CHANGES IN FATTY ACIDS PROFILES AFTER THREE WEEKS OF HIGH-FAT DIET FEEDING IN OBESITY-PRONE RATS

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By TZU-WEN LIU

Dr. John P. Thyfault, Thesis Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

CHANGES IN FATTY ACIDS PROFILES AFTER THREE WEEKS OF HIGH-FAT DIET FEEDING IN OBESITY-PRONE RATS

presented by Tzu-Wen Liu,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. John Thyfault

Dr. Victoria Vieira-Potter

Dr. Kevin Fritsche

Dedicate to…

My Beloved Parents and Brother

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Changes In Fatty Acids Profiles After Three Weeks Of High-Fat Diet Feeding In Obesity-Prone Rats

Tzu-Wen Liu

Dr. John P. Thyfault, Thesis Supervisor

Abstract

High-fat diet (HFD) feeding is commonly used in animal models to induce obesity and metabolic diseases. However, the effect of HFD on serum fatty acid profiles remains unclear. Changes in serum fatty acid profiles due to HFD may be a factor in lipotoxicity in various organs. Historically, in vitro studies have utilized individual non-esterified fatty acids (NEFA) to study lipid exposure, potentially ignoring the importance of fatty acid combinations on cellular lipid metabolism. **OBJECTIVE:** To accurately characterize the proportion of circulating fatty acids entering and exiting the liver in obese-prone rats fed with HFD in both fasted and fed state. Our ultimate goal is to create a physiological relevant fatty acid mixture to investigate lipid exposure in a cell culture system. **METHODS:** Obesity prone rats were fed a HFD (60%kcal fat) or a control diet (10%kcal fat) for 3 weeks; liver, portal and systemic blood samples were collected. Triglycerides (TG) and NEFA in the serum, TG, diglycerides (DG) and phospholipids (PL) in the liver were extracted and analyzed using gas chromatography. **RESULTS:** HFD group was heavier and had bigger fat pad compared to control diet fed animals. Both systemic and portal serum TG were ~40% lower in HFD. In contrast, liver had higher fasting TG (~2-fold) and DG (~1.3-fold) in the HFD group compared to control group. Total serum NEFA levels were not affected by diet in the fasted state, but

increased in the HFD group compared to the control group under fed state. In the control group, monounsaturated fatty acids (MUFA) were the predominant fatty acids in serum TG, whereas polyunsaturated fatty acids (PUFA) were the dominant fatty acids in the HFD group. The elevations of PUFA were mostly attributed to the increased of n-6 PUFA, linoleic acid and arachidonic acid. Similar shift from MUFA to n-6 PUFA also occurred in the serum NEFA fraction. **CONCLUSION:** HFD shifted predominate fatty acids of serum TG fraction from MUFA in the control diet to n-6 PUFA. A more physiologically relevant fatty acid mixture to mimic HFD conditions for in vitro studies may include increased linoleic acid and arachidonic acid relative to saturated fatty acids and MUFA.

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1. Introduction

The World Health Organization (WHO) indicates that nearly 500 million people are obese and 1.4 billion overweight in 2008. Moreover, 43 million children under the age of five were overweight in 2010 [1]. The estimated medical costs of obesity related diseases in 2008 were twice as much as in 1998 in the United States [2]. It is apparent that obesity is a serious health issue leads to increased cost of health care. The associated adverse health problem and complications of obesity are usually the causes of morbidity and mortality, including insulin resistance, Type 2 diabetes (T2D), and the metabolic syndrome [3, 4].

Except genetic factors, the prevalence of obesity is mostly caused by overnutrition and sedentary lifestyle. The high proportion of fat and simple sugar in the Western diet are one of the major causes of overnutrition in modern society. With the occurrence of obesity, dyslipidemia and ectopic lipid accumulation appear. These two main symptoms are believed to be the major drivers of insulin resistance and T2D. Lipid exposure has therefore been used to induce excess lipid storage and insulin resistance in various cell culture models of tissues known to participate in the development of metabolic diseases associated with obesity (muscle, liver, adipose, endothelial, pancreas). Induction of insulin resistance via lipid exposure in cell culture provides scientists a useful isolated system with which to test a myriad of nutraceutical/pharmaceutical treatments for obesity-related diseases.

Obesity, Fatty Acids And Insulin Resistance

Obesity has been linked to elevated circulating fatty acids levels, including both NEFA [5] and TG [6-8], as well as insulin resistance[4]. Under normal circumstances, fatty acids are stored in the adipocytes as TG and then undergo liberation by lipolysis during fasting that leads to elevated circulating NEFA levels. The existence of adipose tissues protects non-adipose tissues such as liver, muscle, heart and pancreas that have limited capacity for TG storage from excessive lipid accumulation. Lipotoxicity is induced when excessive lipid storage in these tissues occur, so called ectopic lipid accumulation that is associated with metabolic syndrome, insulin resistance and dyslipidemia [9]. Elevated circulating TG is well-known to be positively associated with insulin resistance and has been commonly used as a clinical marker [10], however, the evidences and mechanism of how NEFA associated with insulin resistance are still not completely understood [11].

Despite the lack of solid consensus on how elevated circulating NEFA induce insulin resistance, evidence shows that obese people have increased plasma NEFA level due to the elevated NEFA secretion from enlarged and stressed adipocytes and/or reduced NEFA clearance [5]. Lowering elevated plasma NEFA levels in both lean and obese patients has been shown to improve insulin resistance and glucose tolerance [12]. Moreover, portal NEFA levels appear to be negatively correlated with hepatic insulin clearance in obese rats [13]. Circulating NEFA and TG appear to play an important role in obesity and insulin sensitivity.

Hepatic and Circulating Lipid Metabolism

Lipid metabolism is mostly dominated by liver, the main organ that takes up the majority of the lipids absorbed in the gut and a) uses them as an energy source, b) stores them or c) repackages them into lipoproteins. As mentioned above, excessive lipid accumulation in the liver causes cellular toxicity in which the liver fails to suppress VLDL secretion due to increased apolipoprotein B level and eventually leads to dyslipidemia [14, 15]. These elevated lipids travel through the circulation and may further cause lipotoxicity in various organs and lead to inflammation.

Liver receives exposure to fatty acids from the blood stream of both systemic circulation and the portal vein. In the fasted state, fatty acids in the portal vein are a result of lipolysis in visceral fat [16, 17]. To our best knowledge, the differences of fatty acid profiles of TG and NEFA between systemic circulation and portal blood in relation to a high fat diet or obesity are still unclear.

Fatty Acid Usage In Cell Culture

Due to the important regulatory roles of liver, primary hepatocytes and immortalized hepatocyte cell lines have been widely used to investigate how fatty acids induce lipid accumulation and hepatic insulin resistance [18, 19]. Many different fatty acid species or different fatty acid mixtures have been used to demonstrate lipid exposure to induce insulin resistance. Previous evidence showed that multiple fatty acid species have the same ability in inducing insulin resistance in primary hepatocytes [20], however, palmitic acid (C16:0) and oleic acid (C18:1, n-9) are two of the favorite species to most

of the researchers [21, 22]. Palmitic acid and oleic acid are the dominant saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA) in the circulation and most tissues, respectively [23]. Additionally, some research teams believe that only SFA have the ability to induce intracellular lipid accumulation followed by the development of insulin resistance, therefore, palmitic acid is the species that has been used more commonly for these purposes [24-26]. Despite the intense lipotoxicity induced by palmitic acid, adding oleic acid into the fatty acid mixtures increase the physiological relevance even though it acts almost in an opposite sense with palmitic acid to cellular lipid metabolism.

A study conducted by Chabowski et al. [27] suggested that oleic acid was contributed to the increasing amount of diglycerides (DG) fraction, whereas palmitic acid was the one that can induced the accumulation of TG. Palmitic acid has the ability to increase saturation in ceramide fraction, whereas oleic acid incubation could prevent inflammation, insulin resistance and apoptosis by a sequestration of palmitic acid into TG pool, thus preventing its conversion into ceramides [9]. Apparently every researcher has their favorite fatty acid species for different reasons; a standard fatty acid mixture does not exist, ergo making it harder to compare the responses obtained from each study.

Liver is exposed to more than one single fatty acid *in vivo* [23], therefore the current method may potentially ignore the importance of fatty acid combinations in cellular lipid metabolism. It has been shown that different fatty acid species cause various toxicity and/or inflammation reactions to cells that may eventually alter cell functions. For instance, a study aiming to determine the toxicity of NEFA on two human leukemic cell lines by the loss of cell membrane integrity and/or DNA fragment showed

that of the SFA, the longer the carbon chain length the higher the cytotoxicity (stearic acid (C18:0)> palmitic acid (C16:0) > myristic acid (C14:0) > lauric acid (C12:0)) [28]. In the same study, the ranking of toxicity of the monounsaturated fatty acids (MUFAs) was: palmitoleic acid (C16:1n7) > vaccenic acid (C18:1n7) > oleic acid (C18:1n9) > elaidic acid (C18:1n9 trans) [28]. Furthermore, the cells challenged with polyunsaturated fatty acids (PUFA) has been shown to have membrane modifications due to the fast assimilation of PUFA in neutral or polar lipids. The effects of n-3 and n-6 PUFA on membrane signaling of immune response have also been widely documented [29-31]. Due to the specificity of some fatty acids on regulating cell metabolism, it is important to create more physiological relevant fatty acid mixtures to mimic *in vivo* response. To accomplish this goal, we first need to examine the fatty acid profile in the circulating serum. Moreover, this should be done in an *in vivo* model in which lipid accumulation and insulin resistance is induced by a HFD in order to adequately reflect the obese, high-energy (HE) diet condition.

Obesity-prone Sprague-Dawley Rat Model

In the outbred Sprague-Dawley rats fed with HE diet that contains 31% kcal fat, approximately half of the rats became hyperphagic and developed diet induced obesity (DIO), while the rest of them were resistant to the HE diet [32, 33]. The high responding substrain, known as obesity-prone rats, have been inbred over several generations in an effort to retain this obese phenotype. Similar to human obesity, the bimodal pattern of this DIO model has proven to be due to a polygenic pattern of inheritance [34]. This

polygenic obesity-prone substrain has common features with human obesity that serves as a suitable model for studying the effects of a high-energy/high-fat diet on circulating fatty acid levels [35]. Most importantly, they ensure that we will have consistent hyperphagic and weight gain response to a high-energy/ high-fat diet.

Effects of High-Fat Diet on Serum Fatty Acid Profile

High-fat diet (HFD) has been widely used to develop a diet-induced obesity model for animal researches. The Open Source Research Diets (Research Diet, Inc., New Brunswick, NJ, USA) is one of the most commonly used commercial diet in rodent studies nowadays that provides diets with various proportion of macronutrients to fit different research needs. Among all the HFD products in this company, the lard-base HFD that contains 45% kcal (24% by weight) or 60% kcal (35% by weight) fat has been used most frequently. Despite the widely usage of these HFDs, how they affect serum fatty acids profile in different lipid classes is still unclear.

In order to create a more physiological relevant fatty acid mixture to study lipid exposure in a cell culture system, we first need to accurately characterize the proportion and concentration of circulating fatty acids entering and exiting the liver in obese-prone Sprague-Dawley rats fed with HFD in both fasted and fed states. We fed the obesity-prone rats a lard-base HFD with 60% kcal fat for three weeks and analyzed the fatty acids profiles in different lipid classes from their portal and systemic circulating serum samples. In the future, fatty acid mixtures will be made based on the data we collect in this study

and incubated with HepG2 cells to compare the cell responses with the conventional fatty acids treatments.

2. Materials and Methods

Animals and Study Design

Thirty-four 5-week-old obesity-prone Sprague-Dawley rats were purchased from Taconic (Hudson, NY, USA) and housed in a room controlled for temperature (75°F), humidity (50%), and light cycle (12 hour light/dark) in Harry S Truman Memorial Veterans Medical Center. 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 (10% kcal fat (half of the fat came from lard, half of them came from soybean oil), 20% kcal protein, 70% kcal carbohydrate, D12450B, Research Diet, Inc., New Brunswick, NJ, USA) for three weeks. Rats were then randomly separated into two groups with equal average body weight. One group was continuing to be fed the control diet while the other group was fed the HFD (60% kcal fat (92% of the fat came from lard, 8% from soybean oil), 20% kcal protein, 20% kcal carbohydrate, D12492, Research Diets Inc., New Brunswick, NJ, USA) for three weeks. Diets compositions and fatty acid profiles are shown in **Table 1** and **Table 2**. Fourteen rats (7 in control group, 7 in HFD group) were euthanized at 9am after a 12-h food withdraw whereas the other twenty rats (10 in control group, 10 in HFD group) were continue to be fed *ad libitum* until euthanasia at 7am in the morning under fed state. More rats were used in the fed state group because more variations were expected due to

ad libitum feeding prior to sacrifice. Rats were anesthetized with pentobarbital (100mg/kg) and then exsanguinated by removal of heart. Systemic circulation and portal blood samples were collected and sit under room temperature for 20 min before centrifuged (7000*g*, 10 min, 4°C). Serum were aliquoted and stored in -80°C for further analysis. Study timeline is shown in **Figure 1**.

	Control Diet		HFD	
	%g	%kcal	%g	%kcal
Protein	19.2	20	26.2	20
CHO	67.3	70	26.3	20
Fat	4.3	10	34.9	60
Total		100		100
kcal/g	3.85		5.24	

	Control Diet		HFD	
	g	kcal	g	kcal
Sucrose	350	1400	68.8	275.2
Soybean oil	25	225	25	225
Lard	20	180	245	2205
Total	1055	4057	774	4057

Table 1. Diets compositions. The upper part of this table shows the proportion of macronutrients in both the control and high-fat diets. The lower part of this table includes the important constituents in the diets and the actual amount of food that animals need to ingest for the same caloric consumption.

	Control Diet (D12450B)		HFD (D12492)	
Fatty acid	g/kg diet	%	g/kg diet	%
16:0	6.5	15.1	49.9	20.3
16:1	0.3	0.7	3.4	1.4
18:0	3.1	7.2	26.9	10.9
18:1n9	12.6	29.2	86.6	35.2
18:1n7	0.0	0.0	0.0	0.0
18:2n6	18.3	42.5	73.1	29.7
18:3n3	2.2	5.1	5.2	2.1
20:4n6	0.1	0.2	0.7	0.3
20:5n3	0.0	0.0	0.0	0.0
22:4n6	0.0	0.0	0.0	0.0
22:5n3	0.0	0.0	0.2	0.1
22:6n3	0.0	0.0	0.0	0.0
Total	43.1	100.0	246.0	100.0
SFA		22.0		31.0
MUFA		30.0		37.0
PUFA		48.0		32.0

Table 2. Fatty acid profile of the diets.

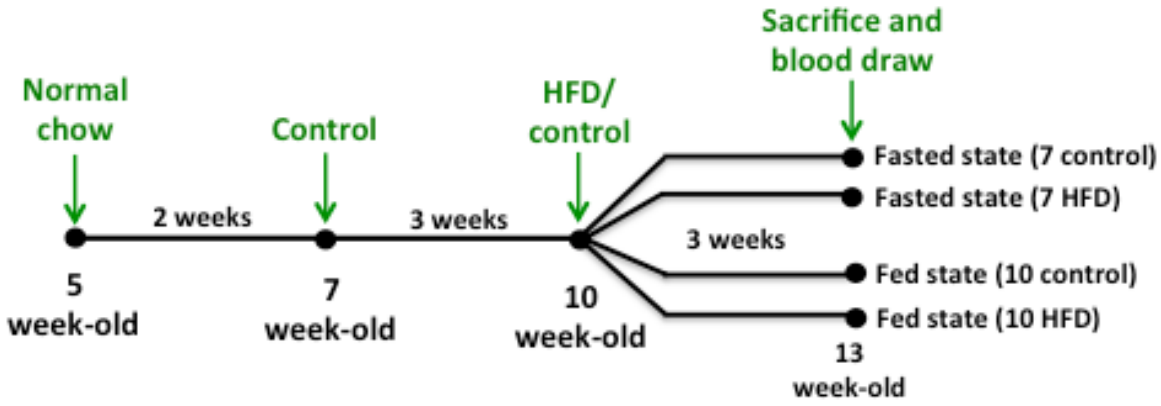


Figure 1. Study timeline.

Lipid Extraction

Serum: 0.5mL of serum from each sample was pipetted into a 15 mL screw-capped glass conical tube and mixed with 1.5 mL of ice-cold aqueous buffer Tris[hydroxymethyl]aminomethane hydrochloridef[50 mmol/L] and Na_2EDTA [1 mmol/L]; pH, 7.4). Twenty μg of C17:0 NEFA (Sigma, St. Louis, MO, USA) in chloroform and 25 μg of C17:0 TG (Nu-chek, Elysian, MN, USA) were added into each sample as internal standards.

Liver: Approximately 100mg of liver from each sample was homogenized in 2mL of Trizma/EDTA buffer. One hundred μg of C17:0 TG, 25μg of C17:0 DG (Nu-chek, Elysian, MN, USA), and 100μg of C17:0 phospholipids (PL; Avanti, Alabaster, AL, USA) were added into each sample as internal standards.

Lipids were extracted by the method of Folch et al.[(36)] with slight modifications. Samples were mixed with 3ml of 0.5% of acetic acid in methanol, and then 6mL of chloroform was added into the mixtures. The mixtures were sitting under room temperature for 10 minutes for phase separation before being centrifuged at 190*g* for 10 minutes. The lower organic phase containing lipids were transferred into another glass conical tube through 1PS filter (Whatsman, Piscataway, NJ, USA) and evaporated under a nitrogen stream and then reconstituted with 0.1mL of 2:1 chloroform: methanol.

Separation of Different Lipid Classes

Individual lipid classes of the extracted lipid were separated using thin-layer chromatography (TLC). A 20 × 10 cm silica gel 60Å analytical plate (Sigma, St. Louis,

MO, USA) was washed with a 1:1 chloroform: methanol solvent before spotted with extracted lipid. Lipid class standards were also spotted for detecting target bands. Total lipid classes were separated by developing the plate for about 20 min in a solvent system containing hexane, diethyl ether, and acetic acid in the ratio of 70:30:1 (v/v/v). The TLC plate was then sprayed with 0.1% of 2', 7'- dichlorofluorescein in methanol. Individual lipid bands on the plate were detected under a UV lamp and the margins were marked with a thumbtack. TG and NEFA bands in the serum samples and the TG, DG and PL bands in the liver samples were scraped with a razor blade and collected into round bottom screw-capped glass tubes.

Fatty acid Methyl Esters (FAME)

Toluene (0.5 mL) was added into the round-bottom glass tubes with collected lipid fractions. ***For TG, DG and PL fractions:*** 1mL of 0.5M sodium methoxide in anhydrous methanol was mixed with the each sample for 1 min, 0.75mL of distilled deionized water and 2 mL of iso-octane were then added into the glass tube. The samples were vortexed thoroughly and sat 10 min for phase separation before being centrifuged under 190*g* for 10 min. After centrifuged, the upper layer was transferred into another 15mL conical glass tube through a sodium sulfate-filled glass pipette before blowing down under a nitrogen stream. The FAME of TG, DG and PL were reconstituted with 0.1 mL of heptane and transferred into auto-injector vial. ***For the NEFAs fractions:*** 1.2 mL of methanol and 0.1 mL of acetyl chloride were added into the glass tube, the mixture was heated up in the water bath for 60 min in 100°C. Two ml of hexane and 0.75mL of

distilled deionized water were added to the NEFAs mixture; mix thoroughly and wait 10 min for phase separation. The upper phase was transferred into another conical glass tube through a sodium sulfate-filled glass pipette before blowing down under a nitrogen stream. The FAME of NEFAs were reconstituted with 0.1mL of heptane and transferred into auto-injector vials for analysis by gas chromatography.

GC Data Analysis

Commercially available FAME standards were used to identify target fatty acids by comparing the chromatograms of sample FAME with the FAME standards. The peak area of each fatty acid was normalized to the peak area and concentration of the internal standard. The percentage and total μg of each fatty acid species were calculated. Moreover, the molar concentration of the each fatty acid was calculated and then converted to moles of the corresponding lipid classes through molecular weight calculations. The total concentration of lipid classes was normalized to serum volume to obtain concentration (mg/dL) in each sample.

Stearoyl-CoA desaturase 1 (SCD-1) indices

SCD-1, also called delta-9 desaturase, is an enzyme that convert SFA (C16:0 and C18:0) to MUFA (C16:1 and C18:1). The indices of SCD-1 was calculated by dividing the total concentration of C16:1 with C16:0 (SCD-16) and C18:1/C18:0 (SCD-18) in each fraction of serum samples and liver.

Statistic Analysis

One-way ANOVA was performed using SPSS software to obtain the comparison of body weight, body weight gain, fat pad weight, food intake, and each fatty acid in the liver between control diet and HFD group. The differences between SFA, MUFA and PUFA in each diet group were also determined by one-way ANOVA. Two-way ANOVA was performed using SPSS software to obtain the comparison of serum fatty acids in control diet versus HFD and portal versus systemic blood samples in either fasted state or fed state. Post hoc analysis was performed by LSD comparisons where significant differences were found. GraphPad PRISM (version 5.0, GraphPad Software Inc.) was used to graph the results. Data are shown as the mean ± SEM. Statistical significance was considered when $p<0.05$.

3. Result

Body, fat pad weight and food intake

Animal characteristics are shown in **Table 3**. The final body weight in the HFD group was ~40g (9.8%) higher than the control diet group ($p<0.05$). Similarly, the body weight change was also significant higher in the HFD group compared to the control diet group. Fat pads were dissected and weighted; total fat pad weight was the sum of epididymal and retroperitoneal fat pads. Animals in HFD group also had significantly bigger fat pad compared to the control group. Food intake by weight in HFD group was markedly lower than the control diet group, but the actual calories intake was higher in the HFD group compared to the control animals because the energy density of HFD was higher ($p<0.05$).

Total serum TG and NEFA concentrations

Total serum concentrations of TG and NEFA are shown in **Figure 2**. Total serum TG level was significantly lower in the HFD fed group compared to the control diet under both fasted and fed states. Moreover, total TG level was significantly higher in the portal serum compared to systemic serum in the fed state, but no difference was found in the fasted state. This finding was not surprising since the absorbed lipids drain into liver through portal vein under fed state. In the fasted state, no significant difference was found between the diets and the serum sources of total NEFA level. However, HFD group had markedly higher total NEFA concentration compared to the control diet in the fed state, indicating that the ability of suppressing lipolysis during a fed state was likely

impaired in the HFD fed animals. Furthermore, their total NEFA level in the portal serum was significantly higher than systemic serum.

Fatty acid profile in TG and NEFA fraction

Complete serum fatty acid profile in percent fatty acid of TG is shown in **Table 4**. The table includes data in both systemic and portal serum under fasted and fed states in control and HFD groups. The predominant fatty acids in the TG fractions (oleic acid, palmitic acid, and linoleic acid) and some other fatty acids with known physiological functions were graphed and discussed in the following figures. Complete serum fatty acid profile in percent fatty acid of NEFA fraction is shown in **Table 5**. The dominant fatty acids in NEFA fraction include oleic acid, stearic acid, palmitic acid, and linoleic acid.

Proportion of SFA, MUFA and PUFA in TG fraction of systemic serum

Total SFA, MUFA and PUFA have been calculated to evaluate the changes of fatty acid composition. The fatty acid composition of TG in the systemic serum is shown in **Figure 3**. Control diet fed animals sacrificed under fasted state had 54% of MUFA, 23% of PUFA and 23% of SFA of total fatty acids in their systemic serum samples Whereas systemic serum of HFD group had 32% of MUFA, 48% PUFA and 21% SFA in total fatty acids (**Fig. 3A**). HFD apparently shifted the dominant fatty acids from MUFA to PUFA in the systemic serum samples after three weeks of HFD feeding. Seventy-two percent of the serum PUFA in HFD group came from linoleic acid, 15% were composed

by arachidonic acid. The majority of the TG in the serum comes from liver in the fasted state, indicating that the liver was synthesizing and releasing more n-6 PUFA than MUFA.

In the fed state, control group had 51% of MUFA, 19% of PUFA and 30% of SFA of the total fatty acids in their systemic serum. Whereas HFD group had 42% MUFA, 36% PUFA and 22% SFA in the TG fraction of their systemic serum (**Fig. 3B**). 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 what we have seen in the fasted state. This might due to the fact that serum TG fraction reflects the fatty acid composition of dietary fat in the fed state, and the amount of oleic acid in the HFD led to a MUFA dominant profile.

Proportion of SFA, MUFA and PUFA in TG fraction of portal serum

Figure 4 shows the fatty acid composition in the portal serum. Similar with what we saw in the systemic serum, the control diet fed animals displayed a fatty acid profile with 53% of the total fatty acids MUFA, 18% PUFA and 29% SFA in the portal serum under fasted state. HFD also shifted the predominant fatty acids to PUFA in the portal serum under fasted state that composed by 32% of MUFA, 39% of PUFA and 29% of SFA in the total fatty acids (**Fig. 4A**).

In the fed state, the majority of fatty acids in the control diet fed animals were still MUFA (42% of total fatty acid), PUFA accounted for 22% of the total fatty acid, and 36% were SFA. HFD fed animals had 42% MUFA of total fatty acid, 34% of PUFA and

24% of SFA in their portal serum (**Fig. 4B**). The overall profile in the portal serum was very similar with systemic serum; the HFD had similar degree of shifting from MUFA to PUFA in both portal serum and systemic serum. The proportion of SFA in the fasted state did not change between control diet and HFD in both serum sources. In the fed state, however, HFD group tended to have less proportion of SFA and higher PUFA compared to control diet.

Proportion of SFA, MUFA and PUFA in NEFA fraction of systemic serum

Compared to serum TG fraction, systemic serum NEFA fractions between groups showed distinctly different fatty acid profiles **(Figure 5)**. 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 of animals fed with control diet. The majority of the fatty acids in the SFA were stearic acid (50%) and palmitic acid (40%). In the systemic serum of animals fed with HFD, SFA was still the dominant fatty acids that represented 47% of the total fatty acids. However, there were more PUFA (31% of total fatty acids) in the HFD group compared to the control group. MUFA only accounted for 22% of the total NEFA in the systemic serum of the animals fed with HFD under fasted state (**Fig. 5A**). This finding suggested that a similar shift from MUFA to PUFA with HFD feeding still existed in the NEFA fraction of systemic serum samples.

In the fed state, 61% of the total fatty acids were SFA in the NEFA fraction of systemic serum; 21% were MUFA; 18% were PUFA in the control diet fed animals. Whereas in the rats fed with HFD, 45% of the total fatty acids in the NEFA fraction of

systemic serum were SFA, 25% were MUFA, and 30% were PUFA (**Fig. 5B**). The proportion of PUFA in both fasted and fed states seemed to increase with HFD feeding.

Proportion of SFA, MUFA and PUFA in NEFA fraction of portal serum

The pattern of fatty acids composition in the NEFA fraction of portal serum, as shown in **Figure 6**, was similar with what we have seen in the systemic serum. In the control diet fed group, the predominant fatty acids in the NEFA fraction of portal serum were still SFA, represented 53% of the total fatty acids in the fasted state; 32% of the total fatty acids were MUFA; 15% were PUFA. In the HFD group, 50% of the total fatty acids in the portal serum were SFA, 26% were MUFA, and 24% were PUFA under fasted state (**Fig. 6A**). The percentage of MUFA decreased in the HFD group and shifted to PUFA compared to control diet group.

In the fed state, 59% of the total fatty acids in the NEFA fraction of portal serum were SFA, 24% were MUFA, and the rest 17% were PUFA in the control diet group (Fig. 6C). In the HFD fed rats, 48% of the total fatty acids were SFA, 27% were MUFA, and 25% were PUFA in the NEFA fraction of their portal serum under fed state (**Fig. 6B**).

Palmitic acid (C16:0) in the serum

Percent palmitic acid in the TG fraction is shown in **Figure 7A**. There was a diet effect showed that the HFD group had significantly lower palmitic acid in the TG fraction under both fasted and fed states. Additionally, portal serum had markedly higher palmitic acid under both fasted and fed states. An interaction between diet interventions

and serum sources was been observed in the fed state. The increased palmitic acid in the control diet might indicate an increase *de novo* lipogenesis from carbohydrates.

Figure 7B showed the percent palmitic acid in the NEFA fraction. Similar with TG fraction, HFD group had significantly lower palmitic acid in the NEFA fraction in both fasted and fed state. The portal serum had higher palmitic acid under fasted state; no serum source effect has been found in the fed state.

Palmitoleic acid (C16:1) in the serum

Percent palmitoleic acid in the TG fraction is shown in **Figure 8**. The HFD fed animals had significantly lower palmitoleic acid in their serum under both fasted and fed states. Portal serum had higher percentage of palmitoleic acid compared to the systemic serum samples in the fasted state. Interactions between diet intervention and serum source has been found in both fasted and fed states. No palmitoleic acid was detected in the NEFA fraction of systemic and portal serum.

Stearic acid (C18:0) in the serum

Figure 9A showed the percentage of stearic acid in the TG fraction. The HFD group had markedly higher percent stearic acid in both fasted and fed states. The percent of stearic acid did not differ between serum sources.

Percent stearic acid in the NEFA fraction is shown in the **Figure 9B**. No diet effect was found in either fasted or fed state. However, portal serum had lower stearic acid in the NEFA fraction in fasted state.

Oleic acid (C18:1, n-9) in the serum

Percent oleic acid in the TG fraction is shown in **Figure 10A**. The HFD group had lower oleic acid in the TG fraction in the fasted state, but higher in the fed state. There was an interaction between diet interventions and serum sources in the fed state.

Figure 10B showed the percentage of oleic acid in the NEFA fraction. No difference between diet interventions or serum types was found in the fasted state. In the fed state, HFD group had higher oleic acid in the NEFA fraction compared to the control diet group.

Linoleic acid (C18:2, n-6) in the serum

Figure 11A shows the percent linoleic acid in the TG fraction. The percentages of linoleic acid in the serum of HFD fed animals were significantly higher compared to the control diet group in both fasted and fed state. Portal serum had higher linoleic acid than systemic serum in the fed state.

The percent linoleic acid in the NEFA fraction (**Figure 11B**) was higher in the HFD group in both fasted and fed states compared to control diet fed animals. No difference between two serum sources was found.

Arachidonic acid (C20:4, n-6) in the serum

Significantly higher percent of arachidonic acid in the total fatty acids have been found in the HFD fed animals compared to control diet group in both fasted and fed states

(**Figure 12A**). Portal serum has lower percentage of arachidonic acid compared to systemic serum in the fasted state.

The percent of arachidonic acid in the portal serum was lower than systemic serum in the NEFA fraction under both fasted and fed states (**Figure 12B**). No diet effect was found in the percent of arachidonic acid in the NEFA fraction.

Liver TG, DG and PL

In the fasted state, total liver TG level was higher in the HFD group compared to the control diet fed animals (**Figure 13A**). In contrast, total liver TG level did not differ between diet groups under fed state. However, liver TG was higher in the fed state compared to the fasted state. Diet interventions did not change the level of total DG in the liver, but the total liver DG in the fed state was higher than the fasted state (**Figure 13B**). Total PL concentration in the liver did not differ between diet groups or fasting status (**Figure 13C**).

Fatty acid profile in the liver TG fraction

Table 6 shows the fatty acid profile in the liver TG fraction. In both fasted and fed states, HFD groups had lower percent plamitic acid and palmitoleic acid than control diet groups. Additionally, palmitoleic acid was ~10 times lower in the HFD group. HFD also lowered the percent oleic acid in the liver TG under fasted state. In contrast, HFD fed animals had significantly higher stearic acid, linoleic acid, and arachidonic acid in the liver TG under both fasted and fed states. Moreover, linoleic acid was 3-fold and 6-fold

higher in the HFD group under fasted state and fed state, respectively. Arachidonic acid also elevated in similar degree in the HFD group compared to control diet fed animals.

Fatty acid profile in the liver DG fraction

Fatty acid profile in the liver DG fraction is shown in **Table 7**. The percent of palmitic acid, palmitoleic acid and vaccenic acid were lower in the HFD group under both fasted and fed states. Oleic acid was 30% lower in the HFD under fasted state but not fed state. On the other hand, stearic acid in the liver DG was approximately 2-fold higher in the HFD group compared to control diet under both fasted and fed state. Linoleic acid in liver DG fraction was three times higher in the HFD group in the fasted state, and four times higher in the fed state. Arachidonic acid in liver DG was about 2-fold higher in the HFD group than control diet group in both fasted and fed states.

Fatty acid profile in the liver PL fraction

In the fasted state, the percentage of palmitic acid in the liver PL fraction did not differ between control diet and HFD group; it was ~20% lower in the HFD group under fed state (**Table 8**). There was however a dramatic (~88%) decrease in the percent palmitoleic acid in the liver PL fraction in the HFD under both fasted and fed states compared to control diet. Similarly, the percent stearic acid was increased in the HFD groups but both oleic acid and vaccenic acid were decreased. The percentage of linoleic acid and arachidonic acid in the liver PL fraction did not change between control and HFD.

SCD1 indices in the serum and liver

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 (C18:1/C18:0) is associated with increased plasma TG levels [37-39]. SCD1 desaturase indices are a good biomarker for actual enzyme activity and has also been proposed as a good clinical indicator of SCD1 gene expression. Hence, we here calculated the SCD1 indices to represent the SCD1 activity indirectly. The SCD-16 (C16:1/C16:0) and SCD-18 indices were decreased in the liver TG, DG, and PL fractions as well as fasting systemic serum TG (**Figure 14**). SCD16 in the systemic serum NEFA fraction was not calculable because C16:1 was not detectable in most of our samples. SCD18 in fasting systemic serum NEFA fraction did not change with HFD feeding.

	Control diet	HFD
Terminal body weight (g)	440.1 ± 8.9	479 ± 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*
Food intake (g/day)	25.4 ± 0.6	21.4 ± 0.7*
Energy intake (kcal/day)	97.7 ± 2.1	111.9 ± 3.7*

Table 3. Animal Characteristics. 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 compare to the control diet group.

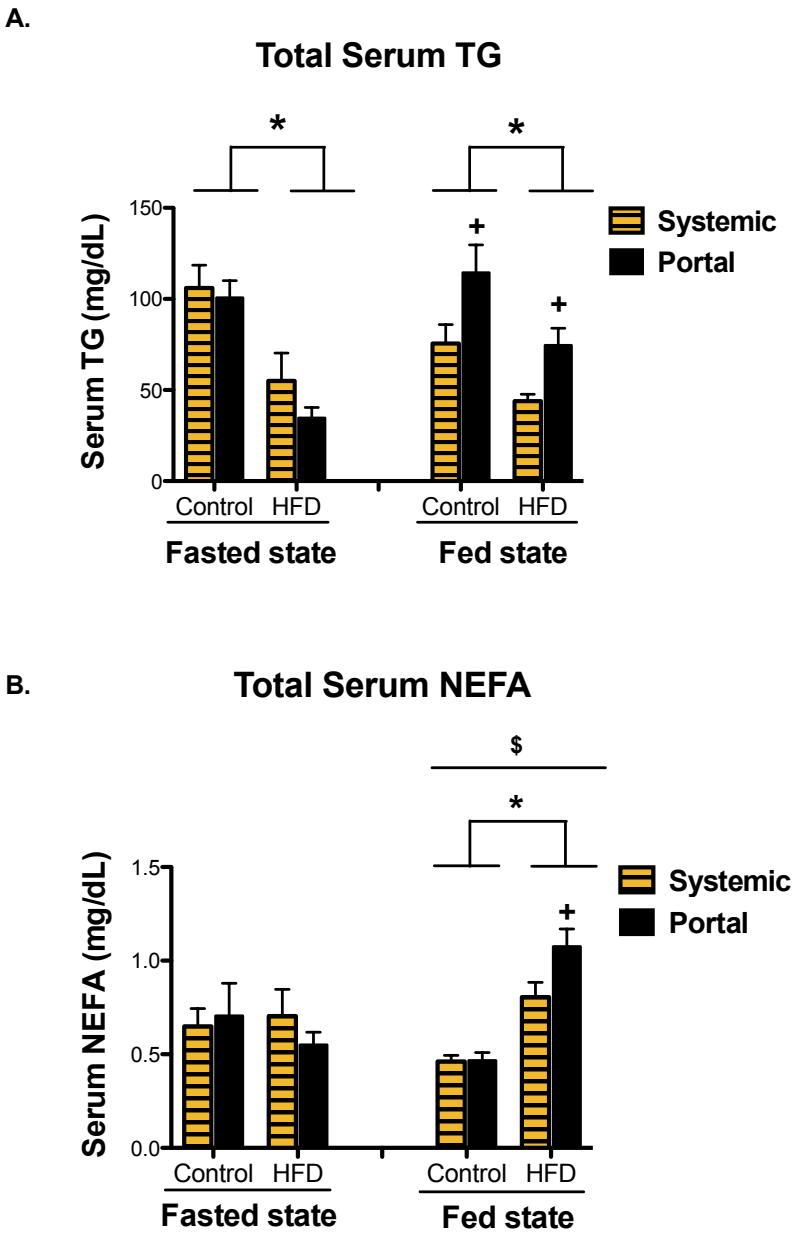


Figure 2. Total serum TG and NEFA concentrations. Data are shown as mean ± SEM, n=5-10 per group. * denoted diet effect (control vs. HFD); + denoted serum source effect (systemic vs. portal) within the diet group; $ denoted interaction, $p<0.05$.

Fatty acids in the serum TG (%)								
	Control Diet				HFD			
	Systemic		Portal		Systemic		Portal	
	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed
14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:0	17.4	26.3	27.0	31.6	12.5	15.5	21.6	15.6
16:1	5.7	6.8	10.7	7.9	0.5	0.7	1.1	0.3
18:0	2.6	3.3	2.0	4.4	8.1	7.9	7.3	7.9
18:1n9	39.7	35.0	34.1	28.0	28.4	38.4	28.5	39.2
18:1n7	8.6	9.2	7.7	6.1	2.2	2.8	2.1	2.9
18:2n6	15.9	15.7	14.3	19.4	29.6	26.5	28.3	27.9
18:3n6	0.9	1.5	0.0	0.8	1.2	1.4	0.0	0.0
18:3n3	0.2	0.0	0.0	0.0	0.3	0.0	0.7	0.0
20:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:1	0.6	0.5	0.3	0.0	0.5	0.8	0.3	0.0
20:2n6	0.8	0.3	0.3	0.0	1.0	0.9	0.2	0.0
20:3n6	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0
20:4n6	3.3	0.7	2.0	1.0	7.9	3.3	5.9	3.9
20:3n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:5n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:1n9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:2n6	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
23:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:4n6	1.3	0.2	0.7	0.2	3.4	0.7	2.1	1.0
24:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:5n3	1.1	0.6	0.9	0.7	0.9	1.0	1.5	1.3
22:6n3	2.0	0.0	0.0	0.0	3.2	0.0	0.5	0.0
24:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 4. Circulating systemic and portal serum fatty acid profiles of triglyceride in control and HFD group under both fasted and fed states. Data are shown as mean, n=5-10 per group. Statistic differences were denoted in the graphs below.

Fatty acids in the serum NEFA (%)								
	Control Diet				HFD			
	Systemic		Portal		Systemic		Portal	
	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed
14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16:0	18.7	25.8	34.0	19.8	15.1	14.4	25.8	16.1
16:1	0.7	0.0	4.8	0.0	0.0	0.0	1.0	0.0
18:0	22.4	33.9	17.2	36.4	27.6	29.5	22.0	30.8
18:1n9	24.4	16.7	19.8	17.6	20.3	23.4	22.4	26.2
18:1n7	5.3	2.8	4.2	3.6	1.2	1.5	2.0	0.9
18:2n6	10.5	8.3	9.5	10.8	15.8	17.9	17.5	19.5
18:3n6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18:3n3	0.7	0.0	0.0	0.8	0.2	0.0	0.0	0.0
20:0	0.0	0.0	0.5	0.0	1.6	0.0	0.0	0.0
20:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:2n6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:3n6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:4n6	8.1	6.3	3.8	5.1	8.8	8.4	5.0	5.0
20:3n3	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
20:5n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:0	3.5	0.6	1.9	2.8	2.9	0.8	2.1	1.5
22:1n9	1.4	1.8	2.4	2.9	0.0	0.2	0.3	0.0
22:2n6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23:0	0.0	0.2	0.0	0.0	0.0	0.2	0.0	0.0
22:4n6	0.0	2.9	0.0	0.0	1.7	1.8	0.0	0.0
24:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:5n3	4.2	0.7	1.7	0.2	4.6	2.1	1.9	0.0
22:6n3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24:1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 5. Circulating systemic and portal serum fatty acid profiles of triglyceride in control and HFD group under both fasted and fed states. Data are shown as mean, n=5-10 per group. Statistic differences were denoted in the graphs below.

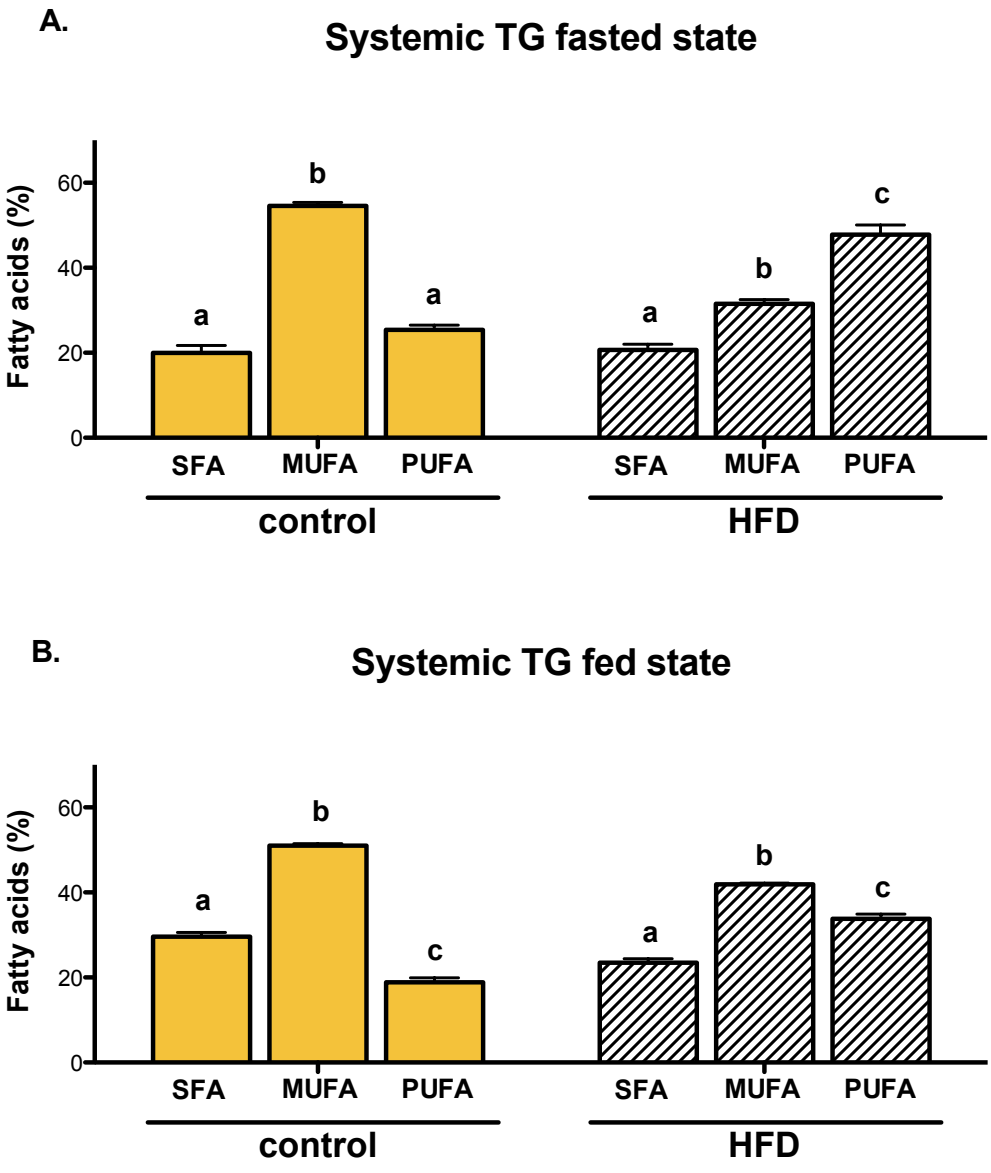


Figure 3. Fatty acid composition of systemic serum in TG fraction under (A) fasted and (B) fed state in animals fed with control diet or HFD groups. Data are shown as mean ± SEM, n=5-10 per group. Means with different subscripts within each diet group differ by $p<0.05$.

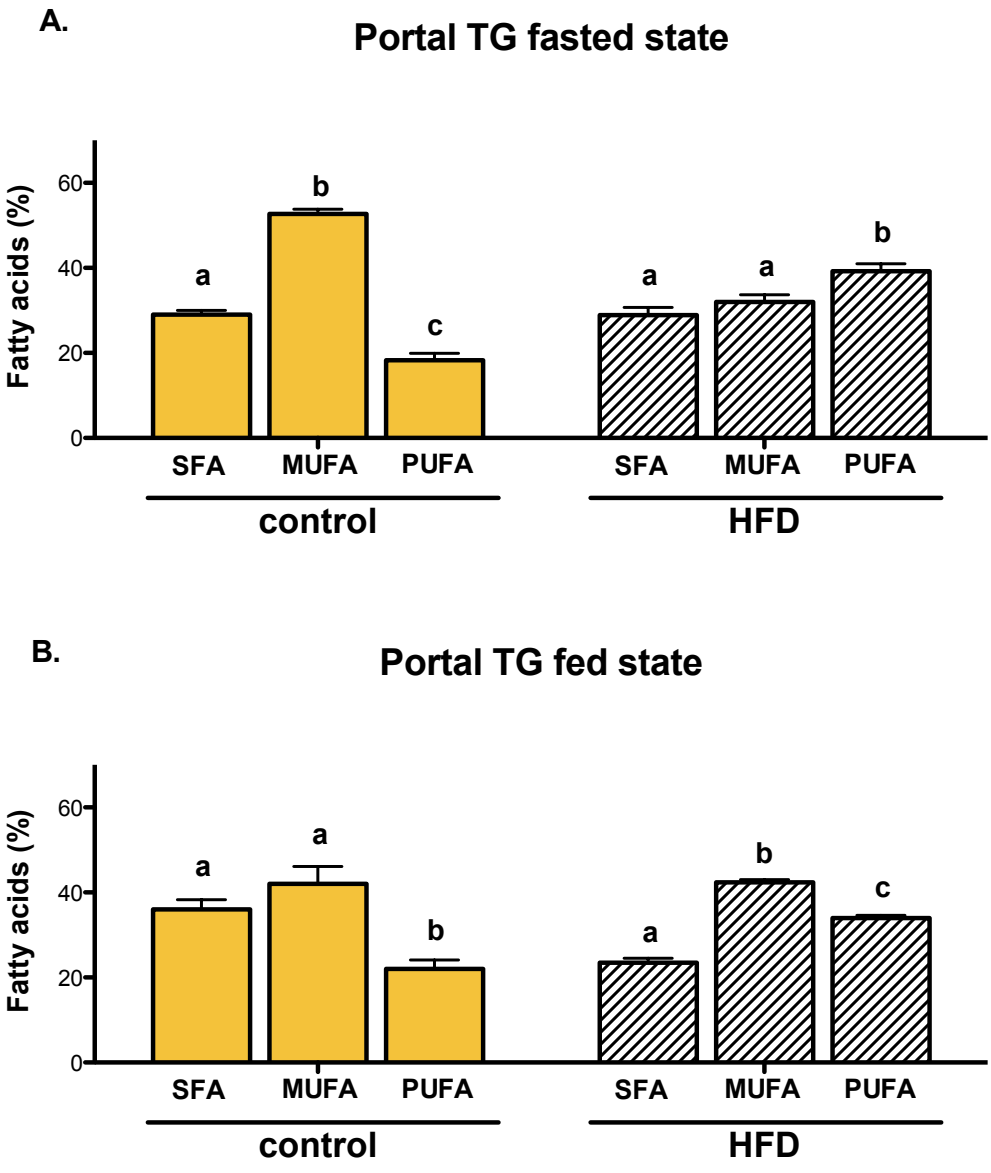


Figure 4. Fatty acid composition of portal serum in TG fraction under (A) fasted and (B) fed state in animals fed with control diet or HFD groups. Data are shown as mean ± SEM, n=5-10 per group. Means with different subscripts within each diet group differ by $p<0.05$.

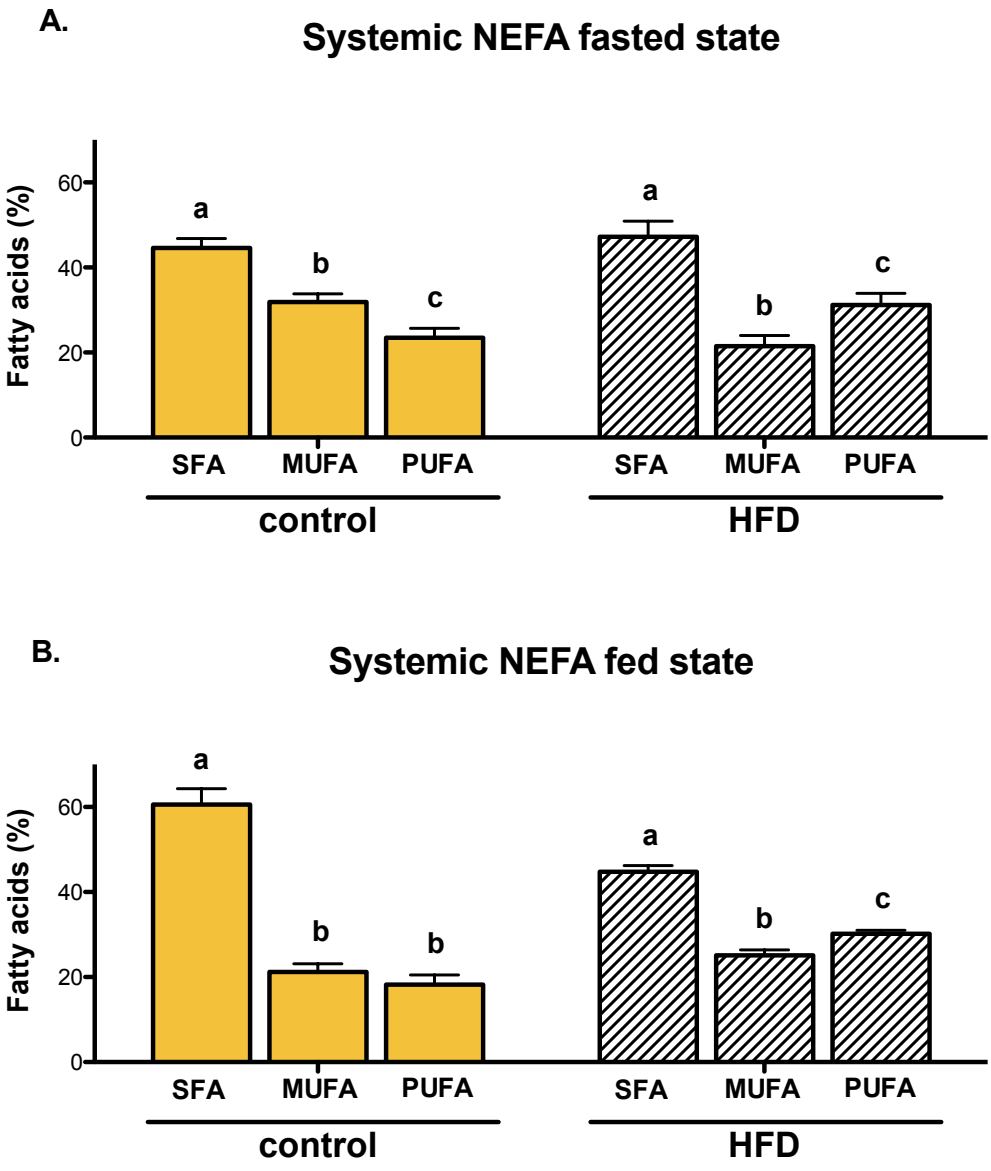


Figure 5. Fatty acid composition of systemic serum in NEFA fraction under both (A) fasted and (B) fed state in animals fed with control diet or HFD groups. Data are shown as mean ± SEM, n=5-10 per group. Means with different subscripts within each diet group differ by $p<0.05$.

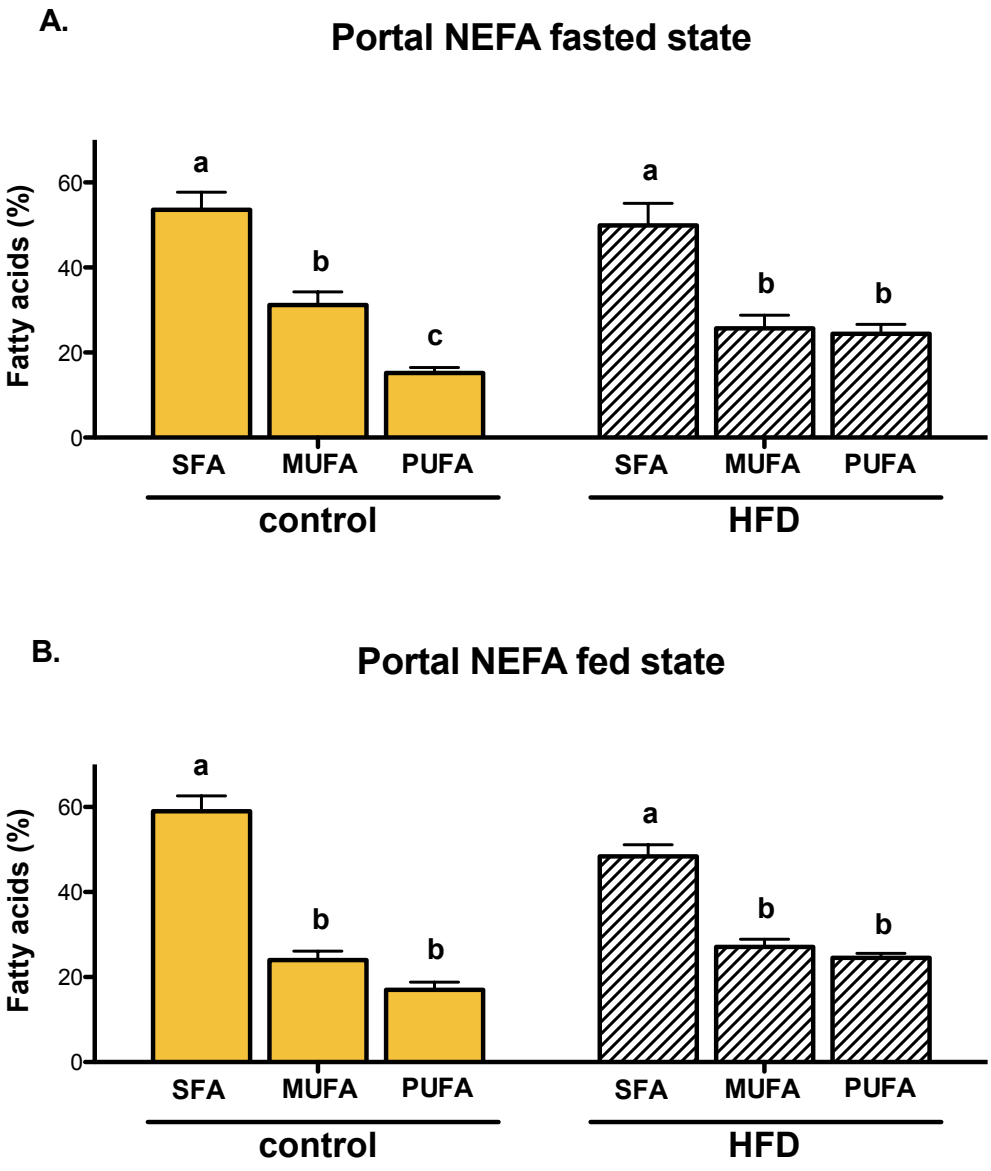
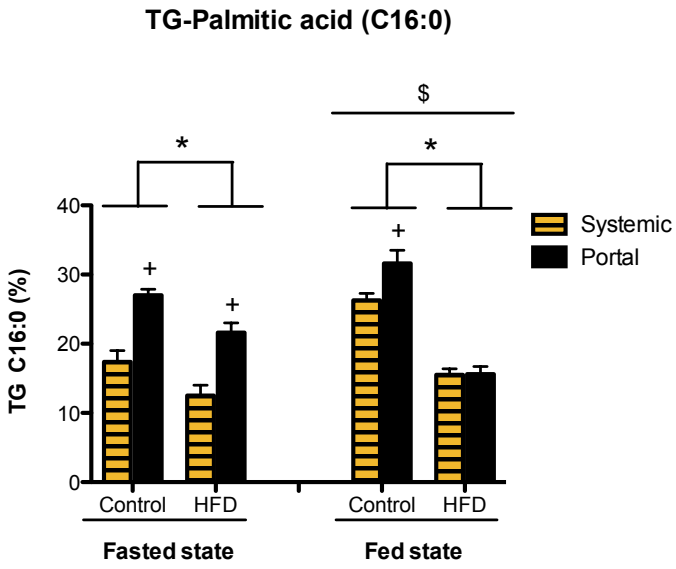


Figure 6. Fatty acid composition of portal serum in NEFA fraction under (A) fasted and (B) fed state in animals fed with control diet or HFD groups. Data are shown as mean ± SEM, n=5-10 per group. Means with different subscripts within each diet group differ by $p<0.05$.

A.



B.

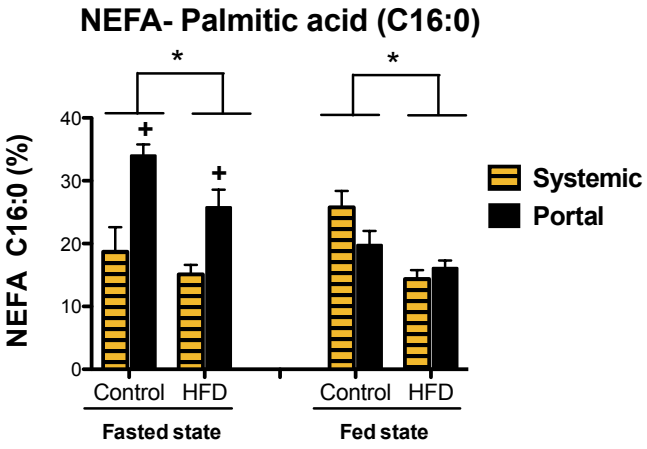


Figure 7. Percent palmitic acid in A) TG and B) NEFA fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum) within the diet group, $denotes interaction, $p<0.05$

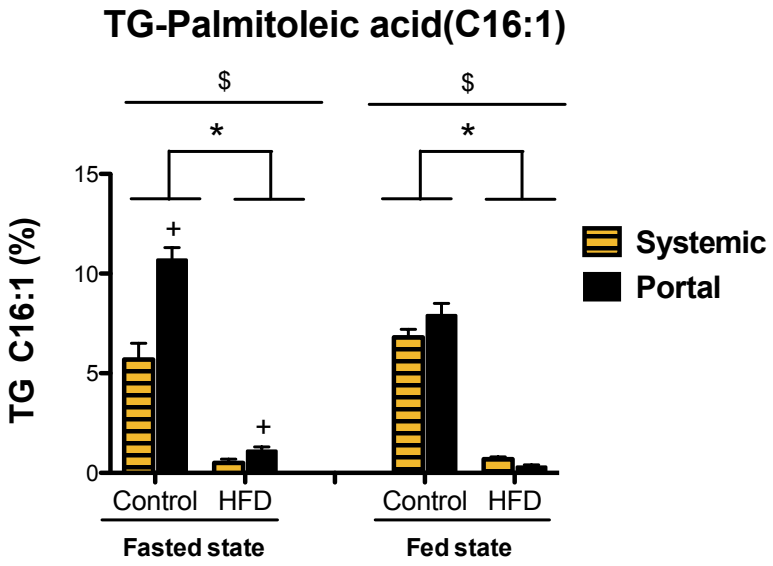


Figure 8. Percent palmitoleic acid in TG fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum), $denotes interactions, $p<0.05$

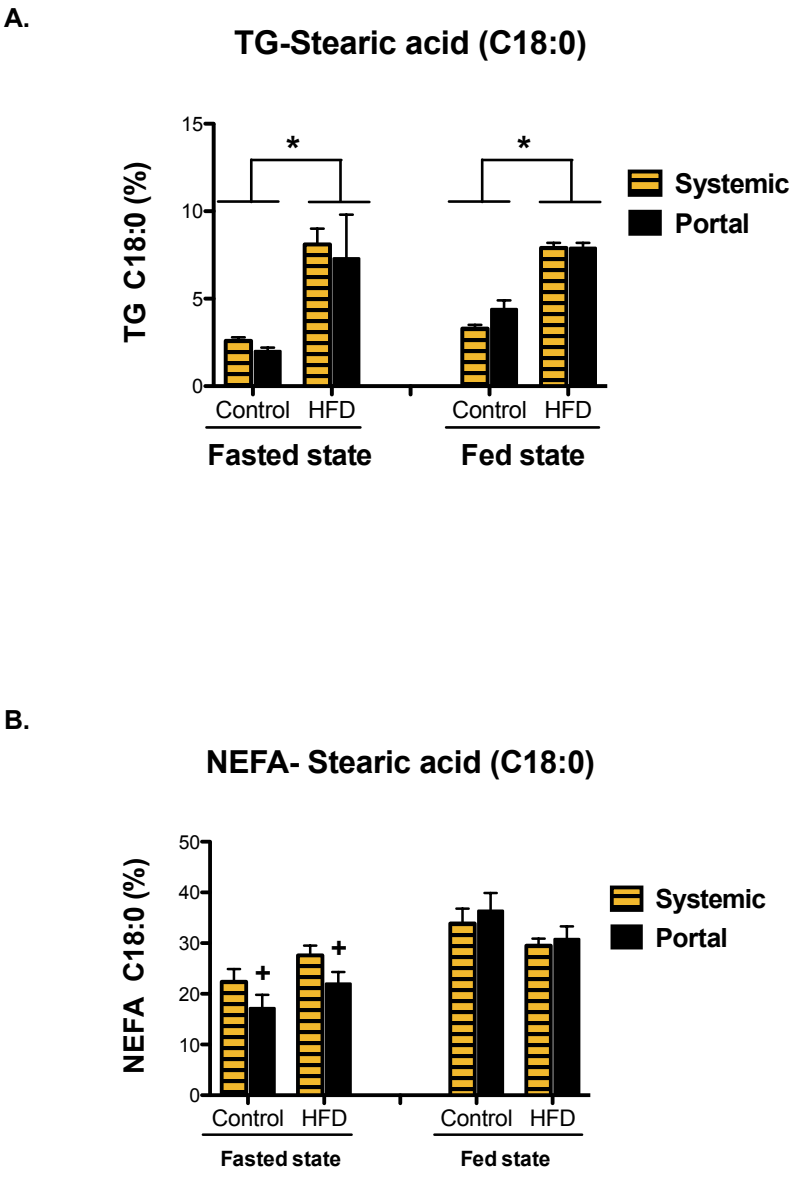
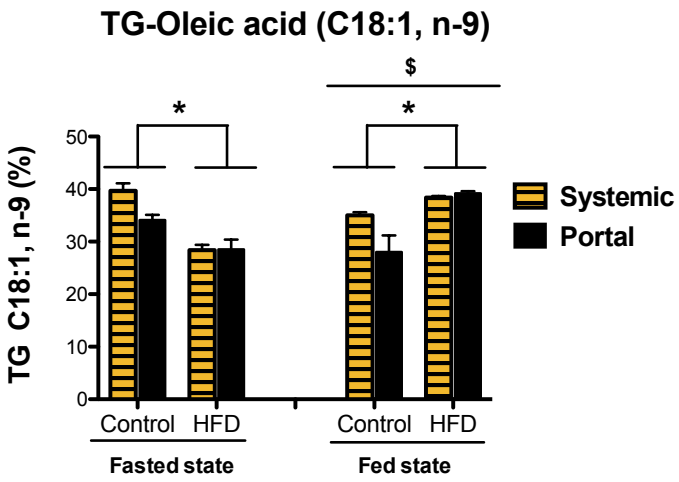


Figure 9. Percent stearic acid in A) TG and B) NEFA fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum), $p<0.05$

A.



B.

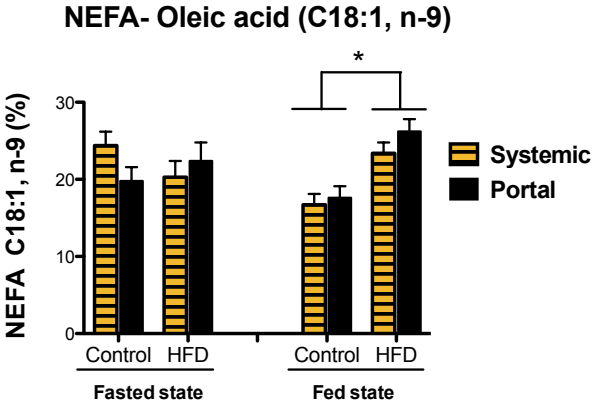
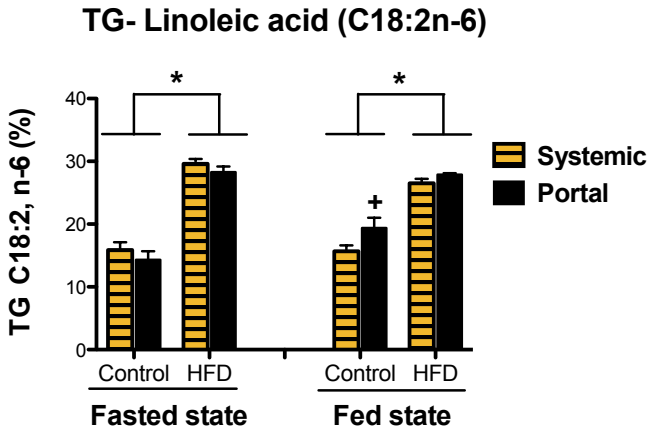


Figure 10. Percent linoleic acid in A) TG and B) NEFA fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum), $denotes interactions, $p<0.05$

A.



B.

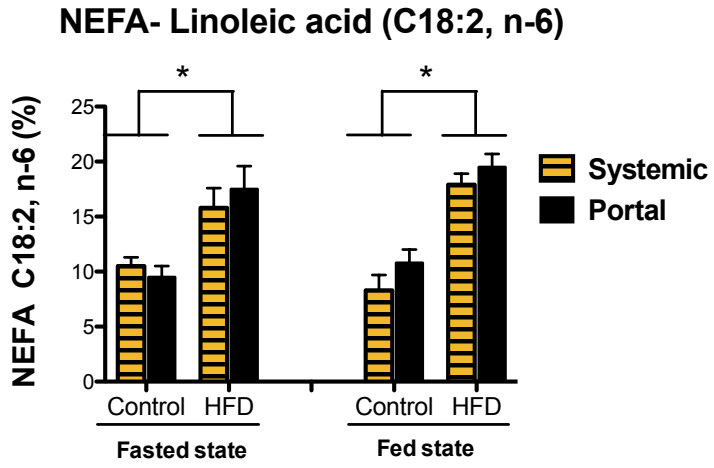


Figure 11. Percent linoleic acid in A) TG and B) NEFA fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum), $p<0.05$

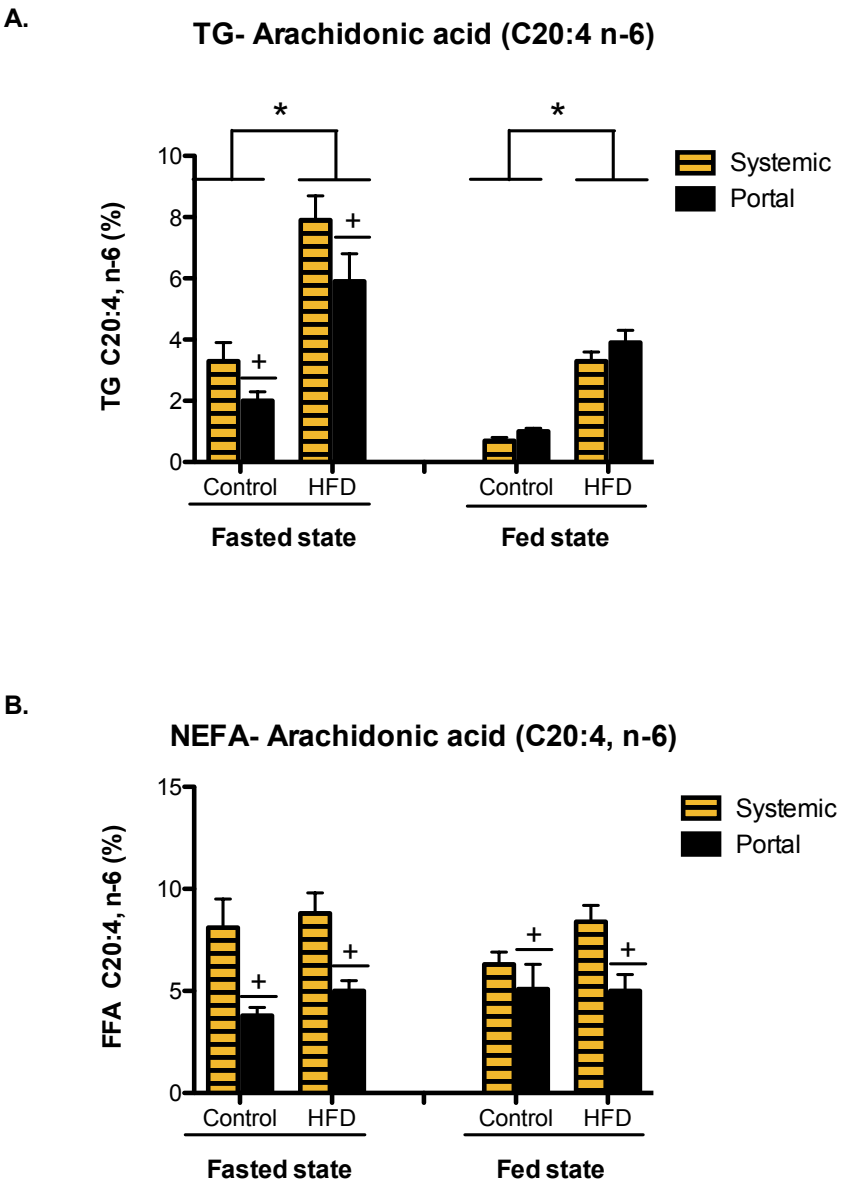


Figure 12. Percent arachidonic acid in A) TG and B) NEFA fraction in systemic and portal serum under fasted and fed state. Data are shown as mean ± SEM, n=5-10 per group. *denotes diet effect (control versus HFD), +denotes serum source effect (systemic versus portal serum), $p<0.05$

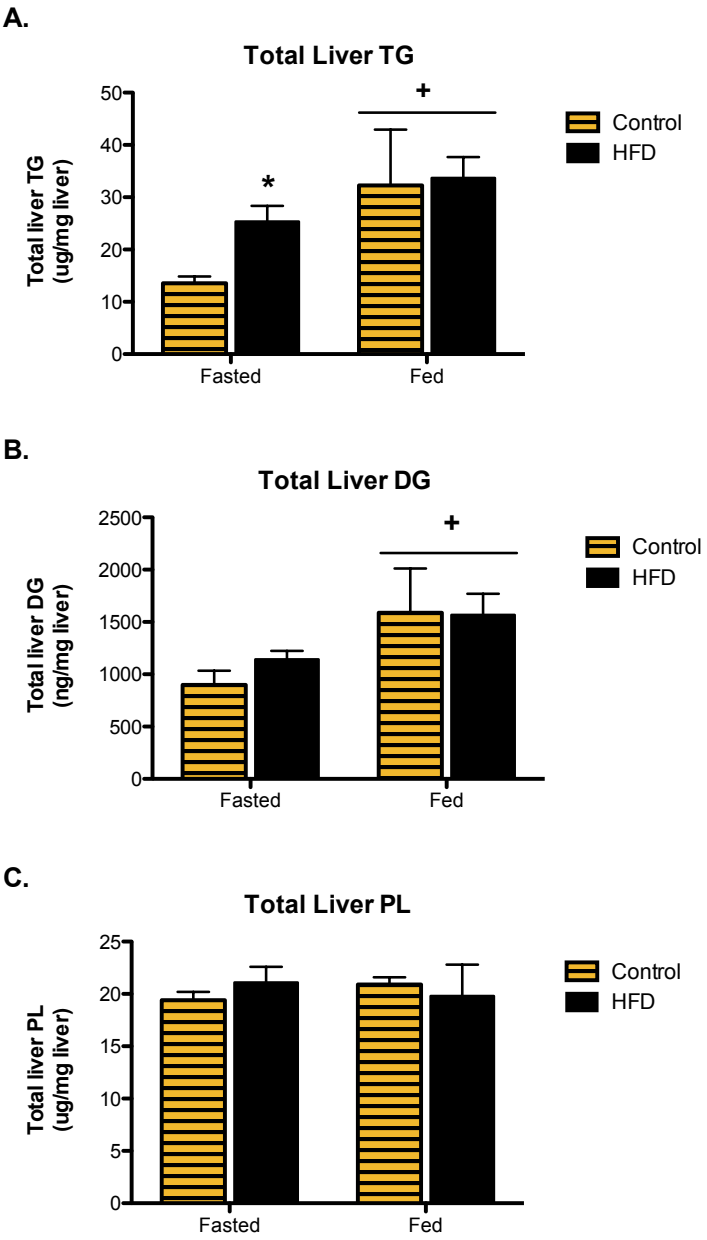


Figure 13. Total liver A) TG, B) DG and C) PL concentrations. Data are shown as mean ± SEM, n=4 per group. * denotes diet effect (control vs. HFD) within the state ; + denoted states differences (fasted vs. fed), $p<0.05$

Fatty acids in the liver TG (%)				
	Fasted State		Fed State	
	Control Diet	HFD	Control Diet	HFD
14:0	0.7 ± 0.1	0.2 ± 0.0	1.7 ± 0.1	0.5 ± 0.0
14:1	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
16:0	29.3 ± 1.2	25.0 ± 0.3*	40.6 ± 2.2	29.7 ± 2.6*
16:1	10.7 ± 1.0	0.6 ± 0.1*	12.0 ± 0.4	1.4 ± 0.3*
18:0	1.6 ± 0.1	3.7 ± 0.1*	2.0 ± 0.1	3.8 ± 0.4*
18:1n9	39.7 ± 0.9	27.4 ± 0.7*	32.2 ± 0.5	32.0 ± 3.3
18:1n7	7.4 ± 0.3	1.8 ± 0.0*	6.7 ± 0.6	3.7 ± 0.4
18:2n6	9.4 ± 1.4	33.7 ± 0.2*	4.4 ±1.0	24.4 ± 3.2*
18:3n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:3n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:2n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n6	0.5 ± 0.1	3.9 ± 0.7*	0.1 ± 0.1	2.9 ± 0.2*
20:3n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:5n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:2n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
23:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:4n6	0.2 ± 0.0	1.3 ± 0.2*	0.1 ± 0.1	0.7 ± 0.1
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n3	0.3 ± 0.1	2.3 ± 0.3*	0.0 ± 0.0	0.9 ± 0.1*
22:6n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sum	100.0	100.0	100.0	100.0

Table 6. Fatty acid profiles in the liver triglyceride in control and HFD group under both fasted and fed states. Data are shown as mean ± SEM, n=4 per group. The symbol *indicated a significant difference between control diet and HFD within the fasted state or fed state, $p<0.05$

Fatty acids in the liver DG (%)				
	Fasted State		Fed State	
	Control Diet	HFD	Control Diet	HFD
14:0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.2	0.4 ± 0.0*
14:1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
16:0	25.5 ± 2.2	17.6 ± 0.7*	42.0 ± 2.9	26.8 ± 1.1*
16:1	8.5 ± 1.7	0.9 ± 0.2*	9.5 ± 0.2	1.0 ± 0.2*
18:0	4.4 ± 0.6	8.5 ± 0.3*	3.7 ± 0.4	7.9 ± 0.3*
18:1n9	36.2 ± 0.6	24.1 ± 0.9*	28.7 ± 0.9	29.4 ± 0.6
18:1n7	7.0 ± 0.3	2.5 ± 0.2*	5.6 ± 0.6	2.7 ± 0.3*
18:2n6	11.3 ± 1.2	32.4 ± 0.8*	5.6 ± 1.0	22.3 ± 1.6*
18:3n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:3n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:2n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n6	5.3 ± 1.0	8.6 ± 0.6*	3.0 ± 0.5	7.4 ± 0.5*
20:3n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:5n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:2n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
23:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:4n6	0.0 ± 0.0	1.5 ± 0.2*	0.1 ± 0.0	0.3 ± 0.2*
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n3	1.8 ± 0.4	3.7 ± 0.3*	0.4 ± 0.2	1.9 ± 1.1*
22:6n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sum	100.0	100.0	100.0	100.0

Table 7. Fatty acid profiles in the liver diglyceride in control and HFD group under both fasted and fed states. Data are shown as mean ± SEM, n=4 per group. *indicated a significant difference between control diet and HFD within the fasted state or fed state, $p<0.05$.

Fatty acids in the liver PL (%)				
	Fasted State		Fed State	
	Control Diet	HFD	Control Diet	HFD
14:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
16:0	19.1 ± 0.4	16.8 ± 2.0	21.7 ± 0.9	17.6 ± 1.4*
16:1	2.4 ± 0.3	0.3 ± 0.1*	2.3 ± 0.1	0.3 ± 0.0*
18:0	17.6 ± 2.5	31.3 ± 0.6*	23.9 ± 1.5	30.3 ± 1.3*
18:1n9	6.1 ± 0.6	3.4 ± 0.2*	5.0 ± 0.2	3.6 ± 0.4*
18:1n7	6.7 ± 0.7	1.2 ± 0.1*	5.0 ± 0.7	1.7 ± 0.1*
18:2n6	11.3 ± 0.6	10.9 ± 0.7	9.2 ± 0.8	9.3 ± 1.5
18:3n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:3n3	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:2n6	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.1*
20:3n6	2.0 ± 0.3	0.7 ± 0.1*	1.7 ± 0.2	0.8 ± 0.1*
20:4n6	25.6 ± 1.3	24.9 ± 4.4	20.8 ± 2.1	26.2 ± 3.3
20:3n3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:5n3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0
22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:2n6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
23:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:4n6	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.1
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n3	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.2
22:6n3	7.1 ± 0.7	8.9 ± 1.0	8.8 ± 0.9	8.4 ± 1.2
24:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sum	100.0	100.0	100.0	100.0

Table 8. Fatty acid profiles in the liver phospholipid in control and HFD group under both fasted and fed states. Data are shown as mean ± SEM, n=4 per group. *indicated a significant difference between control diet and HFD within the fasted state or fed state, $p<0.05$.

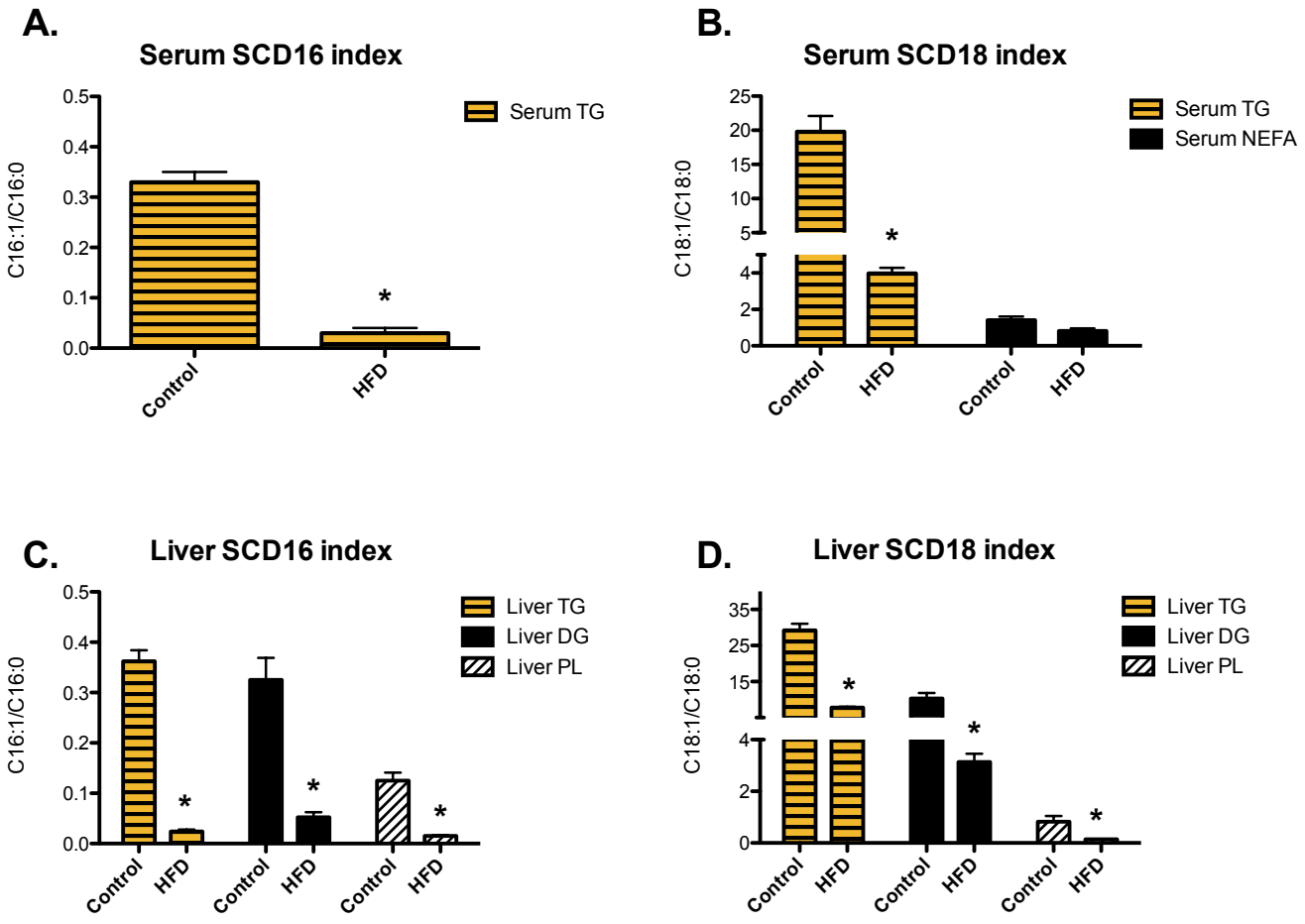


Figure 14. SCD16 and SCD18 indices in A, B) fasting systemic serum and C, D) liver. Data are shown as mean ± SEM, n=4 per group. * indicated a significant difference in the HFD group compared to control diet group, $p<0.05$

4. Discussion

Current study found that 3 weeks of HFD feeding shifted the dominant fatty acids of serum TG fraction from MUFA in the control diet group to n-6 PUFA in the obesity-prone rats. Linoleic acid and arachidonic acid account for the elevation of the n-6 PUFA in the serum of HFD fed animals, suggesting an increased inflammatory status. Moreover, similar shift after occurred in the serum NEFA fraction. Unexpectedly, total serum TG was significantly lower in the HFD group compared to control group under both fasted and fed groups; however, the higher liver TG in the HFD group might explain the lower serum TG observation. The total NEFA level did not change with the HFD feeding under fasted state, yet it increased under fed state in both systemic and portal serum, suggesting impaired ability of suppressing lipolysis.

60% HFD vs. fatty acids intake in the US population

The 60% HFD used in the current study was comprised of 31% SFA, 37% MUFA and 32% PUFA (% fat by weight). The US Population, according to the data from the National Health and Nutrition Examination Survey (NHANES), 1999-2000, consumes approximately 37% of the total fat from SFA, 41% from MUFA, and 22% from PUFA [(40)]. Compared to the human consumption data, the HFD used in the present study has slightly lower SFA and MUFA, but higher PUFA. The same study also estimated the fatty acid species in the dietary fat intake of the US population. Most of the fatty acids profiles were comparable in the HFD used in current study with the human dietary consumptions (palmitic acid: 20.3% vs. 21.4%; stearic acid: 10.9% vs. 9.8%; linolenic

acid: 2.1% vs. 2.0%; arachidonic acid: 0.3% vs. 0.2%), whereas oleic acid was higher in the estimated human consumption (40.2%) compared to the 35.2% in the rodent HFD. Moreover, the HFD used in the current study has higher proportion of linoleic acid (29.7%) compared to the estimated dietary human consumption (20.7%). Overall, the NHANES 1999-2000 data suggested that the HFD current study chose to use had somewhat comparable fatty acid profiles with slightly higher linoleic acid and lower oleic acid.

Body weight and fat pad weight

In present study, the change of body weight in HFD group was slightly (~15g) but significantly higher than control group. HFD group had expending fat pads compared to control diet fed OP rats. Compare to a longer term HFD intervention study using the same diets, the body weight and fat pad weight of the animals in the current study differed in a lower degree between control diet and HFD groups. This suggested that the changes of fatty acid level and profile with 3 weeks HFD feeding might attribute to more of a diet effect instead of causing by diseases.

Triglycerides

It is well established that obesity is positively correlated with hypertriglyceridemia [(5)]; therefore it was surprising that the total serum TG of the HFD fed animals in the present study was lower than the control diet group. Because the size of the fat pads were not expended to maximum after 3 weeks of HFD feeding, we

believed that we caught a moment that these animals were trying to oxidize and store all the excess dietary fat. Previous study has shown that OP rats have the phenotype that tends to store fat into adipose tissue instead of oxidizing them in the skeletal muscle [41]. The larger fat pad and 2-fold higher TG content under fasted state in the liver of HFD group support our speculation.

Non-esterified fatty acid

Total serum NEFA level did not change between control diet and HFD in the fasted state. This finding was not consistent with previous studies showing that obese subjects had elevated NEFA levels in the serum [5]; however, the animals in current study received HFD in a relatively shorter period and the obesity might not be severe enough to display abnormal NEFA levels under fasted state. In contrast, there was a diet effect on the total serum NEFA level in the fed state. The serum NEFA level in the postprandial state is affected by insulin; therefore the increased serum NEFA level in the HFD here indicated HFD fed animals had impaired ability of suppressing lipolysis through insulin.

Actual concentration of each fatty acid or percent of fatty acid?

Fatty acid profile data can be interpreted based on the actual concentration of each fatty acid and/or the percent of each individual fatty acid species within the total fatty acid load. If the total mass of fatty acids entering liver changed, it could lead to changes in lipid metabolism, storage or even down stream inflammatory pathways. Therefore, the bioactive effects from the changes of actual mass of each fatty acid should not be

neglected. However, because purpose in the current study was to make a more physiological fatty acid mixture to treat the cells, the proportion of each fatty acid in total fatty acid was the more appropriate way to report our data.

Palmitic acid, stearic acid and palmitoleic acid

The percent of palmitic acid was decreased in liver TG, liver DG, serum TG and serum NEFA with HFD feeding when the proportion of palmitic acid in the diet was increased, indicating that palmitic acid might have been elongated into stearic acid through Elovl6 or desaturated into palmitoleic acid through SCD-1 (**Figure 16**). However, the palmitoleic acid significantly decreased in the liver TG as well as serum TG in the current study, meanwhile there was an approximately 3-fold increased of stearic acid in the serum TG, indicating that the majority of palmitic acid might become stearic acid through elongation.

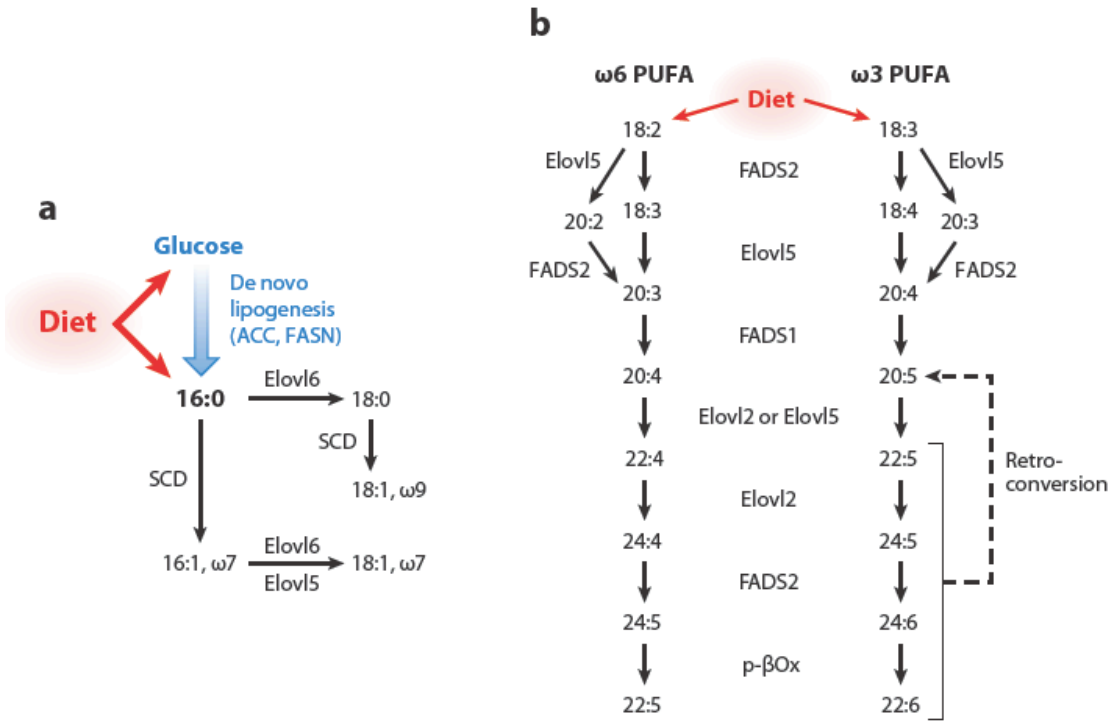


Figure 15. Pathways for hepatic fatty acid synthesis (FASN). Adapted from a recent review published by Jump et al. [(42)]. (a) The pathway describes the conversion of dietary glucose to palmitic acid by de novo lipogenesis using acetyl CoA carboxylase (ACC)-1 and fatty acid synthase. Palmitic acid is a product of de novo lipogenesis but is also derived from the diet. Palmitic acid is subsequently elongated [fatty acid elongase (Elov)l5 and Elovl6] and desaturated [SCD] to form C16−18 saturated and monounsaturated (n7 and n9) fatty acids. (b) The essential fatty acids, linoleic acid and α-linolenic acid (C18:3n3), are derived from the diet. These fatty acids are desaturated [fatty acid desaturases (FADS)1 and FADS2] and elongated (Elovl2 and Elovl5) to form the major C20−22 PUFAs appearing in cells, i.e., arachidonic acid (ARA; 20:4, n6) and docosahexaenoic acid (DHA; 22:6, ω3).

SCD1 indices

The enzyme SCD1, predominantly expressed in the liver, converts dietary or *de novo* lipogenesis saturated fatty acids (C16:0 and C18:0) into MUFA (C16:1 and C18:1) which could be incorporated into phospholipids, triglycerides and cholesterol esters. Numerous studies have suggested that SCD-1 indices have been found to correlate positively with obesity and the development of metabolic syndrome [(43, 44)]. Moreover, high SCD-1 activity is associated with decreased fat oxidation and increased fatty acid synthesis [(45, 46)]. Hence, we were surprised to find that both of the SCD-1 indices, SCD-16 (C16:1/C16:0) and SCD-18 (C18:1/C18:0), in the serum and liver samples of current study were all decreased in the HFD group.

Studies have shown that SCD-1 activity has a positive correlation with serum triglyceride level which makes it a good indicator of hypertriglyceridemia [(39)]. It is possible that the decreased SCD-1 indices that the current study found was associated with the lower serum triglyceride level in the HFD fed animals. Additionally, a previous study has shown that a 3-day higher carbohydrate and lower 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 [(47)]. The control diet that has been used in the present study used has 70%kcal carbohydrates, which might be part of the reason that SCD-1 indices were higher in the control diet group than the HFD fed animals. Furthermore, a recent study conducted by Li et al. [(48)] suggested that the elevated SFA caused by decreased SCD-1 activity in the liver resulting in ongoing hepatocellular apoptosis and liver damage that

develops steatohepatitis and fibrosis, whereas increased SCD-1 activity in the liver actually partitioned excess SFA into MUFA that can be safely stored and leads to hepatic steatosis. Their finding suggested that low SCD-1 activity had higher lipotoxicity to hepatocytes compared to high SCD-1 activity, which might explain our finding in the current study. A recent study also showed that diets rich in MUFA led to decreased SCD-1 indices in the plasma and muscle of healthy human subjects [(49)].

It has been shown that dietary oleic acid, an end product of SCD1 enzyme, increased SCD1 mRNA expression in the liver and primary hepatocytes of liver [(50)]. In contrast, *in vivo* and *in vitro* studies have shown that linoleic acid and arachidonic acid reduced SCD1 activity [(50-52)]. Linoleic acid and arachidonic acid are far more potent in down-regulating the expression of hepatic SCD1 than oleic acid [(50)]. The HFD used in the study has higher linoleic acid levels (73.1g/kg diet vs. 18.3 g/kg diet) and arachidonic acid (0.7g/kg diet vs. 0.1g/kg diet) compared to control diet. Moreover, the actual concentration of linoleic acid in the liver TG fraction was significant higher in the fasted HFD group compared to the control diet group (8569.5±1010.7ng/mg liver vs. 1251.2±139.5ng/mg liver). The increased amount of linoleic acid and arachidonic acid in the diet might contribute to the reduced SCD1 desaturase indices.

Despite the nice correlation of SCD1 mRNA gene expression and the SCD1 desaturase indices, Choi et al. [(53)] reported that SCD1 enzyme activity might be inhibited while SCD1 mRNA gene expression is not, i.e. SCD1 activity could be regulated by posttranslational mechanisms. These data suggest that actual SCD1 enzyme activity should be measured in order to correctly interpret impact of a HFD on SCD1 activity.

MUFA and PUFA

In the current study, HFD altered serum fatty acid composition from one where MUFAs predominant to PUFAs in rats. The majority of the PUFA were n-6 PUFA, including linoleic acid and arachidonic acid. The HFD used in the present study was a MUFA predominant diet that had 31.2% SFA, 36.6% MUFA, and 32.3% PUFA, suggesting that the liver was making more PUFA from MUFA through elongation and desaturation. Based on this finding, the presents of linoleic acid and arachidonic acid were necessary for mimicking an *in vivo* environment.

It has been shown that 1 and 10μM of linoleic acid has the ability to increase adiponectin and resistin secretions from 3T3L1 adipocytes and possible trigger the downstream pathways [(54)]. However, a study conducted by Lee et al. [(55)] has reported that 75μM of linoleic acid is unable to induce COX-2 expression through Toll-like receptor 4 (TLR4) in the RAW 264.7 cells (a murine macrophage-like cell line), suggesting that linoleic acid might not induce inflammation through NFκB pathway. However, n-3 PUFA has displayed its ability to decrease inflammatory status through inhibition of COX-2 expression which mediated by the modulation of TLR-mediated signaling pathways [(56)]. As our best knowledge, the roles of linoleic acid and arachidonic acid on affecting lipid metabolism and inflammatory pathways in the liver cells have not yet been elucidated.

Linoleic acid and arachidonic acid

Dramatic increases in percent linoleic acid and arachidonic acid in the serum and liver of HFD fed animals were found in the current study. The fact that the there was barely any arachidonic acid in the diet suggesting that the majority of the arachidonic acid might be converted from linoleic acid. Arachidonic acid has been suggested as a pro-inflammatory fatty acid that plays an important role in regulating inflammation status [(57)]. Arachidonic acid is an important precursor of pro-inflammatory eicosanoids as it is the primary substrate for the synthesis of the 4-series leukotrienes (Leukotriene B4, C4, E4), the 2-series prostaglandins (prostaglandins E2, prostacyclin I2), and thromboxane A2 [(58)]. The syntheses of these eicosanoids are mediated by cyclooxygenase (COX) and 5-lipooxygenase and could be reduced by EPA as it serves as a substrate of the same enzymes [(58)]. Besides inducing inflammatory response through eicosanoids, the peroxidation of arachidonic acid and linoleic acid form bioactive products that also play a role in regulating inflammation (discuss below) [(59)]. Arachidonic acid is known to be associated with Alzheimer's disease by, at least in part, increasing the inflammation status in the brains [(60)].

Linoleic acid is an essential fatty acid that usually found abundantly in the diet. Linoleic acid is a major precursor of arachidonic acid that can be converted to arachidonic acid through detla-6 desaturase (D6D) *in vivo*. This conversion mostly occurs in liver but also in other tissues. D6D is the rate-limiting enzyme that catalyzes the first reaction of n-6 and n-3 PUFA synthesis that mostly regulates by dietary fat [(61)]. Previous studies suggested that diets high in saturated fatty acid, trans fatty acid and

linoleic acid are associated with increased D6D activity [62, 63]. In addition, D6D activity is upregulated by insulin, growth hormone, inflammatory stimuli, peroxisome proliferators, and sterol regulatory element binding protein-1 (SREBP-1) [64-66]. Similar to SCD-1, D6D also been found to correlate positively with obesity and insulin resistance [43, 44]. The increases in the percent linoleic acid in the serum and liver of HFD group were most likely due to the increased amount of linoleic acid in the diet, especially the changes of fatty acids in the serum TG fraction since it reflects the fatty acid profile in the diet [67]. Similar increase of plasma linoleic acid in the TG fraction has also been found in the diabetic human subjects [68]. Linoleic acid is a direct precursor to the bioactive oxidized linoleic acid metabolites (OXLAMs) that have been mechanistically linked to pathological conditions such as cardiovascular disease, pathogenesis of atherosclerotic, formation of foam cells, and chronic pain [69]. Additionally, plasma OXLAMs has been report to be elevated in the patients with Alzheimer's dementia and non-alcoholic steatohepatitis (NASH) [70, 71]. The increased serum and liver linoleic acid found in the current study might potentially increased inflammation and the risks of the diseases described above.

TG or NEFA?

Although we analyzed TG and NEFA fractions in the serum of an *in vivo* model in the present study, a potential concern of using *in vivo* fatty acid profile of TG to treat isolated cells exists because TG are not readily taken up by *in vitro* cells. However, TG account for a huge proportion of lipids expose in the liver that should not be ignored. Previous studies have shown that liver has the ability of hydrolyzing TG via lipoprotein lipase [(72)]. These LPL has been speculated to locate in the Kupffer cells in the liver [(73)]. Moreover, HFD feeding is known to upregulate the liver LPL activity in mice [(74)]. Similarly, morbidly obese patients also have elevated LPL expression [(72)]. In our opinion, creating an appropriate fatty acid mixture to mimic in vivo environment should consider fatty acid profiles of both TG and NEFA fractions.

In conclusion, 3 weeks of HFD alters serum fatty acid composition of TG from one where MUFAs predominate to n-6 PUFAs in the obesity-prone rats. The increased linoleic acid and arachidonic acid contributed to the majority of the elevation of n-6 PUFA, potentially increased inflammation status, lipotoxicity and related diseases such as NASH and cardiovascular disease. A more physiologically relevant fatty acid mixture to mimic HFD conditions for *in vitro* studies may include increased LA and AA relative to saturated fatty acids and MUFAs.

In the future, we are planning on creating a new fatty acid mixture in the *in vitro* system using palmitic acid, oleic acid, linoleic acid, and arachidonic acid with a proportion calculated from the fatty acid profile in the fed state, because fed state condition might play a more important role for obese people due to their over-eating

pattern. We are expecting to see a different fatty acid profile in the cells compares with the conventional fatty acid mixture, as well as increased inflammation status.

5. Reference

1. WHO. (May 2011).
2. Finkelstein, E. A., Trogdon, J. G., Cohen, J. W., and Dietz, W. (2009) Annual medical spending attributable to obesity: payer-and service-specific estimates, *Health affairs 28*, w822-831.
3. Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S., and Marks, J. S. (2003) Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001, *Jama 289*, 76-79.
4. Reaven, G. M. (2005) The insulin resistance syndrome: definition and dietary approaches to treatment, *Annu Rev Nutr 25*, 391-406.
5. Bjorntorp, P., Bergman, H., and Varnauskas, E. (1969) Plasma free fatty acid turnover rate in obesity, *Acta medica Scandinavica 185*, 351-356.
6. Yuan, G., Al-Shali, K. Z., and Hegele, R. A. (2007) Hypertriglyceridemia: its etiology, effects and treatment, *Cmaj 176*, 1113-1120.
7. Brunzell, J. D. (2007) Clinical practice. Hypertriglyceridemia, *N Engl J Med 357*, 1009-1017.
8. Lee, H., Lee, H., Cho, J., Stampfer, M., Willett, W., Kim, C., and Cho, E. (2013) Overall and abdominal adiposity and hypertriglyceridemia among Korean adults: the Korea National Health and Nutrition Examination Survey 2007-2008, *Eur J Clin Nutr 67*, 83-90.
9. Savary, S., Trompier, D., Andreoletti, P., Le Borgne, F., Demarquoy, J., and Lizard, G. (2012) Fatty acids-induced lipotoxicity and inflammation, *Curr Drug Metab*.
10. Grundy, S. M. (1999) Hypertriglyceridemia, insulin resistance, and the metabolic syndrome, *The American journal of cardiology 83*, 25F-29F.
11. Boden, G. (2011) Obesity, insulin resistance and free fatty acids, *Curr Opin Endocrinol Diabetes Obes 18*, 139-143.
12. Santomauro, A. T., Boden, G., Silva, M. E., Rocha, D. M., Santos, R. F., Ursich, M. J., Strassmann, P. G., and Wajchenberg, B. L. (1999) Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects, *Diabetes 48*, 1836-1841.
13. Stromblad, G., and Bjorntorp, P. (1986) Reduced hepatic insulin clearance in rats with dietary-induced obesity, *Metabolism 35*, 323-327.
14. Cusi, K. (2012) Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications, *Gastroenterology 142*, 711-725 e716.
15. Mortiaux, A., and Dawson, A. M. (1961) Plasma free fatty acid in liver disease, *Gut 2*, 304-309.
16. Iozzo, P., Bucci, M., Roivainen, A., Nagren, K., Jarvisalo, M. J., Kiss, J., Guiducci, L., Fielding, B., Naum, A. G., Borra, R., Virtanen, K., Savunen, T., Salvadori, P. A., Ferrannini, E., Knuuti, J., and Nuutila, P. (2010) Fatty acid metabolism in the

liver, measured by positron emission tomography, is increased in obese individuals, *Gastroenterology 139*, 846-856, 856 e841-846.
17. Bergman, R. N., and Ader, M. (2000) Free fatty acids and pathogenesis of type 2 diabetes mellitus, *Trends in endocrinology and metabolism: TEM 11*, 351-356.
18. Ontko, J. A. (1972) Metabolism of free fatty acids in isolated liver cells. Factors affecting the partition between esterification and oxidation, *The Journal of biological chemistry 247*, 1788-1800.
19. Patsch, W., Tamai, T., and Schonfeld, G. (1983) Effect of fatty acids on lipid and apoprotein secretion and association in hepatocyte cultures, *The Journal of clinical investigation 72*, 371-378.
20. Svedberg, J., Björntorp, P., Smith, U., and Lönnroth, P. (1990) Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes, *Diabetes 39*, 570-574.
21. de Almeida, I. T., Cortez-Pinto, H., Fidalgo, G., Rodrigues, D., and Camilo, M. E. (2002) Plasma total and free fatty acids composition in human non-alcoholic steatohepatitis, *Clin Nutr 21*, 219-223.
22. Bysted, A., Holmer, G., Lund, P., Sandstrom, B., and Tholstrup, T. (2005) Effect of dietary fatty acids on the postprandial fatty acid composition of triacylglycerol-rich lipoproteins in healthy male subjects, *Eur J Clin Nutr 59*, 24-34.
23. Firl, N., Kienberger, H., Hauser, T., and Rychlik, M. (2012) Determination of the fatty acid profile of neutral lipids, free fatty acids and phospholipids in human plasma, *Clin Chem Lab Med 0*, 1-12.
24. Chavez, J. A., and Summers, S. A. (2003) Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes, *Arch Biochem Biophys 419*, 101-109.
25. Gorgani-Firuzjaee, S., Bakhtiyari, S., Golestani, A., and Meshkani, R. (2012) Leukocyte antigen-related inhibition attenuates palmitate-induced insulin resistance in muscle cells, *J Endocrinol 215*, 71-77.
26. Jung, T. W., Lee, K. T., Lee, M. W., and Ka, K. H. (2012) SIRT1 attenuates palmitate-induced endoplasmic reticulum stress and insulin resistance in HepG2 cells via induction of oxygen-regulated protein 150, *Biochem Biophys Res Commun 422*, 229-232.
27. Chabowski, A., Zendzian-Piotrowska, M., Konstantynowicz, K., Pankiewicz, W., Miklosz, A., Lukaszuk, B., and Gorski, J. (2013) Fatty acid transporters involved in the palmitate and oleate induced insulin resistance in primary rat hepatocytes, *Acta Physiol (Oxf) 207*, 346-357.
28. Lima, T. M., Kanunfre, C. C., Pompéia, C., Verlengia, R., and Curi, R. (2002) Ranking the toxicity of fatty acids on Jurkat and Raji cells by flow cytometric analysis, *Toxicology in vitro : an international journal published in association with BIBRA 16*, 741-747.

29. Bonilla, D. L., Ly, L. H., Fan, Y. Y., Chapkin, R. S., and McMurray, D. N. (2010) Incorporation of a dietary omega 3 fatty acid impairs murine macrophage responses to Mycobacterium tuberculosis, *PLoS One 5*, e10878.
30. Fan, Y. Y., Ly, L. H., Barhoumi, R., McMurray, D. N., and Chapkin, R. S. (2004) Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production, *J Immunol 173*, 6151-6160.
31. Kim, W., Khan, N. A., McMurray, D. N., Prior, I. A., Wang, N., and Chapkin, R. S. (2010) Regulatory activity of polyunsaturated fatty acids in T-cell signaling, *Prog Lipid Res 49*, 250-261.
32. Levin, B. E., Finnegan, M. B., Marquet, E., Triscari, J., Comai, K., and Sullivan, A. C. (1984) Effects of diet and obesity on brown adipose metabolism, *The American journal of physiology 246*, E418-425.
33. Levin, B. E., Triscari, J., Hogan, S., and Sullivan, A. C. (1987) Resistance to diet-induced obesity: food intake, pancreatic sympathetic tone, and insulin, *The American journal of physiology 252*, R471-478.
34. Levin, B. E., Dunn-Meynell, A. A., Balkan, B., and Keesey, R. E. (1997) Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats, *Am J Physiol 273*, 725-730.
35. Levin, B. E., and Routh, V. H. (1996) Role of the brain in energy balance and obesity, *The American journal of physiology 271*, R491-500.
36. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues, *J Biol Chem 226*, 497-509.
37. Paton, C. M., and Ntambi, J. M. (2009) Biochemical and physiological function of stearoyl-CoA desaturase, *American journal of physiology. Endocrinology and metabolism 297*, E28-37.
38. Hofacer, R., Magrisso, I. J., Jandacek, R., Rider, T., Tso, P., Benoit, S. C., and McNamara, R. K. (2012) Omega-3 fatty acid deficiency increases stearoyl-CoA desaturase expression and activity indices in rat liver: positive association with non-fasting plasma triglyceride levels, *Prostaglandins, leukotrienes, and essential fatty acids 86*, 71-77.
39. Attie, A. D., Krauss, R. M., Gray-Keller, M. P., Brownlie, A., Miyazaki, M., Kastelein, J. J., Lusis, A. J., Stalenhoef, A. F., Stoehr, J. P., Hayden, M. R., and Ntambi, J. M. (2002) Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia, *Journal of lipid research 43*, 1899-1907.
40. Ervin, R. B., Wright, J. D., Wang, C. Y., and Kennedy-Stephenson, J. (2004) Dietary intake of fats and fatty acids for the United States population: 1999-2000, *Advance data*, 1-6.
41. Jackman, M. R., Kramer, R. E., MacLean, P. S., and Bessesen, D. H. (2006) Trafficking of dietary fat in obesity-prone and obesity-resistant rats, *American journal of physiology. Endocrinology and metabolism 291*, E1083-1091.

42. Jump, D. B., Tripathy, S., and Depner, C. M. (2013) Fatty Acid-Regulated Transcription Factors in the Liver, *Annu Rev Nutr*.
43. Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendziorski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M., and Attie, A. D. (2002) Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity, *Proceedings of the National Academy of Sciences of the United States of America 99*, 11482-11486.
44. Warensjo, E., Riserus, U., and Vessby, B. (2005) Fatty acid composition of serum lipids predicts the development of the metabolic syndrome in men, *Diabetologia 48*, 1999-2005.
45. Bjermo, H., and Riserus, U. (2010) Role of hepatic desaturases in obesity-related metabolic disorders, *Current opinion in clinical nutrition and metabolic care 13*, 703-708.
46. Flowers, M. T., and Ntambi, J. M. (2009) Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity, *Biochimica et biophysica acta 1791*, 85-91.
47. Chong, M. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N., and Fielding, B. A. (2008) Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding, *The American journal of clinical nutrition 87*, 817-823.
48. Li, Z. Z., Berk, M., McIntyre, T. M., and Feldstein, A. E. (2009) Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase, *J Biol Chem 284*, 5637-5644.
49. Vessby, B., Gustafsson, I. B., Tengblad, S., and Berglund, L. (2013) Indices of fatty acid desaturase activity in healthy human subjects: effects of different types of dietary fat, *Br J Nutr*, 1-9.
50. Landschulz, K. T., Jump, D. B., MacDougald, O. A., and Lane, M. D. (1994) Transcriptional control of the stearoyl-CoA desaturase-1 gene by polyunsaturated fatty acids, *Biochem Biophys Res Commun 200*, 763-768.
51. Poudyal, H., Kumar, S. A., Iyer, A., Waanders, J., Ward, L. C., and Brown, L. (2013) Responses to oleic, linoleic and alpha-linolenic acids in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats, *The Journal of nutritional biochemistry*.
52. Ntambi, J. M., Sessler, A. M., and Takova, T. (1996) A model cell line to study regulation of stearoyl-CoA desaturase gene 1 expression by insulin and polyunsaturated fatty acids, *Biochem Biophys Res Commun 220*, 990-995.
53. Choi, Y., Park, Y., Pariza, M. W., and Ntambi, J. M. (2001) Regulation of stearoyl-CoA desaturase activity by the trans-10,cis-12 isomer of conjugated linoleic acid in HepG2 cells, *Biochem Biophys Res Commun 284*, 689-693.
54. Schaeffler, A., Gross, P., Buettner, R., Bollheimer, C., Buechler, C., Neumeier, M., Kopp, A., Schoelmerich, J., and Falk, W. (2009) Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity, *Immunology 126*, 233-245.

55. Lee, J. Y., Sohn, K. H., Rhee, S. H., and Hwang, D. (2001) Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4, *J Biol Chem 276*, 16683-16689.
56. Lee, J. Y., Plakidas, A., Lee, W. H., Heikkinen, A., Chanmugam, P., Bray, G., and Hwang, D. H. (2003) Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids, *Journal of lipid research 44*, 479-486.
57. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., and Moussignac, R. L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, *The Journal of experimental medicine 196*, 1025-1037.
58. Le, H. D., Meisel, J. A., de Meijer, V. E., Gura, K. M., and Puder, M. (2009) The essentiality of arachidonic acid and docosahexaenoic acid, *Prostaglandins, leukotrienes, and essential fatty acids 81*, 165-170.
59. Kubala, L., Schmelzer, K. R., Klinke, A., Kolarova, H., Baldus, S., Hammock, B. D., and Eiserich, J. P. (2010) Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation, *Free radical biology & medicine 48*, 1311-1320.
60. Sanchez-Mejia, R. O., and Mucke, L. (2010) Phospholipase A2 and arachidonic acid in Alzheimer's disease, *Biochimica et biophysica acta 1801*, 784-790.
61. Sprecher, H. (1981) Biochemistry of essential fatty acids, *Prog Lipid Res 20*, 13-22.
62. Garg, M. L., Sebokova, E., Thomson, A. B., and Clandinin, M. T. (1988) Delta 6-desaturase activity in liver microsomes of rats fed diets enriched with cholesterol and/or omega 3 fatty acids, *The Biochemical journal 249*, 351-356.
63. Das, U. N. (2010) A defect in Delta6 and Delta5 desaturases may be a factor in the initiation and progression of insulin resistance, the metabolic syndrome and ischemic heart disease in South Asians, *Lipids in health and disease 9*, 130.
64. Mercuri, O., Peluffo, R. O., and Brenner, R. R. (1966) Depression of microsomal desaturation of linoleic to gamma-linolenic acid in the alloxan-diabetic rat, *Biochimica et biophysica acta 116*, 409-411.
65. Nakamura, M. T., Phinney, S. D., Tang, A. B., Oberbauer, A. M., German, J. B., and Murray, J. D. (1996) Increased hepatic delta 6-desaturase activity with growth hormone expression in the MG101 transgenic mouse, *Lipids 31*, 139-143.
66. Nara, T. Y., He, W. S., Tang, C., Clarke, S. D., and Nakamura, M. T. (2002) The E-box like sterol regulatory element mediates the suppression of human Delta-6 desaturase gene by highly unsaturated fatty acids, *Biochem Biophys Res Commun 296*, 111-117.
67. Ma, J., Folsom, A. R., Shahar, E., and Eckfeldt, J. H. (1995) Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study Investigators, *The American journal of clinical nutrition 62*, 564-571.

68. Hallgren, B., Stenhagen, S., Svanborg, A., and Svennerholm, L. (1960) Gas chromatographic analysis of the fatty acid composition of the plasma lipids in normal and diabetic subjects, *J Clin Invest 39*, 1424-1434.
69. Ramsden, C. E., Ringel, A., Feldstein, A. E., Taha, A. Y., MacIntosh, B. A., Hibbeln, J. R., Majchrzak-Hong, S. F., Faurot, K. R., Rapoport, S. I., Cheon, Y., Chung, Y. M., Berk, M., and Mann, J. D. (2012) Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans, *Prostaglandins, leukotrienes, and essential fatty acids 87*, 135-141.
70. Yoshida, Y., Yoshikawa, A., Kinumi, T., Ogawa, Y., Saito, Y., Ohara, K., Yamamoto, H., Imai, Y., and Niki, E. (2009) Hydroxyoctadecadienoic acid and oxidatively modified peroxiredoxins in the blood of Alzheimer's disease patients and their potential as biomarkers, *Neurobiology of aging 30*, 174-185.
71. Feldstein, A. E., Lopez, R., Tamimi, T. A., Yerian, L., Chung, Y. M., Berk, M., Zhang, R., McIntyre, T. M., and Hazen, S. L. (2010) Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, *Journal of lipid research 51*, 3046-3054.
72. Pardina, E., Baena-Fustegueras, J. A., Llamas, R., Catalan, R., Galard, R., Lecube, A., Fort, J. M., Llobera, M., Allende, H., Vargas, V., and Peinado-Onsurbe, J. (2009) Lipoprotein lipase expression in livers of morbidly obese patients could be responsible for liver steatosis, *Obesity surgery 19*, 608-616.
73. Camps, L., Reina, M., Llobera, M., Bengtsson-Olivecrona, G., Olivecrona, T., and Vilaro, S. (1991) Lipoprotein lipase in lungs, spleen, and liver: synthesis and distribution, *Journal of lipid research 32*, 1877-1888.
74. Ahn, J., Lee, H., Chung, C. H., and Ha, T. (2011) High fat diet induced downregulation of microRNA-467b increased lipoprotein lipase in hepatic steatosis, *Biochem Biophys Res Commun 414*, 664-669.

APPENDIX A

EXTENDED LITERATURE REVIEW

Diseases prevention has been a major concern in the modern society due to the dramatic increased rate of obesity and metabolic diseases. Obesity is known to be associated with numerous diseases including Type 2 diabetes (T2D), cardiovascular disease, stroke, non-alcoholic fatty liver disease (NAFLD), chronic kidney disease and certain types of cancer [1]. The high fat contents of westernized diet plays a critical role in the progression of obesity—along with sedentary life style, metabolic diseases become inevitable. Hence, high fat diets (HFD) are widely used in basic scientific research for inducing obesity and metabolic diseases in animal models. *In vitro* culture system has also been intensively used in obesity related research to decode the mechanistic questions at a molecular level. When using cell culture systems to study hepatic lipid metabolism, scientists often treat hepatocytes with single or dual non-esterified fatty acids to induce hepatic insulin resistance through lipid accumulation. An apparent disconnect existing in these methods is the fact that the liver is commonly exposed to multiple fatty acid species *in vivo* as opposed to single fatty acids species. The following review will discuss the current commonly used methods and the disconnect between *in vivo* and *in vitro* studies.

Obesity

Obesity is defined as excessive accumulation of body fat [2]. Body mass index (BMI), calculated as weight in kilogram divided by height in meter squared, is commonly used clinically to identified obesity. BMI is known to be associated with morbidity/mortality in a J-shape relationship that both lower end and higher end of BMI have higher risks of morbidity/mortality [3]. The classification of health risk according to the BMI is shown in Table 1 [2, 4].

Table 1. Health risk classification and comorbidities by body mass index

Classification	BMI (kg/m2)	Risk of comorbidities
Underweight	<18.5	Low (but risk of other clinical problems increased)
Normal range	18.5 – 24.9	Average
Overweight	≥ 25	
Pre-obese	25.0 – 29.9	Increased
Obese class I	30.0 – 34.9	High
Obese class II	35.0 – 39.9	Very high
Obese class III	≥ 40.0	Extremely high

Current epidemic of obesity

The prevalence of obesity in the United States increased in the past decades. According to the Centers for Disease Control and Prevention (CDC), more than one-third of adults and almost 17% of youth were obese in 2009-2010 [(5)]. The growing rate of obesity is associated with a rise in medical expenditures. In 2008, the medical costs of obesity related diseases were estimated to be approximately $147 billion in the United States—twice as much as in 1998 [(6)]. The phenomenon is not any better in the other countries of the world, especially the large emergent economies such as Asia, Southeast Asia and Latin America [(7)]. Although the prevalence rates for overweight and obese people differ in each region in the world, the increasing obese population is a global issue. Despite the efforts that we have placed upon obesity research over the last many years, the obesity trend unfortunately did not decline. Obesity will continue to impose the

major costs on health system in the foreseeable future if the growing epidemic cannot be stopped.

Dietary factors associated with obesity

Three major risk factors contribute to the etiology of obesity including metabolic factors, diet, and physical inactivity. Overconsumption of calories leads to obesity due to energy imbalance. The fat content in the diet has been emphasized due to its higher energy density and metabolic consequences. Additionally, the body has limited capacity to oxidized dietary fat compared to carbohydrate and protein. Fat is also more efficient at expending fat mass than other macronutrients [(8)]. Saturated fatty acids (SFAs) content has raised the highest interest since it has the ability to induce lipotoxicity and insulin resistance compare to most of the unsaturated fatty acids [(9)]. Besides fat, sucrose has also been recognized as a risk factor to obesity as it can induce hepatic lipogenic enzyme activity, elevate circulating insulin, lead to impaired glucose clearance and weight gain [(10)]. Westernized diets are commonly high in both lipids and sucrose content and thus are believed to play a role in increased obesity rates.

Murine and Rodent Models for Obesity Researches

It is apparent that obesity is a critical problem that needs to be solved. From a scientific point of view, animal models are needed to unwrap the secrets behind the causes, developments, etiology as well as possible treatments of obesity. There are numerous animal models available for obesity research; choosing an appropriate

phenotype is important for researchers. Most of the animal models that are currently used are genetically defect monogenic animals, such as the *ob/ob* mice, Zucker-(*fa/fa*) fatty rats (ZFR) and Otsuka Long Evans Tokushima Fatty (OLETF) rats. The *ob/ob* mutation is recessive, and the mutation in the leptin gene is responsible for the *ob* mutation. The *ob/ob* mice spontaneous developed obesity because they fail to secrete leptin, an important protein secreted mostly by adipose tissues that is responsible for appetite regulation [(1, 11)]. Leptin deficiency results in hyperphagia, reduce energy expenditure and extreme obesity [(12)]. ZFR is another well-characterized monogenic rodent model that has been used in obesity research since 1961 [(13)]. Similar with *ob/ob* mice ZFR has an autosomal recessive mutation in the leptin receptor gene that affects leptin signaling and leads to obesity [(11)]. OLETF rodent model lacks of cholecystokinin-1 receptor expression, which leads to meal feedback defect for satiety that results in hyperphagia and obesity [(14)]. OLETF rats are a valuable model on studying insulin resistance, hypertriglyceridemia, obesity, Type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) [(1, 11)]. However, OLETF rats develop these metabolic related diseases just by over-eating low fat Purina chow diet, and high fat content in the Westernized diet is one of the major cause of human obesity as mentioned earlier. These monogenic models provide important information on the general biology of obesity, and have been widely used due to the consistent data researchers obtained.

Despite the advantages of using monogenic animal models, the results obtained from these monogenic phenotypes need to be interpreted with care because human obesity is known to be a polygenic disease that involved more than a single gene

mutation. Polygenic models of obesity may be able to provide more insights of human obesity condition. Therefore, polygenic animal models have been selectively bred to used in obesity research, such as Kuo Kondo (KK) mice and New Zealand Obese (NZO) mice. KK mouse is a polygenic model that developed in Japan with selective inbreeding for large body size [(15)]. This strain displays hyperphagia, hyperinsulinemia, insulin resistance and T2D. NZO strain is also a polygenic mouse model that exhibits T2D only in males [(16)]. NZO mice are hyperphagic but they have normal levels of leptin and leptin receptors. Additionally, NZO mice have decreased exercise activity compared to control or even *ob/ob* mice [(17)]. Polygenic models may be more suitable to study human obesity, but diets do not necessary play a role in the development of obesity in these models. Looking at the polygenic nature of human obesity, a diet-induced polygenic obese rodent model may also be an appropriate system to study the etiology of human obesity.

Different animal strains respond differently with HFD feeding. Some strains develop obesity but not diabetes with HFD; some develop severe hyperinsulinemia; some even develop severe hypoglycemia due to marked hyperinsulinemia that leads to sudden death [(18)]. Interestingly, distinct differences also exist among substrains in response to the HFD.

Among the various mouse strains, C57BL/6J mice have been widely used for HFD-induced obesity due to the similar abnormalities to human metabolic syndrome they exhibit. Several HFD-induced obesity rats model have been developed these years that demonstrates special phenotype mimic human obesity. The diet-induced obesity (DIO) prone rats developed by Dr. Barry E. Levin at New Jersey Medical School are one of the

strains that depict the polygenic phenotype of human obesity[19]. These obesity prone rats were being selectively bred from outbred Sprague-Dawley rats. Original outbred male and female Sprague-Dawley rats breeding stock was fed one month when they were 5-week-old with high-energy (HE) diet, which was made by 8% of corn oil, 44% of sweetened condensed milk, and 48% Purina rat chow. This HE diet contained 4.47 kcal/g, with 21%kcal of protein, 31%kcal of fat, and 48%kcal of carbohydrate—half of which was sucrose. After one month on the HE diet, several highest and lowest weight gainers were selected as breeding stock. DIO prone phenotype and diet resistance (DR) phenotype were then inbred for several generations; a firmly separation of obesity-prone and DR phenotype established by the F_3 generation. According to Dr. Levin, the phenotypes differences were strongly expressed by the HE diet exposure. Although sex differences exist, the obesity-prone male rats were still 31% heavier than respective DR rats.

High-Fat Diet

About 60 years ago, Samuels described that rats fed with a diet containing 70% energy of fat developed obesity and elevated basal and postprandial blood glucose levels [20]. HFD has been rigorous used in obesity research as a non-leptin deficient model ever since—with different fat type, macronutrient composition, feeding length and for different animal strains. Under normal circumstances, most of the ingested lipids store in the adipose tissues if body does not use them as energy source. However, if the amount of fat hit the limit of what body can handle for a certain period of time, ectopic lipid

depositions may occur which are part of the mechanism by which obesity leads to metabolic disorders.

Despite the wide usage of HFD, the exact fat content and fatty acid acids composition of HFD varies. The term "HFD" apparently does not have a solid definition, which makes the results of feeding HFD inconsistent and hard to compare between studies. The fat content in the HFDs that has been used in researches ranges from 20%kcal to 60%kcal, and the constituent of fat also varies between animal-derived fat and plant-based oil, e.g., lard vs. corn oil and soybean oil [(21)]. Without a standardized diet, every scientist has their own opinions and preferences on choosing an appropriate diet to experimental animals. Some scientists chose to use semi-purified HFDs that are well refined and have comparable control diet that substitute fat with carbohydrate or protein. However, some researchers simply add fat or oil into standard chow diet, which may dilute other nutrients (e.g. vitamins, minerals, protein…etc.) and non-nutrients (e.g. phytochemicals). Moreover, the "standard chow diets" that each animal facility choose to use are manufactured by different companies, which may increase the variability between studies. Some of these chow diets are variable formula, such as LabDiet 5001, in which the ingredients used in the diets change from batch to batch—depending on the source of raw ingredients manufacturer can get. Standard chow diet also has various levels of phytoestrogens that may affect lipid metabolism or atherosclerosis, which may influence the physiological function and diseases outcome [(22)]. When the sources of the diet and the compositions change among each lot, the variations between control group and HFD group may increase.

Commonly used commercial semi-purified high-fat rodent diets

More and more researchers nowadays are willing to use commercially made semi-purified diets due to their conveniences, and the availability of the control diet with matched macronutrient. Moreover, using the same diets make it easier to compare observations across the studies between different research teams. There are several companies manufacturing open source diets using different lipid sources and various proportions of lipids that have been commonly used in obesity related researches. The following are four major companies that produce open source diets and have been widely used in the United States and all over the world.

I. Research Diets, Inc. (http://www.researchdiets.com/)

Research Diet, Inc. manufactures several DIO series diets; two of the most popular formulas are the D12492 and D12451 diets (**Supplemental 1.**). This series of HFD uses lard as their major source of fat. Diet D12492 is the HFD that contains 60%kcal of fat, 20%kcal of carbohydrate (35% of the carbohydrates are from sucrose), and 20%kcal of protein. Approximately 92% of fat in this diet comes from lard, and the rest of 8% comes from soybean oil. There is 300.8mg of cholesterol in one kilogram of diet in the D12492 diet.

Another HFD in the same DIO series, D12451, has 45%kcal of fat, 35%kcal of carbohydrate (50% of the carbohydrates are from sucrose), and 20%kcal of protein. Lard contributes to 88% of the fat in this diet, 12% are from soybean oil. There is

196.5mg of cholesterol in one kilogram of diet in the D12451 diet. This company manufactures various matched macronutrients control diets with different levels of sucrose for scientists to choose. Their control diets use approximately half soybean oil and half lard as their fat sources. In general, these control diets have 20%kcal of protein, match with HFD, 10%kcal of fat, and 70%kcal of carbohydrate.

II. Bio-Serv (http://www.bio-serv.com)

Another commonly used commercial semi-purified HFD is the #F3282 that manufactured by Bio-Serv (**Supplemental 2.**). This lard based high-fat formula has 59%kcal from fat, 26%kcal from carbohydrate, and 15%kcal from protein. The control diet that this company formulizes, F4031, also uses lard as its fat source. The control diet has 21%kcal of protein, 16%kcal of fat, and 63%kcal of carbohydrate.

III. Harlan Laboratories, Inc. (http://www.harlan.com)

Adjusted calories diet TD.88137 manufactures by Harlan Laboratories provides a HFD that uses milk fat as its major fat source (**Supplemental 3.**). Because dairy and sucrose attributes to a marked proportion in the Western diet, this formula uses high fat (42%kcal) and sucrose (35% by weight) compositions to mimic Western Diet. Another 42.7%kcal of this diet is made by carbohydrates, 15.2%kcal by protein. On top of the existing cholesterol in the milk fat, this company had added cholesterol into this diet to make it contains 0.2% of cholesterol.

IV. TestDiet (http://www.testdiet.com)

Numerous HFDs have been formulated and manufactured by TestDiet. The Van Heek diet-induced obesity series is one of the most common series that has been used by many research teams (**Supplemental 4.**). Similar with the DIO series manufactured by ResearchDiet, Inc., this series has 45%kcal fat (#58V8) and 60%kcal fat (#58Y1) high fat formulas that made with lard and soybean oil (88% of total fat from lard, 12% from, soybean oil), and their comparable control diet with 10%kcal fat (58Y2). Diet#58V8 has 18.3%kcal if protein, 45.7%kcal of fat, and 35.5%kcal of carbohydrates (half of the carbohydrates are sucrose). Diet# 58Y1 has 18.3%kcal if protein, 60.9%kcal of fat, and 20.1%kcal of carbohydrates (one-third of the carbohydrates are sucrose). TestDiet also manufactures HFDs that uses corn oil (#58G8) or coconut oil (#58R3) as their lipid sources.

Composition and major lipid sources of HFD

I. Macronutrient distribution

As mentioned above, the macronutrient compositions vary in almost every HFD formula. Typically, the fat contributes to 40-60% of the total calories of the diet; about 20%kcal comes from protein, and 20-40%kcal from carbohydrates in the HFD. The proportion of carbohydrate or protein needs to be decrease to compromise the increased proportion of fat while making HFDs. Since protein usually contributes to a relatively small proportion of diets and its important role in physiological functions,

formulas often substituted carbohydrate with fat to increase fat content. Details of some of the often-used formulas have been previously discussed (see above).

II. Fatty acid profiles of several commonly used fat sources

A. Lard

Lard-based HFD is known to cause diet-induced obesity, increase serum glucose and insulin level, and also lead to higher serum triglycerides [(23)]. Based on United States Department of Agriculture (USDA) National Nutrient Database, there are 41% of total saturated fatty acids (SFA), 47% of total monounsaturated fatty acids (MUFA), and 12% of total polyunsaturated fatty acids (PUFA) in the total lipid of lard [(24)]. Lard generally contains 95mg/100g of cholesterol.

Magidman et al. [(25)] analyzed the fatty acid profiles of three different sources of lard back in 1962, and reported similar proportion of fatty acid shown in USDA database—41% of SFA, 46% of MUFA, and 13% of PUFA. In the same report, the majority of SFA is palmitic acid (C16:0) that contributes to approximately 26% of total fatty acid of lard. Stearic acid (C18:0) consists 10 to 14% of lard. Oleic acid (C18:1), in the other hand, is the major fatty acids in MUFA; it composes 42 to 46% of total fat in lard. Two percent of total fat comes from palmitoleic acid (C16:1). Additionally, the major PUFA source is linoleic acid (C18:2, n-6, ~10%).

The lard that Bio-Serv uses to formulate their F3282 HFD also has similar fatty acid composition as what USDA reported. Despite the similar fatty acid

profile results shown in the several reports mentioned above, the lard that Research Diet Inc. uses has distinct fatty acid profile according to the data analyzed by the company. Their data showed that SFA accounts for 34% of the lipid in their lard source, 38% comes from MUFA, and 28% from PUFA. Based on their report, the lard they used has lower proportion of SFA and MUFA, higher PUFA—mostly from linoleic acid (C18:2, n-6).

B. Milk fat

Based on USDA National Nutrient Database, whole milk with 3.25% milk fat contains 10mg/100g of cholesterol. 65% of calories in the milk fat comes from SFA, 28 %kcal from MUFA and 7 %kcal from PUFA [(24)].

Chouinard et al. [(26)] analyzed the fatty acid profile of milk fat and found similar fatty acid profiles as USDA database. Palmitic acid (C16:0) and oleic acid (18:1, n-9) are two of the major fatty acids that contribute 27% and 24% of total lipid in the milk fat, respectively. There are also 10.9% of stearic acid (C18:0) and 11% of myristic acid (C14:0) in the fat milk according to the same study.

C. Soybean oil

According to the USDA National Nutrient Database, 16 % of the total lipid of soybean oil are SFA, 24% are MUFA, 60% are PUFA. This plant base oil does not contain any cholesterol [(24)].

Chowdhury et al. [(27)] reported similar SFA, MUFA and PUFA composition as shown in the USDA National Nutrient Database. More

specifically, there are 14% of palmitic acid, 4% of stearic acid, 23% of oleic acid, 52% of linoleic acid, and 6% of linolenic acid (C18:3, n-3) in the soybean oil.

Outcomes of long-term HFD in animals

Long term-HFD feeding is known to induce obesity and its related diseases in animal models with some exceptions of diet rich in long chain n-3 PUFAs. Some evidences suggested that n-3 PUFA has protective effects against obesity and insulin resistance, but this statement remain inconclusive due to inconsistent results obtained from different research teams [28]. The fat sources used to make these diets could lead to different results because of the distinct fatty acid profiles of each lipid source. A long term (9-10 month) HFD feeding study fed male Wistar rats with safflower oil-based HFD that contained 59% of fat and 20% carbohydrate (cornstarch: sucrose=2:1 wt/wt) found that the total body weight of HFD group was 22% higher than the control group [29]. Additionally, the total fat weight and intra-abdominal fat (sum of the weights of epididymal, retroperitoneal, and mesenteric fat depots) were 51% and 41% higher in the HFD group, respectively. The increased fat consumption also leads to changes in lipid metabolism that will be discussed in the following paragraph.

I. Essential fatty acid functions and abnormal lipid metabolism

Dietary fatty acids are essential for normal physiological functions because fatty acids not only play important roles in maintaining cell structure and fluidity, but also act as ligands of several nuclear receptors [30, 31] (peroxisome proliferator active

receptor (PPAR) family, liver X receptor α, and hepatocyte nuclear factor-4α[HNF-4α]) and transcription factors [31] (sterol regulatory element-binding protein 1 (SREBP-1), carbohydrate response element-binding protein) involve in lipid metabolism. Certain fatty acids (eicosapentaenoic acid [EPA, C20:5, n-3], docosahexaenoic acid [DHA, C22:6, n-3); arachidonic acid [AA, C20:4, n-6]) also serve as precursors for "hormonal" molecules (eicosanoids/docosanoids) that play important roles in regulating inflammation status [32].

Despite these crucial role of fatty acids and lipid, excess fat may be associate with enlarged adipocytes and increased ectopic fat deposition in liver, beta cells and skeletal muscles when adipocytes cannot store all the exogenous and endogenous lipid. Enlarged adipocytes and the ectopic lipid infiltration lead to insulin dysregulation that may affect lipid metabolism [33]. Elevated circulating lipid happened when adipocytes failed to buffer and store fat as neutral lipid and cause dyslipidemia.

II. Effects of SFA and MUFA on inflammation and insulin resistance

For many years, lipotoxicity triggered by excess free fatty acids was restricted to SFAs [34]. However, it has been suggested that all fatty acids are toxic in different degree. The degree of toxicity of fatty acids reflected differently depending on the cell type, the chain length of fatty acids and the number of double bonds [34]. Evidence suggested that high levels of circulating SFAs are associated with apoptosis, insulin resistance, diabetes, obesity and hyperlipidemia [9]. Higher lipotoxicity of

SFAs has been observed compare to MUFAs, and it has been casually assumed that cells generate more apoptotic related lipid molecules and/or signaling molecules in response to SFA species [35].

Sphingolipids ceramide accumulation has been shown to contribute to insulin resistance and often associated with multiple obesity related diseases including diabetes, cardiomyopathy, and atherosclerosis [36]. Stress stimuli such as SFAs, inflammatory agonists and chemotherapeutics have been shown to increase ceramide synthesis. Indeed, palmitic acid and stearic acid (18:0), the predominant SFAs *in vivo*, are the precursors of *de novo* synthesis of ceramides [35]. SFAs have also been proposed as the ligands of the membrane receptor Toll-like receptor 4 (TLR4), which leads to Nuclear factor kappa B (NF-κB) and C-jun N-terminal kinase (JNK) activation as well as cytokine production and inflammation in adipose tissue [37].

It has been suggested that MUFAs have the ability to protect cells against from apoptosis, inflammation, ER stress and insulin resistance when used in combination with SFAs [38]. Although MUFAs may induce modest toxicity in hepatocytes, it is far less toxic compared to the same concentration of SFAs [39]. For instance, oleic acids have been shown to reduce palmitate-mediated ER stress and apoptosis in the H4IIE hepatocytes [40]. The associated mechanism is still unclear, but there are two main hypotheses proposed in which MUFAs protect against SFAs through 1) esterified FAs into neutral lipid TGs for storage and 2) facilitated lipid detoxification by fatty acid oxidation [41].

III. Effects of PUFA on inflammation and insulin resistance

It has been suggested that certain PUFA species have the abilities to affect inflammatory response [32]. PUFAs regulate fuel partitioning within the cells by reducing lipogenic gene expression through PPARs [30] and SREBP pathway [42] and prevent insulin resistance by increasing membrane fluidity and GLUT4 transport [43]. Although parts of the functions still remain unclear, n-3 and n-6 PUFAs are generally being recognized as anti-inflammatory and pro-inflammatory fatty acids, respectively [44].

Obesity is characterized as a state of low-grade of chronic inflammation. Numerous studies have investigated the potential of n-3 PUFAs on lowering inflammatory responses. Mehra et al. [45] have demonstrated that 8 g/day of n-3 PUFAs supplementation (80% of this supplement were n-3 fatty acids ethyl esters, in which 44% were EPA, 24% DHA and the rest 12% were other n-3 fatty acid ethyl esters) reducing inflammatory cytokines including tumor necrosis factor (TNF)- α and interleukin (IL)-1 in patients suffered from severe heart failure. Moreover, EPA and DHA supplementations have been shown to have the abilities of lowering IL-6, IL-9, monocyte chemotactic protein-1 and high sensitivity C-reactive protein. n-3 PUFAs also have physiological effect on lowering oxygen free-radical generation, which may play a role in its function on reducing inflammation [46].

The mechanismn-3 PUFAs might be their anti-inflammatory actions on cyclooxygenase (COX). EPA acts as a competitive inhibitor for AA, an n-6 PUFA, on COX, which produces pro-inflammatory eicosanoids when AA is used as a

substrate (**Fig. 1**) [47]. More specifically, AA is a precursor of 2-series prostaglandins and thromboxanes, such as PGE_2 and TXA_2, respectively. These 2-series pro-inflammatory factors could induce the production of IL-1, IL-6, and TNF-α from macrophage [48]. This means that elevated AA level might induce AA-derived eicosanoids and then further enhance or prolong the inflammatory status. Whereas EPA is known to increase the 3-series prostaglandins and thromboxanes, such as PGE_3 and TXA_3, respectively, which has significant weaker effect of inducing inflammatory cytokines than 2-series [47].

It is obvious that these longer chain n-3 and n-6 PUFAs are critical in affecting inflammatory response. In theory, mammals can synthesize these longer chain PUFAs from the essential fatty acids—linoleic acid (LA, 18:2, n-6) and α-linolenic acid (ALA, 18:3, n-6) (**Fig. 1**). However, some evidences showed that the conversion from ALA to the longer chain n-3 PUFAs could be inefficient in some populations, which means the n-3 PUFAs in the tissue and circulation may reflect dietary fatty acid composition [49].

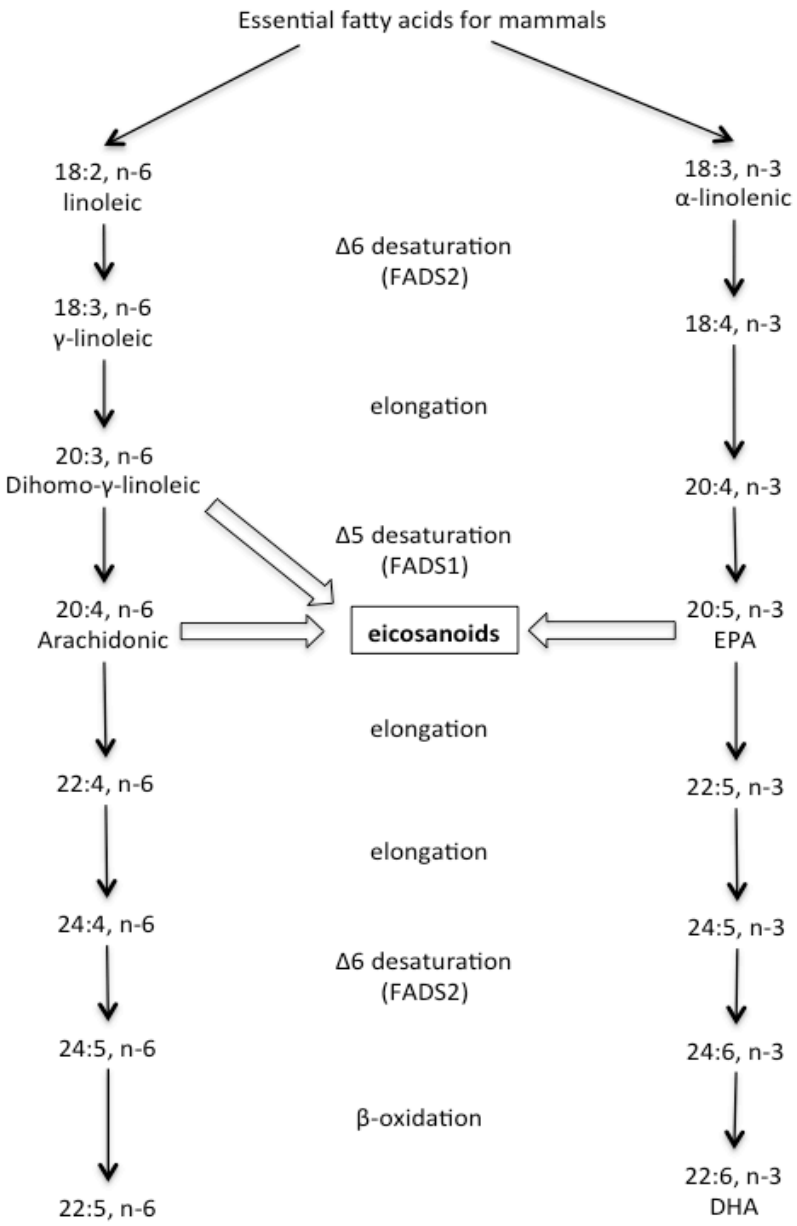


Figure 1. Schematic of the desaturation and elongation biosynthetic pathway of essential dietary fatty acids to longer chain unsaturated fatty acids [(50, 51)]. Precursors of eicosanoids are also highlighted. n-3 fatty acids acronyms: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Enzyme acronyms: FADS1, fatty acid desaturase 1; FADS2, fatty acid desaturase 2.

In vitro model to Study Hepatic lipotoxicity

In vitro cell culture and animal models are both been intensively used in studying molecular mechanisms, drug development, and cellular toxicity…etc. *In vitro* models are essential when the *in vivo* models are limited due to animal welfare/ethical concerns or when tissue specific effects need to be studied in an isolated environment.

Hepatic steatosis and insulin resistance

Hepatic lipid accumulation is known to be associated with hepatic insulin resistance and systemic insulin resistance [(52)], increased endogenous glucose production [(53, 54)], and mitochondrial dysfunctions [(55)]. Whether the correlation between ectopic lipid accumulation in liver and insulin resistance is due to a causal relationship between the conditions have long been debated. Several pathways that have been proposed to be associated with the link of these two conditions. Increased lipid metabolites such as diglycerides (DG) in the hepatocytes can cause hepatic insulin resistance by activating protein kinase Cε (PKCε) [(56)]. Activated PKCε [1] binds to insulin receptors and inhibits its tyrosine kinase activity and [2] interferes with the ability of insulin to phosphorylate IRS-2, both affect phosphatidylinositol 3-kinase (PI3K)-AKT pathway and eventually lead to increased hepatic glucose production (**Fig. 2**). Cholesterol and certain non-esterified fatty acids (NEFA) species have also been shown to downregulate hepatic IRS-2 expression through c-Jun NH2-terminal kinase (JNK) activation [(57)].

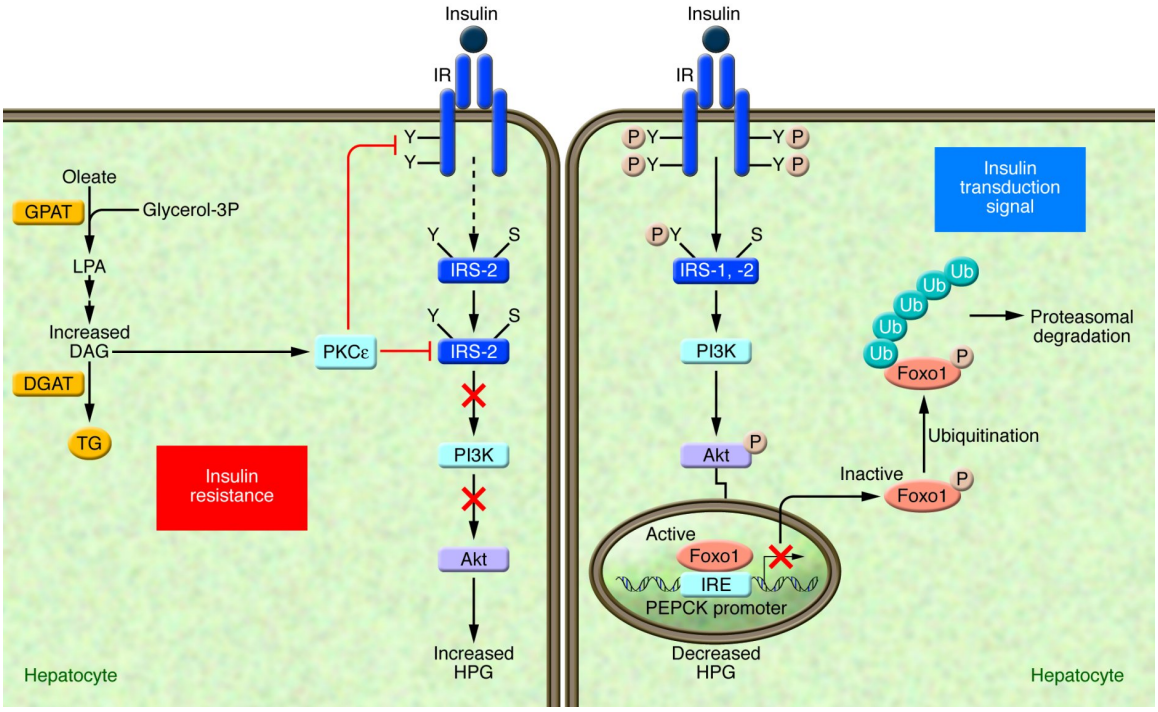


Figure 2. Lipid metabolites affect insulin sensitivity in the hepatocytes; adapted from Postic et al.[56]. When an adequate transduction signal is present (right panel), insulin binding to the insulin receptor results in the phosphorylation of tyrosine residues (Y) on insulin receptor substrates (IRS-1 and -2), which leads to the activation of PI3K and the subsequent phosphorylation of Akt, which are involved in mediating the metabolic effects of insulin. On the other hand, excess lipid metabolites such as DAG can cause insulin resistance by activating PKCε (left panel). The activated PKCε binds to the insulin receptor and inhibits its tyrosine kinase activity. The activation of PKCε may also interfere with the ability of insulin to phosphorylate IRS-2 on tyrosine residues. IRE, insulin-responsive element; S, serine; Ub, ubiquitination.

Increased oxidative stress has also been suggested to play a role in the induction of hepatic and systemic insulin resistance. NEFA accelerates oxidation in the hepatocytes triggered by upregulation of carnitine palmitoyltransferase-1a (CPT-1a) that causes excess electron flux in the mitochondrial respiratory chain, resulting in reactive oxygen species (ROS) overproduction[57]. ROS then activate JNK which inhibits insulin signaling through IRSs. Excessive substrate acetyl CoA derived from glucose and fatty acid metabolism may also induced ROS production [58]. The excess acetyl CoA flows into mitochondrial β-oxidative phosphorylation (OXPHOS) to generate ATP that would stimulate gluconeogenic enzyme such as phosphoenolpyruvate carboxykinase (PEPCK), eventually lead to increased hepatic glucose production (**Fig.3**).

Hepatic insulin resistance is crucial and could potentially leads to systemic insulin resistance. A study conducted by Michael et al. [59] showed that the disruption of hepatic insulin signaling in the liver-specific insulin receptor knock-out (LIRKO) mice resulting in hyperinsulinemia, fasting and postprandial hyperglycemia and the subsequent development of peripheral (muscle) insulin resistance. Moreover, the mice with disruptive insulin signaling in the skeletal muscle and adipose tissue remained normal glucose homeostasis [60]. These findings suggested a direct role of insulin signaling in liver in the regulation of postprandial glucose homeostasis and whole-body insulin sensitivity.

Furthermore, since fatty liver is generally correlated with obesity, the changed of metabolism associated with obesity and metabolic syndrome may also contribute to the impaired insulin signaling. The potentially increased NEFA, especially SFA, in

circulation of obese patients could stimulate TLR4 that would activate both Ikkb/NFκB and JNK signaling, not only potentially inhibit PI3K/Akt pathway but also lead to the expression and secretion of proinflammatory cytokines such as I11b, IL-6, TNF-α, MCP1…etc [(61)]. Additionally, the hypertrophy and hyperplasia adipocytes of obese patients outstrip the local oxygen supply that leads to cell autonomous hypoxia with activation of cellular stress pathways, which leads to inflammation response of cells that release cytokines and other pro-inflammatory factors. All these produced cytokines could travel through circulation and affect hepatic insulin signaling. For example, tumor necrosis factor-α (TNF-α) could activate TNF-α receptors resulting in stimulation of NFκB signaling via Ikkb that inhibits IRS-1 expression (**Fig. 4**).

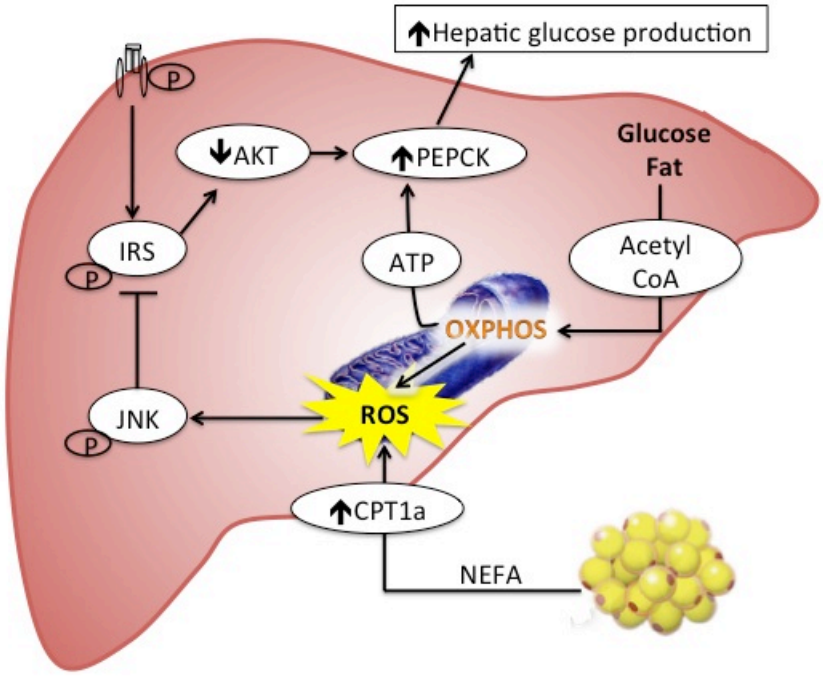


Figure 3. The role of mitochondrial ROS and excessive substrate acetyl CoA in the development of hepatic insulin resistance through interfering with PI3K/AKT pathway [57, 58]. Saturated NEFA has been shown to have the ability to induce CPT-1a expression, provide excess electrons for mitochondrial OXPHOS and generate ROS. Excess substrate acetyl CoA derived from glucose and fat flow into mitochondrial OXPHOS also generate ROS. ROS then desensitizes the insulin signaling pathway by activating JNK, impairing tyrosine phosphorylation of IRS-2, and causing hepatic insulin resistance. Moreover, the excess substrate acetyl CoA flow into mitochondrial OXPHOS to generate ATP, that stimulates gluconeogenic enzymes.

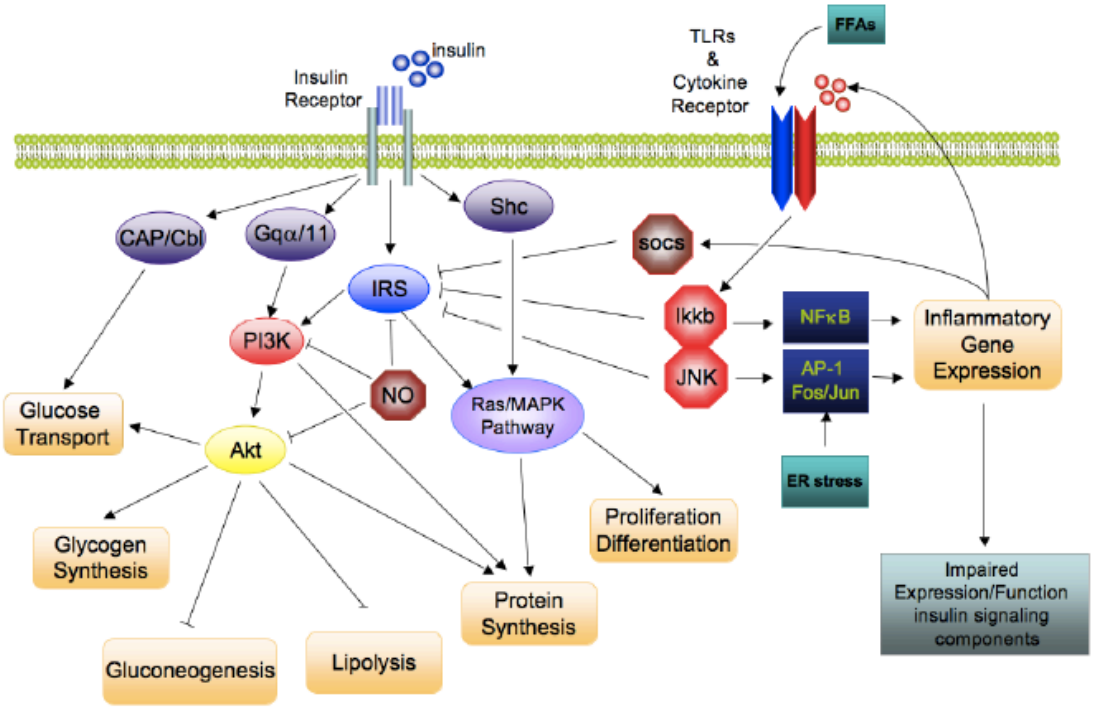


Figure 4. Direct interaction of insulin signaling and inflammatory pathways. The insulin signaling cascade branches into two main pathways. The PI3K/AKT pathway mediates insulin action on nutrient metabolism including glucose uptake. The Ras/MAPK pathway mediates insulin's effect on gene expression, but also interacts with the PI3K-AKT pathway to control cell growth and differentiation. Activation of the insulin receptor leads to tyrosine phosphorylation of IRS1 thereby initiating signal transduction. Stimulation of the NFκB and AP-1 Fos/Jun inflammatory pathways results in the activation of the serine kinases, Ikkb and Jnk1, which reduce the signaling ability of IRS1. Additional inflammation-related negative regulators of IRS proteins include the Socs proteins and NO, which are induced in inflammation, and promote IRS degradation.

That is to say, induction of lipid accumulation in hepatocyte could lead to hepatic insulin resistance and other complications that serve as a great model to study the mechanism associated with obesity. Thus, liver cells are often being exposed to fatty acids to induce hepatic lipid storage and then study resulting metabolic effects.

Commonly used *in-vitro* model for studying hepatic steatosis

Isolated primary hepatocytes and HepG2 cells have both been used to examine hepatic metabolism *in-vitro*. The transformed, proliferating cell line such as HepG2 and the highly differentiated, non-proliferating primary hepatocytes likely have some differences on gene expressions and enzymatic functions [(62)]. Primary hepatocytes can be isolated from mice or rats through perfusion methods; they can also be cultured from the liver samples come from human donors. Primary human hepatocytes are considered the gold standard to study human related liver metabolism. The applicability of cultured primary hepatocytes makes it a great model to obtain species specific response from liver. The down side of using this primary *in vitro* model is that the idiosyncratic responses of donors display by the hepatocytes may cause certain variable responses.

HepG2 is an immortalized cell line that derived from a liver hepatoma of a 15-year-old Caucasian male [(63)]. This established cell line with hepatic characteristics is very convenient for molecular and cellular researches on investigating human liver response due to the availability of unlimited quantity. Although isolated primary hepatocytes and HepG2 cells have similar glucuronidation and cytochrome reductase activities, they are differ in microsomal epoxide hydrolase activity [(64)]. In addition, lack of many cytochrome

P450s expression of HepG2 cells might affect the differential responses to chemical exposures [65].

Hepatic lipid exposure

Elevated plasma NEFAs level is associated with intra-abdominal obesity [66]. It has been widely believed that enlarged visceral fat depot of obese subjects undergo lipolysis and the resulting NEFAs are exposed to the liver through the portal vein [66] The liver is also exposed to NEFA through systemic circulation by the hepatic artery. Liver is one of the most crucial metabolic regulation organs that involves in synthesis and degradation of lipids. Hepatocytes uptake NEFAs and convert them to fatty acyl CoA before they undergo metabolism through either 1) partially or wholly oxidation to carbon dioxide and ketone bodies or 2) esterification to form TGs, phospholipids (PL), and other fatty acyl esters [67, 68]. The rates of esterification and the formation of TG appear to be unsaturable [68]. Needless to say that increased NEFAs uptake by liver leads to excess lipid formation, resulting in ectopic lipid accumulation, increased lipolysis of excess lipid, increased lipid secretion into circulation which may cause dyslipidemia [69]. Hepatic steatosis is a term used to describe fat infiltration of the liver or otherwise termed fatty liver. The definition of fatty liver is when over 5% of the liver weight is made up of stored lipids.

Use of fatty acid species in cell culture

While demonstrating lipid exposure on cultured cells, non-esterified fatty acids are usually used because they can be readily uptake by cells whereas TGs need to be hydrolyzed by lipases prior to be uptake. An older study from Svedberg et al. [66] evaluated the abilities of several fatty acid species in inhibiting insulin binding in primary rat hepatocytes. They showed that 0.4 mM of oleic acid, palmitic acid, stearic acid, palmitoleic acid and eicosapentaenoic acid (EPA) achieved same level (~40%) of inhibition ability on insulin binding.

Despite of the similar abilities of inhibiting insulin binding, some researchers believed that only saturated fatty acid could induce insulin resistance and downstream (post receptor) insulin signaling via intracellular lipid accumulation [70]. Palmitic acid is predominately used in most studies because it is the dominant fatty acid in SFA and it is a species that leads to TG formation [71, 72]. On the other hand, evidence showed that oleic acid (C18:1, n-9), the dominant MUFA *in vivo*, contributes to the accumulation of diglyceride (DG) fraction. DG have become the stored lipid most tightly linked to the progression of insulin resistance because it has bioactive signaling properties, while TG are inert and have not been shown to directly stimulate pathways [72]. Although oleic acid is less toxic to hepatocytes and is able to reduce palmitate-mediated ER stress and apoptosis, the steaotosis induced by oleic acid is believed to be associated with apoptosis in hepatocytes. Due to its abundance and apparent effects on cellular metabolism, some studies tend to use palmitic acid and oleic acid mixtures in a 1:1 or 2:1 proportion to induce lipid accumulation in a more physiological sense in the *in vitro* models [53, 72-74].

There is also evidence that supplying oleic acid in the mix allows you to use higher palmitic acid concentrations before apoptosis is activated.

Non-esterified fatty acids have to be dissolved in ethanol and then conjugated with albumin before mixing with medium to treat the cells. In the hepatocytes, cells are usually incubated with the medium free of fetal bovine serum (FBS) 1-hour prior to the fatty acids treatment to demonstrate FBS deprivation. After several hours of incubating with the targeted fatty acids, the cells are chased with or without insulin before being harvested for insulin signaling studies [(72)].

It has been shown that non-esterified fatty acids are rapidly taken up into cultured primary hepatocytes in 1-hour exposure to a 1:2 palmitic acid: oleic acid fatty acids mixture [(53)]. Three hours exposure is enough for primary hepatocytes to esterified fatty acids into TGs, and the amount of TGs increased in a time dependent manner [(53)].

Disconnect between *in vivo* and *in vitro* studies

Fatty acids profile of total lipids in the systemic circulation serum in human

Fern´andez-Real et al. [(75)] assessed the fatty acid profiles of total lipids in the serum of lean and overweight men and women under their routine diets (**Table 2**). After analyzing the serum fatty acid profiles in 232 subjects, the proportion of SFA in human serum was approximately 30%, MUFA was about 22%, and the PUFA accounted for 43% of total lipids. The dominant fatty acid species in SFA and MUFA were palmitic acid (16:0) and oleic acid (18:1, n-9) as mentioned above. n-6 PUFA appeared to be the majority source of PUFA (94% of total PUFA) , especially linoleic acid (18:2, n-6; 79%

of total n-6 PUFA), compared to n-3 PUFA. Whereas arachidonic acid (20:4, n-6) accounted for 17% of the total n-6 PUFA in the human serum fatty acid profile found in the same study. Additionally, the dominant n-3 PUFA comes from DHA. This study also reported that the percent palmitic acid and DHA appeared to be significant higher in men than in women.

The same study separated the subjects into two subgroups—lean and overweight by BMI (22.2±1.8 vs. 27.9±2.4). n-3 PUFA was significant lower in the overweight men and women, which was due to decreased EPA in men and DHA in women. However, this result may not be applicable now because a much higher percentage of the population (30% in the United States) is above BMI 30 according to CDC.

In a study conducted by Santomauro et al. [(76)], the total NEFA concentrations of obese nondiabetic subjects (560 ± 52 μmol/l) and obese diabetic subjects (584 ± 39 μmol/l) are significantly higher than lean nondiabetic subjects (329 ± 28 μmol/l). A recent study also showed higher serum NEFA concentration in obese children (422.2 μmol/l) than normal weight children (332.0 μmol/l) [(77)]. However, a steady accumulation of published data supported the fact that increased fat mass in obese patients does not necessarily lead to elevated serum NEFA level. Karpe et al.[(78)] conducted a systemic literature review analyzing 43 original reports including 953 nonobese and 1410 overweight/obese subjects and concluded that although most of the studies showed greater NEFA concentration in the obese/overweight group, the average difference was modest and appeared to be unrelated to the fat mass.

Fatty acids (%)	Lean men	Lean women	Overweight men	Overweight women
SFA	**30.8±1.9**	**29.6±3.2**	**30.3±1.7**	**29.4±3.6**
14:0	0.45±0.26	0.42±0.34	0.34±0.22[a]	0.40±0.24
16:0	20.13±2.20	18.94±3.27	19.63±1.88	18.9±2.95
18:0	7.73±0.76	7.72±0.95	7.82±0.79	7.52±1.11
20:0	0.29±0.06	0.30±0.07	0.30±0.06	0.30±0.08
22:0	0.84±0.18	0.88±0.19	0.86±0.16	0.88±0.20
MUFA	**22.79±4.23**	**22.3±4.73**	**22.35±4.66**	**24.6±9.4**
16:1, n-9	0.37±0.11	0.36±0.09	0.34±0.09	0.34±0.12
18:1, n-9	20.69±3.37	20.95±3.53	20.98±3.58	22.36±8.72
PUFA (n-6)	**40±4.7**	**40.5±5.4**	**40.7±4.6**	**40.5±6.4**
18:2, n-6	31.37±4.52	31.89±5.13	32.12±4.58	32.1±5.80
18:3, n-6	0.46±0.14	0.44±0.19	0.43±0.15	0.42±0.15
20:4, n-6	7.13±1.32	7.10±1.35	7.03±1.41	6.92±1.43
PUFA (n-3)	**3.1±1.0**	**3.01±0.96**	**2.72±0.6**[a]	**2.59±0.93**[b]
18:3, n-3	0.32±0.18	0.34±0.35	0.27±0.09	0.29±0.15
20:5, n-3	0.67±0.43	0.59±0.38	0.44±0.21[a]	0.47±0.33
22:6, n-3	2.11±0.68	2.06±0.55	2.00±0.50	1.82±0.63[b]

Table 2. Serum fatty acid profiles in both lean and overweight men and women. Data are shown as mean±SD. Table generated from data published by Fern´andez-Real et al. *(75)*.

[a] $p<0.05$ compared to lean men; [b] $p<0.05$ compared to lean women.

Fatty acids profile in NEFA, TG, PL and CE fractions

Each lipid class displayed a distinct fatty acid pattern. A recent study reported the fatty acid profiles in TG, PL and CE fractions in the plasma of 22 obese subjects (11males and 11 females). The average age of these subjects were 64±5 years old, and the average BMI was 27.6±2.7. The percent of fatty acid species in each fraction are shown in **Table 3**. The predominant fatty acids in the TG fraction are oleic acid and linoleic acid (both 27%), and then follow by palmitic acid (24.4%). In the PL fraction, palmitic acid is the most abundant fatty acid (30.5%); linoleic acid is the second abundant fatty acid (22.8%) in this fraction. Whereas linoleic acid is dominant in the CE fraction (57.5%), and then followed by palmitic acid and oleic acid (both 11.9%). Goodman et al. [(79)] reported similar fatty acid profiles in two healthy subjects back in 1964, except those subjects had substantial higher percentages of oleic acid and lower linoleic acid. The differences found in these two studies might come from the different dietary fat since TG fraction in the plasma reflects short-term dietary fat intake (discussed in the following paragraph). Goodman et al. [(79)] also analyzed the fatty acid profile in NEFA fraction in two healthy human plasma (a 39-year-old black male and 18-year-old Caucasian male). There was approximately 24% of palmitic acid, 45% of oleic acid, 7~11% of linoleic acid in the NEFA fraction in the plasma of both subjects. However, the older subject had higher percent of steric acid in the NEFA fraction (16.9±3.8) than the younger subjects (9.4±0.49).

Fatty acids (%)	TG	PL	CE
SFA	**29.6±4.9**	**47.2±1.6**	**13.5±1.3**
14:0	2.3±0.7	0.54±0.12	0.75±0.21
16:0	24.4±4.1	30.5±1.1	11.9±1.0
18:0	2.9±0.8	14.0±1.1	0.81±0.24
20:0	-	0.38±0.04	-
22:0	-	1.1±0.1	-
24:0	-	0.74±0.13	-
MUFA	**35.4±4.8**	**11.3±1.4**	**16.1±2.5**
16:1, n-9+n-7	3.3±1.0	0.58±0.14	2.0±0.7
18:1, n-9	27.0±4.5	6.2±1.2	11.9±2.8
18:1, n-7	1.9±0.4	1.2±0.2	1.1±0.2
PUFA	**35.0±5.9**	**42.2±1.5**	**70.4±3.4**
n-6	**31.3±5.6**	**35.7±2.0**	**68.2±3.6**
18:2, n-6	27.0±5.2	22.8±2.6	57.5±4.5
18:3, n-6	0.7±0.3	0.15±0.06	0.87±0.37
20:3, n-6	0.4±0.1	3.5±1.0	0.62±0.19
20:4, n-6	1.5±0.5	9.3±1.9	7.3±2.0
22:4, n-6	-	0.21±0.05	-
n-3	**3.7±1.2**	**5.6±1.4**	**2.2±0.8**
18:3, n-3	2.3±0.9	0.3±0.09	0.76±0.33
20:5, n-3	0.4±0.2	0.98±0.64	0.91±0.53
22:5, n-3	0.3±0.1	0.74±0.19	-
22:6, n-3	0.7±0.6	3.6±0.9	0.52±0.20

Table 3. Serum fatty acid profiles of TG, PL and CE fractions in the fasting serum of overweight human subjects (women, n=18; men, n=18). Data are shown as mean±SD. Table adapted from data published by Matthan et al. [(80)].

Dayton et al. [81] assessed the CE, PL, and TG fractions in serum lipid of elderly men who ingested the mostly SFA (control) vs. unsaturated FA (experimental) in their isocaloric diets that contained 40% fat. According to their data, serum TG fraction appeared to reflect the dietary fatty acid composition (**Fig. 2**). Similar results have been reported in other studies as well [82, 83]. The relationship between the ingested amount of PUFA and the corresponding proportions of the same fatty acids in the plasma lipid ester are often strong [84].

A study conducted by Klein-Platat et al. [85] showed that overweight adolescence (average age 11.6 years old) with metabolic syndrome has higher percentage of SFA in plasma phospholipid compare to the normal weight adolescence. The increased SFA were caused by increased stearic acid (C18:0) rather than palmitic acid (16:0). The higher SFA is positively associated with The increased percentage of SFA led to decreased percentage of PUFA in plasma phospholipid, and the lower percentage of PUFA were due to the lower percentage of linoleic acid (18:2, n-6) and docosahexaenoic acid (DHA; 22-6, n-3).

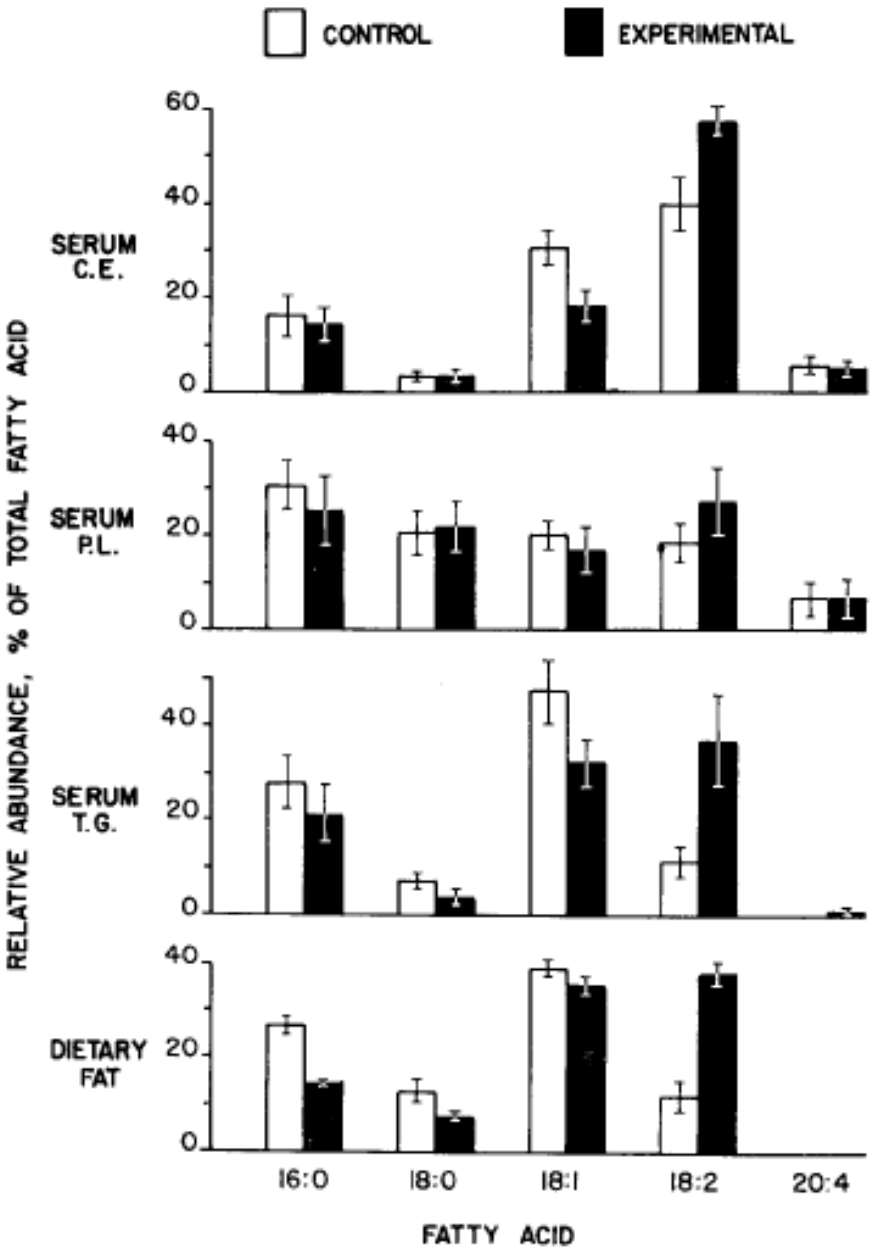


Figure 2. Data from Dayton et al. [81] showed the fatty acid compositions in the major serum lipid fractions, compared with dietary fat. Values are shown as mean ± SD, n=10. Abbreviations: CE, cholesterol ester; PL, phospholipid; TG, triglyceride.

Effects of the combination of fatty acids have been left out in *in vitro* studies

It is apparent that there are more than two fatty acids circulating in the body, and each fatty acid species existing for different purposes. Obtaining fatty acid profiles provide information regarding fatty acid metabolism and storage, degree of *de novo* lipogenesis, dietary lipid source and even desaturase indices. Most of these outcomes are determined by assessing the interactions between fatty acid species.

Physiologically important fatty acid species and their interactions

The chain length of dietary fatty acids, degrees of unsaturation, and double bonds configuration have significant impact on their metabolic fate [(86)]. How specific fatty acids affect in vivo metabolism and overall health are still not fully understood due to the intricate biochemistry of each fatty acid, their unique physiological and molecular actions. Ergo the understanding of the interactions between each fatty acid species are limited [(44)].

Certain fatty acid species have known to compete with each other to bind to receptors that involved in lipid metabolism or to affect the production of inflammatory cytokines. In a compete binding study conducted by Kliewer et al.[(30)], PUFAs such as ALA, LA and AA appeared to have more efficient binding with both PPARα and PPARγ than SFAs and MUFAs. PUFAs are apparently stronger ligands to PPARα and PPARγ that involved in lipid metabolism. In the three MUFA species that the same study tested, petrosellenic acid (18:1, n-6) seemed to have higher binding ability to both PPARα and PPARγ compare to oleic acid. Interestingly, oleic acid demonstrated a unique selectively binding ability with PPARα. These evidences suggested that the increased in the

circulating levels of certain FA species could results in enhancing catabolism and/or storage of FAs via activation of PPARα and PPARγ, respectively.

It is obvious that the interactions between fatty acid species are complicated and relatively unclear. A recent published study investigated the different ratio of SFA: MUFA mixtures affecting the degree of apoptosis in vascular smooth muscle cells [(38)]. SFA such as palimitic acid are known to have pro-apoptosis characteristic, whereas MUFA such as oleic acid are known to be anti-apoptosis. They found that all of these fatty acid mixtures inhibited cells proliferation by their pro-apoptosis potential, however, oleic acid reduces apoptosis in a dose-dependent manner. Additionally, oleic acid has better capacity to reduce apoptosis when combined with a SFA than being used alone. These results highlight the fact that the ratio of fatty acids plays a critical role in affecting cell responses.

Summary

Animal models are valuable in helping researcher to understand whole body metabolism and functions of individual organ; however, *in vitro* cell culture technique is essential when studying molecular mechanism, cellular toxicity and organelle functions. Developing an appropriate *in vitro* system that induces lipid accumulation and insulin resistance is undoubtedly important. The single or dual fatty acids that have been widely used in the *in vitro* system to induce lipid accumulation and insulin resistance do not mimic *in vivo* environment. The disconnect between the *in vivo* model and *in vitro* model

could be resolved by adding fatty acid species in a physiological relevant proportion to treat the cultured cells based on results obtained from *in vivo* studies.

Reference

1. Kanasaki, K., and Koya, D. (2011) Biology of obesity: lessons from animal models of obesity, *J Biomed Biotechnol 2011*, 197636.
2. James, P. T., Leach, R., Kalamara, E., and Shayeghi, M. (2001) The worldwide obesity epidemic, *Obes Res 9 Suppl 4*, 228S-233S.
3. Berrington de Gonzalez, A., Hartge, P., Cerhan, J. R., Flint, A. J., Hannan, L., MacInnis, R. J., Moore, S. C., Tobias, G. S., Anton-Culver, H., Freeman, L. B., Beeson, W. L., Clipp, S. L., English, D. R., Folsom, A. R., Freedman, D. M., Giles, G., Hakansson, N., Henderson, K. D., Hoffman-Bolton, J., Hoppin, J. A., Koenig, K. L., Lee, I. M., Linet, M. S., Park, Y., Pocobelli, G., Schatzkin, A., Sesso, H. D., Weiderpass, E., Willcox, B. J., Wolk, A., Zeleniuch-Jacquotte, A., Willett, W. C., and Thun, M. J. (2010) Body-mass index and mortality among 1.46 million white adults, *N Engl J Med 363*, 2211-2219.
4. Tchernof, A., and Despres, J. P. (2013) Pathophysiology of human visceral obesity: an update, *Physiol Rev 93*, 359-404.
5. Ogden, C. L., Carroll, M. D., Kit, B. K., and Flegal, K. M. (2012) Prevalence of obesity in the United States, 2009-2010, *NCHS Data Brief*, 1-8.
6. Finkelstein, E. A., Trogdon, J. G., Cohen, J. W., and Dietz, W. (2009) Annual medical spending attributable to obesity: payer-and service-specific estimates, *Health Aff (Millwood) 28*, w822-831.
7. Stevens, G. A., Singh, G. M., Lu, Y., Danaei, G., Lin, J. K., Finucane, M. M., Bahalim, A. N., McIntire, R. K., Gutierrez, H. R., Cowan, M., Paciorek, C. J., Farzadfar, F., Riley, L., and Ezzati, M. (2012) National, regional, and global trends in adult overweight and obesity prevalences, *Popul Health Metr 10*, 22.
8. Popkin, B. M., Paeratakul, S., Zhai, F., and Ge, K. (1995) A review of dietary and environmental correlates of obesity with emphasis on developing countries, *Obes Res 3 Suppl 2*, 145s-153s.
9. Galgani, J. E., Uauy, R. D., Aguirre, C. A., and Diaz, E. O. (2008) Effect of the dietary fat quality on insulin sensitivity, *Br J Nutr 100*, 471-479.
10. Kanarek, R. B., and Orthen-Gambill, N. (1982) Differential effects of sucrose, fructose and glucose on carbohydrate-induced obesity in rats, *The Journal of nutrition 112*, 1546-1554.
11. Shafrir, E. (2010) Contribution of animal models to the research of the causes of diabetes, *World J Diabetes 1*, 137-140.

12. Kennedy, A. J., Ellacott, K. L., King, V. L., and Hasty, A. H. (2010) Mouse models of the metabolic syndrome, *Dis Model Mech 3*, 156-166.
13. Zucker, L. M., and Zucker, T. Z. (1961) Fatty, a new mutation in the rat *J Hered 52*, 275-278.
14. Rector, R. S., Thyfault, J. P., Uptergrove, G. M., Morris, E. M., Naples, S. P., Borengasser, S. J., Mikus, C. R., Laye, M. J., Laughlin, M. H., Booth, F. W., and Ibdah, J. A. (2010) Mitochondrial dysfunction precedes insulin resistance and hepatic steatosis and contributes to the natural history of non-alcoholic fatty liver disease in an obese rodent model, *J Hepatol 52*, 727-736.
15. Nakamura, M., and Yamada, K. (1967) Studies on a diabetic (KK) strain of the mouse, *Diabetologia 3*, 212-221.
16. Herberg, L., and Coleman, D. L. (1977) Laboratory animals exhibiting obesity and diabetes syndromes, *Metabolism 26*, 59-99.
17. Jurgens, H. S., Schurmann, A., Kluge, R., Ortmann, S., Klaus, S., Joost, H. G., and Tschop, M. H. (2006) Hyperphagia, lower body temperature, and reduced running wheel activity precede development of morbid obesity in New Zealand obese mice, *Physiological genomics 25*, 234-241.
18. Kanasaki, K., and Koya, D. (2011) Biology of obesity: lessons from animal models of obesity, *J Biomed Biotechnol 2011*, 197636.
19. Levin, B. E., Dunn-Meynell, A. A., Balkan, B., and Keesey, R. E. (1997) Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats, *Am J Physiol 273*, R725-730.
20. Samuels, L. T., Gilmore, R. C., and Reinecke, R. M. (1948) The effect of previous diet on the ability of animals to do work during subsequent fasting, *The Journal of nutrition 36*, 639-651.
21. Buettner, R., Scholmerich, J., and Bollheimer, L. C. (2007) High-fat diets: modeling the metabolic disorders of human obesity in rodents, *Obesity (Silver Spring) 15*, 798-808.
22. Brown, N. M., and Setchell, K. D. (2001) Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones, *Lab Invest 81*, 735-747.
23. Van Heek, M., Compton, D. S., France, C. F., Tedesco, R. P., Fawzi, A. B., Graziano, M. P., Sybertz, E. J., Strader, C. D., and Davis, H. R., Jr. (1997) Diet-induced obese mice develop peripheral, but not central, resistance to leptin, *J Clin Invest 99*, 385-390.
24. USDA. National Agricultural Library.
25. Magidman, P., Herb, S. F., Luddy, F. E., and Riemenscheneider, R. W. (1962) Fatty acids of lard. B. Quantitative Estimation by Silicic Acid and Gas-Liquid Chromatography, *Journal of the American Oil Chemists Society 40*.
26. Chouinard, P. Y., Corneau, L., Barbano, D. M., Metzger, L. E., and Bauman, D. E. (1999) Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows, *J Nutr 129*, 1579-1584.
27. Chowdhury, K., Banu, L. A., and Latif, A. (2007) Studies on the Fatty Acid Composition of Edible Oil, *Bangladesh J. Sci. Ind. Res. 42*.

28. Borengasser, S. J., Rector, R. S., Uptergrove, G. M., Morris, E. M., Perfield, J. W., 2nd, Booth, F. W., Fritsche, K. L., Ibdah, J. A., and Thyfault, J. P. (2012) Exercise and Omega-3 Polyunsaturated Fatty Acid Supplementation for the Treatment of Hepatic Steatosis in Hyperphagic OLETF Rats, *J Nutr Metab 2012*, 268680.
29. Chalkley, S. M., Hettiarachchi, M., Chisholm, D. J., and Kraegen, E. W. (2002) Long-term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats, *Am J Physiol Endocrinol Metab 282*, E1231-1238.
30. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma, *Proceedings of the National Academy of Sciences of the United States of America 94*, 4318-4323.
31. Jump, D. B., Thelen, A., and Mater, M. (1999) Dietary polyunsaturated fatty acids and hepatic gene expression, *Lipids 34 Suppl*, S209-212.
32. Serhan, C. N., Hong, S., Gronert, K., Colgan, S. P., Devchand, P. R., Mirick, G., and Moussignac, R. L. (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, *The Journal of experimental medicine 196*, 1025-1037.
33. Ravussin, E., and Smith, S. R. (2002) Increased fat intake, impaired fat oxidation, and failure of fat cell proliferation result in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus, *Annals of the New York Academy of Sciences 967*, 363-378.
34. Garbarino, J., and Sturley, S. L. (2009) Saturated with fat: new perspectives on lipotoxicity, *Current opinion in clinical nutrition and metabolic care 12*, 110-116.
35. Savary, S., Trompier, D., Andreoletti, P., Le Borgne, F., Demarquoy, J., and Lizard, G. (2012) Fatty acids-induced lipotoxicity and inflammation, *Curr Drug Metab*.
36. Holland, W. L., and Summers, S. A. (2008) Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism, *Endocrine reviews 29*, 381-402.
37. Kennedy, A., Martinez, K., Chuang, C. C., LaPoint, K., and McIntosh, M. (2009) Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications, *J Nutr 139*, 1-4.
38. St-Denis, C., Cloutier, I., and Tanguay, J. F. (2012) Key fatty acid combinations define vascular smooth muscle cell proliferation and viability, *Lipids 47*, 1073-1084.
39. Malhi, H., Bronk, S. F., Werneburg, N. W., and Gores, G. J. (2006) Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis, *J Biol Chem 281*, 12093-12101.

40. Wei, Y., Wang, D., Topczewski, F., and Pagliassotti, M. J. (2006) Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells, *Am J Physiol Endocrinol Metab 291*, E275-281.
41. Nolan, C. J., and Larter, C. Z. (2009) Lipotoxicity: why do saturated fatty acids cause and monounsaturates protect against it?, *Journal of gastroenterology and hepatology 24*, 703-706.
42. Sekiya, M., Yahagi, N., Matsuzaka, T., Najima, Y., Nakakuki, M., Nagai, R., Ishibashi, S., Osuga, J., Yamada, N., and Shimano, H. (2003) Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression, *Hepatology 38*, 1529-1539.
43. Manco, M., Calvani, M., and Mingrone, G. (2004) Effects of dietary fatty acids on insulin sensitivity and secretion, *Diabetes Obes Metab 6*, 402-413.
44. Kim, J., Li, Y., and Watkins, B. A. (2013) Fat to treat fat: Emerging relationship between dietary PUFA, endocannabinoids, and obesity, *Prostaglandins Other Lipid Mediat*.
45. Mehra, M. R., Lavie, C. J., Ventura, H. O., and Milani, R. V. (2006) Fish oils produce anti-inflammatory effects and improve body weight in severe heart failure, *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation 25*, 834-838.
46. Leaf, A., and Weber, P. C. (1988) Cardiovascular effects of n-3 fatty acids, *The New England journal of medicine 318*, 549-557.
47. Bagga, D., Wang, L., Farias-Eisner, R., Glaspy, J. A., and Reddy, S. T. (2003) Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion, *Proc Natl Acad Sci U S A 100*, 1751-1756.
48. Tilley, S. L., Coffman, T. M., and Koller, B. H. (2001) Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes, *J Clin Invest 108*, 15-23.
49. Arterburn, L. M., Hall, E. B., and Oken, H. (2006) Distribution, interconversion, and dose response of n-3 fatty acids in humans, *Am J Clin Nutr 83*, 1467S-1476S.
50. Evans, S. J., Kamali, M., Prossin, A. R., Harrington, G. J., Ellingrod, V. L., McInnis, M. G., and Burant, C. F. (2012) Association of plasma omega-3 and omega-6 lipids with burden of disease measures in bipolar subjects, *Journal of psychiatric research 46*, 1435-1441.
51. Leonard, A. E., Bobik, E. G., Dorado, J., Kroeger, P. E., Chuang, L. T., Thurmond, J. M., Parker-Barnes, J. M., Das, T., Huang, Y. S., and Mukerji, P. (2000) Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids, *The Biochemical journal 350 Pt 3*, 765-770.
52. Tiikkainen, M., Tamminen, M., Hakkinen, A. M., Bergholm, R., Vehkavaara, S., Halavaara, J., Teramo, K., Rissanen, A., and Yki-Jarvinen, H. (2002) Liver-fat accumulation and insulin resistance in obese women with previous gestational diabetes, *Obes Res 10*, 859-867.

53. Budick-Harmelin, N., Anavi, S., Madar, Z., and Tirosh, O. (2012) Fatty acids-stress attenuates gluconeogenesis induction and glucose production in primary hepatocytes, *Lipids Health Dis 11*, 66.
54. Boden, G., and Shulman, G. I. (2002) Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction, *Eur J Clin Invest 32 Suppl 3*, 14-23.
55. Begriche, K., Massart, J., Robin, M. A., Bonnet, F., and Fromenty, B. (2013) Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease, *Hepatology*.
56. Postic, C., and Girard, J. (2008) Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice, *J Clin Invest 118*, 829-838.
57. Nakamura, S., Takamura, T., Matsuzawa-Nagata, N., Takayama, H., Misu, H., Noda, H., Nabemoto, S., Kurita, S., Ota, T., Ando, H., Miyamoto, K., and Kaneko, S. (2009) Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria, *J Biol Chem 284*, 14809-14818.
58. Takamura, T., Misu, H., Ota, T., and Kaneko, S. (2012) Fatty liver as a consequence and cause of insulin resistance: lessons from type 2 diabetic liver, *Endocrine journal 59*, 745-763.
59. Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A., and Kahn, C. R. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction, *Molecular cell 6*, 87-97.
60. Lauro, D., Kido, Y., Castle, A. L., Zarnowski, M. J., Hayashi, H., Ebina, Y., and Accili, D. (1998) Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue, *Nature genetics 20*, 294-298.
61. Reynolds, C. M., McGillicuddy, F. C., Harford, K. A., Finucane, O. M., Mills, K. H., and Roche, H. M. (2012) Dietary saturated fatty acids prime the NLRP3 inflammasome via TLR4 in dendritic cells-implications for diet-induced insulin resistance, *Molecular nutrition & food research 56*, 1212-1222.
62. Harris, A. J., Dial, S. L., and Casciano, D. A. (2004) Comparison of basal gene expression profiles and effects of hepatocarcinogens on gene expression in cultured primary human hepatocytes and HepG2 cells, *Mutation research 549*, 79-99.
63. Knowles, B. B., Howe, C. C., and Aden, D. P. (1980) Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen, *Science 209*, 497-499.
64. Grant, M. H., Duthie, S. J., Gray, A. G., and Burke, M. D. (1988) Mixed function oxidase and UDP-glucuronyltransferase activities in the human Hep G2 hepatoma cell line, *Biochem Pharmacol 37*, 4111-4116.
65. Rodriguez-Antona, C., Donato, M. T., Boobis, A., Edwards, R. J., Watts, P. S., Castell, J. V., and Gomez-Lechon, M. J. (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that

determine lower expression in cultured cells, *Xenobiotica; the fate of foreign compounds in biological systems 32*, 505-520.
66. Svedberg, J., Bjorntorp, P., Smith, U., and Lonnroth, P. (1990) Free-fatty acid inhibition of insulin binding, degradation, and action in isolated rat hepatocytes, *Diabetes 39*, 570-574.
67. Mayes, P. A., and Felts, J. M. (1967) Regulation of fat metabolism of the liver, *Nature 215*, 716-718.
68. Ontko, J. A. (1972) Metabolism of free fatty acids in isolated liver cells. Factors affecting the partition between esterification and oxidation, *The Journal of biological chemistry 247*, 1788-1800.
69. Mooney, R. A., and Lane, M. D. (1981) Formation and turnover of triglyceride-rich vesicles in the chick liver cell. Effects of cAMP and carnitine on triglyceride mobilization and conversion to ketones, *J Biol Chem 256*, 11724-11733.
70. Jung, T. W., Lee, K. T., Lee, M. W., and Ka, K. H. (2012) SIRT1 attenuates palmitate-induced endoplasmic reticulum stress and insulin resistance in HepG2 cells via induction of oxygen-regulated protein 150, *Biochem Biophys Res Commun 422*, 229-232.
71. Firl, N., Kienberger, H., Hauser, T., and Rychlik, M. (2012) Determination of the fatty acid profile of neutral lipids, free fatty acids and phospholipids in human plasma, *Clin Chem Lab Med 0*, 1-12.
72. Chabowski, A., Zendzian-Piotrowska, M., Konstantynowicz, K., Pankiewicz, W., Miklosz, A., Lukaszuk, B., and Gorski, J. (2013) Fatty acid transporters involved in the palmitate and oleate induced insulin resistance in primary rat hepatocytes, *Acta Physiol (Oxf) 207*, 346-357.
73. de Almeida, I. T., Cortez-Pinto, H., Fidalgo, G., Rodrigues, D., and Camilo, M. E. (2002) Plasma total and free fatty acids composition in human non-alcoholic steatohepatitis, *Clin Nutr 21*, 219-223.
74. Chavez, J. A., and Summers, S. A. (2003) Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes, *Arch Biochem Biophys 419*, 101-109.
75. Fernandez-Real, J. M., Broch, M., Vendrell, J., and Ricart, W. (2003) Insulin resistance, inflammation, and serum fatty acid composition, *Diabetes Care 26*, 1362-1368.
76. Santomauro, A. T., Boden, G., Silva, M. E., Rocha, D. M., Santos, R. F., Ursich, M. J., Strassmann, P. G., and Wajchenberg, B. L. (1999) Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects, *Diabetes 48*, 1836-1841.
77. Sabin, M. A., De Hora, M., Holly, J. M., Hunt, L. P., Ford, A. L., Williams, S. R., Baker, J. S., Retallick, C. J., Crowne, E. C., and Shield, J. P. (2007) Fasting nonesterified fatty acid profiles in childhood and their relationship with adiposity, insulin sensitivity, and lipid levels, *Pediatrics 120*, e1426-1433.
78. Karpe, F., Dickmann, J. R., and Frayn, K. N. (2011) Fatty acids, obesity, and insulin resistance: time for a reevaluation, *Diabetes 60*, 2441-2449.

79. Goodman, D. S., and Shiratori, T. (1964) Fatty acid composition of human plasma lipoprotein fractions, *J Lipid Res 5*, 307-313.
80. Matthan, N. R., Ip, B., Resteghini, N., Ausman, L. M., and Lichtenstein, A. H. (2010) Long-term fatty acid stability in human serum cholesteryl ester, triglyceride, and phospholipid fractions, *J Lipid Res 51*, 2826-2832.
81. Dayton, S., Hashimoto, S., Dixon, W., and Pearce, M. L. (1966) Composition of lipids in human serum and adipose tissue during prolonged feeding of a diet high in unsaturated fat, *Journal of lipid research 7*, 103-111.
82. Ma, J., Folsom, A. R., Shahar, E., and Eckfeldt, J. H. (1995) Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study Investigators, *Am J Clin Nutr 62*, 564-571.
83. Nikkari, T., Luukkainen, P., Pietinen, P., and Puska, P. (1995) Fatty acid composition of serum lipid fractions in relation to gender and quality of dietary fat, *Ann Med 27*, 491-498.
84. Vessby, B. (2003) Dietary fat, fatty acid composition in plasma and the metabolic syndrome, *Curr Opin Lipidol 14*, 15-19.
85. Klein-Platat, C., Drai, J., Oujaa, M., Schlienger, J. L., and Simon, C. (2005) Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents, *Am J Clin Nutr 82*, 1178-1184.
86. DeLany, J. P., Windhauser, M. M., Champagne, C. M., and Bray, G. A. (2000) Differential oxidation of individual dietary fatty acids in humans, *The American journal of clinical nutrition 72*, 905-911.

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See next page for low-fat control formulas.

(DIO) Formulas				
Product #	**D12451**		**D12492**	
	gm%	**kcal%**	**gm%**	**kcal%**
Protein	24	20	26	20
Carbohydrate	41	35	26	20
Fat	24	45	35	60
Total		100		100
kcal/gm	4.73		5.24	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	0	0
Maltodextrin 10	100	400	125	500
Sucrose	172.8	691	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Red Dye #40	0.05	0		
FD&C Blue Dye #1			0.05	0
Total	**858.15**	**4057**	**773.85**	**4057**

*Typical analysis of cholesterol in lard = 0.95 mg/gram.
D12451 -
Cholesterol (mg)/4057 kcal = 168.6
Cholesterol (mg)/kg = 196.5
D12492 -
Cholesterol (mg)/4057 kcal = 232.8
Cholesterol (mg)/kg = 300.8

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Product# S3282 - Mouse Diet, High Fat, Fat Calories (60%), 1/2" Soft Pellets, 5 kg/Box - Sterile

Proximate Profile

Protein	%	20.5
Fat	%	36.0
Fiber	%	0.0
Ash	%	3.5
Moisture	%	<10
Carbohydrate	%	35.7

Caloric Profile

Protein	kcal/gm	0.82
Fat	kcal/gm	3.24
Carbohydrate	kcal/gm	1.43
Total	**kcal/gm**	**5.49**

Amino Acids

Alanine	gm/kg	5.3
Arginine	gm/kg	7.3
Aspartic Acid	gm/kg	12.8
Cystine	gm/kg	0.6
Glutamic Acid	gm/kg	40.6
Glycine	gm/kg	4.9
Histidine	gm/kg	5.5
Isoleucine	gm/kg	11.0
Leucine	gm/kg	16.6
Lysine	gm/kg	14.8
Methionine	gm/kg	7.1
Phenylalanine	gm/kg	8.9
Proline	gm/kg	20.5
Serine	gm/kg	11.4
Threonine	gm/kg	8.7
Tryptophan	gm/kg	2.2
Tyrosine	gm/kg	11.4
Valine	gm/kg	13.0

Carbohydrates

Monosaccharides	gm/kg	1.3
Disaccharides	gm/kg	146
Polysaccharides	gm/kg	200

Fatty Acids

C18:2 Linoleic	gm/kg	36.6
C18:3 Linolenic	gm/kg	3.6
Total Saturated	gm/kg	141
Total Monounsaturated	gm/kg	162
Total Polyunsaturated	gm/kg	40.2

Minerals

Calcium	gm/kg	5.6
Chloride	gm/kg	0.86
Copper	mg/kg	3.6
Chromium	mg/kg	0.41
Fluoride	mg/kg	11.0
Iodine	mg/kg	0.31
Iron	mg/kg	40.8
Magnesium	gm/kg	0.49
Manganese	mg/kg	46.7
Phosphorus	gm/kg	5.8
Potassium	gm/kg	5.6
Selenium	mg/kg	0.21
Sodium	mg/kg	571
Sulfur	mg/kg	668
Zinc	mg/kg	21.6

Vitamins

Choline	mg/kg	1148
Folic Acid	mg/kg	0.75
Niacin	mg/kg	15.0
Pantothenic Acid	mg/kg	5.5
Pyridoxine	mg/kg	4.1
Riboflavin	mg/kg	2.3
Thiamin	mg/kg	3.0
Vitamin A	IU/kg	3162
Vitamin B_{12}	mcg/kg	40
Vitamin D_2	IU/kg	1000
Vitamin E	IU/kg	25.7
Vitamin K_3 (Menadione)	mg/kg	0.52

Ingredients

Lard, Casein, Maltodextrin, Sucrose, Mineral Mix, Vitamin Mix, DL-Methionine, Choline Chloride

These are typical amounts of nutrients calculated from available information. Actual assay results may vary.
For more information contact Jaime Lecker, Ph.D.
Phone: 800-996-9908 ext. 112 (U.S. and Canada)
908-996-2155 (International)
Email: jlecker@bio-serv.com.

Revised Date: 1/11

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One 8th Street, Suite 1, Frenchtown, NJ 08828 • Toll-Free: 800-996-9908 (U.S. & Canada)
Phone: 908-996-2155 • Fax: 908-996-4123 • Web: www.bio-serv.com

Supplemental 3. TD.88137—42%kcal HFD from Harlan Laboratories, Inc.

Teklad Custom Research Diet Data Sheet

TD.88137 Adjusted Calories Diet (42% from fat)

Formula	g/Kg
Casein	195.0
DL-Methionine	3.0
Sucrose	341.46
Corn Starch	150.0
Anhydrous Milkfat	210.0
Cholesterol	1.5
Cellulose	50.0
Mineral Mix, AIN-76 (170915)	35.0
Calcium Carbonate	4.0
Vitamin Mix, Teklad (40060)	10.0
Ethoxyquin, antioxidant	0.04

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
- Feed fresh diet at minimum one time per week (discard unused diet)
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Footnote

TD.88137 is often referred to as the Western Diet in the cardiovascular literatures. The overall level of fat and the saturated nature of the fat are representative of diets that are linked to risk of cardiovascular diseases in humans. The formula originated with researchers at Rockefeller University and is used primarily with genetically manipulated mouse models that are susceptible to atherosclerosis. The diet may also be useful in diet-induced obesity, diabetes, and metabolic syndrome models.

Related Diets

There are numerous modifications of TD.88137. Contact a nutritionist for more information about specific modifications, or to develop one that suits your needs.

Product Specific Information

- 1/2" Pellet or Powder (crumbly)
- Minimum order 3 Kg
- Irradiation available upon request

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (1and 2 Kg)

Teklad Diets are designed & manufactured for research purposes only.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	17.3	15.2
Carbohydrate	48.5	42.7
Fat	21.2	42.0

Kcal/g 4.5

Cholesterol [2] 0.2%

[1] Values are calculated from ingredient analysis or manufacturer data

[2] 0.15% added, 0.05% from fat source

International Inquiry

·*Outside U.S.A. or Canada*·

- **askanutritionist@harlan.com**

Speak With A Nutritionist

- **(800) 483·5523**
- **askanutritionist@harlan.com**

Harlan Laboratories · PO Box 44220 · Madison, WI 53744-4220
www.harlan.com

Place Your Order (U.S.A. & Canada)

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- **(800) 483·5523**
- **(608) 277·2066** *facsimile*
- **tekladinfo@harlan.com**

Helping you do research better

03/14/12 SDS

Supplemental 4. Van Heek DIO Series from TestDiet

TestDiet
Fresh from our lab to yours.

Typical Diet Series Spreadsheet

	Van Heek Series					
	10% kcal from fat 58Y2		45% kcal from fat 58V8		60% kcal from fat 58Y1	
	%	% kcal	%	% kcal	%	% kcal
Protein	17.3	18.3	21.3	18.3	23.6	18.3
Carb	67.5	10.2	41.2	45.7	25.9	60.9
Fat	4.3	71.4	23.6	35.5	34.9	20.1
Fiber	4.7		5.8		6.5	
kcal/g	3.78		4.65		5.16	
Ingredient						
Casein, vit free	18.956		23.306		25.845	
Dextrin	29.856		8.483		0	
Sucrose	33.174		20.136		8.891	
Maltodextrin	3.317		11.653		16.153	
Cellulose	4.739		5.827		6.461	
Soy oil	2.37		2.913		3.231	
Lard	1.896		20.684		31.66	
Potassium Citrate	1.564		1.923		2.132	
Mineral Mix	0.948		1.165		1.292	
Vitamin Mix	0.948		1.165		1.292	
Dicalcium Phosphate	1.232		1.515		1.68	
Calcium Carbonate	0.521		0.641		0.711	
L-cystine	0.284		0.35		0.388	
Choline Bitartrate	0.19		0.233		0.258	
Yellow Dye	0.006		0		0	
Red Dye	0		0.006		0	
Blue Dye	0		0		0.006	