modulated by the enzyme SCD1, predominantly expressed in the liver, which converts saturated fatty acids (16:0 and 18:0) derived from dietary fatty acids or from *de novo* lipogenesis into MUFA (16:1 and 18:1). As stated above, previous studies have reported that liver SCD1 mRNA expression is positively correlated with SCD1 desaturase indices in the liver and the plasma triglyceride fractions, and the elevated SCD-18 index is associated with increased plasma TAG levels [25, 26]. Therefore, we used SCD-1 indices to assess serum liver SCD-1 activity. Numerous studies have suggested that the SCD-1 indices have been found to correlate positively with obesity and the development of metabolic syndrome [34, 35]. Moreover, high SCD-1 activity is associated with decreased fat oxidation and increased fatty acid synthesis [36, 37]. Hence, we were surprised to find that both of the SCD-1 indices, SCD-16 (16:1/16:0) and SCD-18 (18:1/18:0), in the serum and liver samples of current study were all decreased in the HFD group. It is possible that the decreased SCD-1 indices found in the current study were associated with the lower serum triglyceride level in the HFD-fed animals. Additionally, a previous study showed that a 3-day high-carbohydrate and low-fat diet (75 % kcal carbohydrate, 10 % kcal fat) feeding increased the SCD-16 index in the VLDL triglycerides in human subjects

compared to a diet with 40 % kcal fat and 45 % kcal carbohydrate [38]. The control diet used in this study had a similar high percentage of carbohydrates (70 % kcal carbohydrates), which possibly explains the SCD-1 indices being higher in the control vs. HFD group. Furthermore, a recent study conducted by Li *et al.* [39] suggested that elevated SFA following decreased SCD-1 activity in the liver results in hepatocellular apoptosis, steatohepatitis and fibrosis. In contrast, increased SCD-1 activity in the liver partitioned excess SFA into MUFA and a benign hepatic steatosis [39]. Overall, these findings suggest that suppression of SCD-1 may lead to greater hepatic lipotoxicity.

Even though the overall profile in the portal serum was identical with systemic serum and the HFD group had a similar degree of shifting from MUFA to PUFA in both portal serum and systemic serum TAG, percentages of certain fatty acid species shifted dramatically when comparing the portal serum vs. systemic serum. For example, the percentage of 16:0 in the portal serum was significantly higher in both the control and HFD groups when compared to systemic serum. Because liver is exposed to fatty acids from the blood stream of both the systemic circulation and portal vein, the differences of fatty acid profile in the systemic circulation and portal vein should be taken into consideration when treating hepatocytes with FFA *in vitro*. On the other hand, the systemic fatty acid profile observed in this study may be used in cell culture studies involving other cell types such as adipocytes, myocytes, endothelial cells, etc.

It is important to mention that ceramide species and other sphingolipids were not measured in this work, some of which have been shown to contribute to insulin resistance and often be associated with multiple obesity-related diseases that deserve further study [40]. Another important question is whether the fatty acid profile measured in this study is human relevant. Certainly, the percent energy coming from fatty acids in the rodent diet (60 %) is much higher than what humans typically consume. However, NHANES data would suggest that the relative percentage of total fat that comes from SFA, MUFA and PUFA sources is quite similar to the rodent HFD utilized here [41]. Thus, there is the possibility that dietary similarities may lead to similar results in circulating lipids. However, digestive and metabolic differences between humans and rodents may ultimately lead to different circulating fatty acids. We would posit that the fatty acid profile described here, while not confirmed in humans, would still be a more physiologically relevant method than using single fatty acids for *in vitro* studies.

In conclusion, 3 weeks of HFD alters the serum fatty acid composition of TAG and FFA from predominantly MUFA to predominantly n-6 PUFA. The increased linoleic acid and arachidonic acid contributed to the majority of the elevation of n-6 PUFA. Therefore, a more physiologically

relevant *in vitro* fatty acid mixture to mimic the initial stage of HFD-induced obesity should include increased linoleic acids and arachidonic acids relative to SFA and MUFA. Further studies are needed to determine whether *in vitro* studies using these FFAs would induce meaningful alterations in inflammation, ectopic lipid storage and insulin signaling.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest, financial or otherwise, to disclose for this research.

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