Untargeted lipidomic analysis of plasma from high fat diet-induced obese rats using UHPLC-linear trap quadrupole-Orbitrap MS

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Abstract

High fat diet (HFD)-induced obesity is a primary risk factor for serious health problems. Although much research has been performed at the genomic level, lipidomic studies were limited. In this study, we aim to obtain the comprehensive profile of circulating plasma lipids, which are altered in rodent rat obesity by untargeted liquid chromatography-mass spectrometry. Rats were fed with HFD for 8 weeks have the increased body weight, liver and adipose tissue weight. The analysis results revealed that polyunsaturated fatty acids (PUFAs) and their corresponding phosphatidylcholine, phosphatidylinositol, and phosphatidylserine were significantly decreased in rats fed with HFD. In contrast, less unsaturated and ether type phosphatidylglycerols were increased. The triacylglycerides (TAGs) having saturated FA were increased in HFD condition, whereas TAGs having PUFA were decreased. The levels of many plasma lipids were altered, interestingly PUFAs derived lipids were negatively associated with obesity signifies the importance of PUFAs enriched diet to overwhelm obesity associated diseases.

Keywords: Plasma lipids, high fat diet, obesity, liquid chromatography, mass spectrometry, hierarchical cluster analysis.
Introduction

Over the past few decades, the incidence of overweight and obesity has dramatically increased across all genders and age groups and is closely linked with the rising prevalence of serious health problem\(^1\)-\(^3\). Obesity is known to be associated with the development of arthritis\(^4\), cardiovascular disease\(^5\), cancer\(^6\), nonalcoholic fatty liver disease\(^7\), and type 2 diabetes mellitus\(^8\). It is commonly known that individual genetic background and lifestyle factors such as excessive intake of fat and decreases in physical activity is the crucial factors in determining its manifestation\(^9,10\). Moreover, the molecular mechanisms connecting obesity to metabolic disorders are not well known. In United States itself >70 % of the adult population is considered as overweight\(^1\). Due to the increasing number of obese populations worldwide, there is a growing need for both clinically and experimentally to identify the blood-based biomarkers for diagnosis of obesity and associated disorders\(^11\). In this regard, several \textit{in vivo} models have been established to study the pathogenesis of this metabolic syndrome, and there are significant experimental evidences that shows impaired lipid metabolism plays a crucial role therein\(^12,13\). Many studies were conducted the analysis at genomic\(^14\) and proteomic level\(^15\), however comprehensive lipidomic studies were limited. To acquire the global lipid profile in biological samples, liquid chromatography-mass spectrometry (LC/MS) based analytical methods are preferable, due to the less requirement of sample, high sensitivity, large dynamic range and the ability to analyze multiple samples in a short duration. And recently developed MS-based lipidomic techniques show a high intricacy of the many circulating plasma lipidome\(^16\).

Although, several targeted approaches using gas-chromatography-mass spectrometry and LC/MS were reported to profile and quantify the lipids in plasma of obese mice\(^17\), rats\(^18\), macaque\(^18\) and human\(^19\), they are limited to a specific group or subgroup of lipid species. Therefore, the identification of more lipid metabolites related to obesity is necessary to further our understanding of obesity metabolism. In other words, untargeted lipidomics allows for the rapid identification and relative quantification of multiple lipid classes at a time. In this regard, in the present study an ultra-high-performance liquid chromatography (UHPLC)-linear trap quadrupole (LTQ)-Orbitrap mass spectrometry was applied to profile the total lipids in plasma of HFD-induced obese rats. Representative structures from each identified lipid classes were shown...
in Fig. 1 and their hierarchical cluster analysis was performed to identify the class specific biomarkers for obesity.

**Experimental**

**Chemicals**
The LC/MS grade isopropanol, methanol, and chloroform, were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). The mobile phase additive ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO). The internal standards such as, phosphatidylethanolamine (PE 15:0-15:0), phosphatidylinositol (PI 8:0-8:0), phosphatidylcholine (PC 13:0-13:0), lysophosphatidylcholine (LPC) 15:0, lysophosphatidylethanolamine (LPE) 13:0, and lysophosphatidylinositol (LPI) 13:0, were obtained from Avanti Polar Lipids (Alabaster, AL). Triacylglyceride (TAG) 11:0-11:0-11:0 and fatty acid 17:0 were obtained from Sigma-Aldrich (St. Louis, MO). TK-16 (Dextran) from Mastutani Chemical Industry Co., Ltd., (Hyogo, Japan), NZMP acid casein from Fonterra Co-operative Group Ltd. (Auckland, New Zealand), Sucrose from Nippon Beet Suagr Manufacturing Co., Ltd. (Tokyo, Japan), Soybean oil from J-Oil Mills, Inc., (Tokyo, Japan), Crystalline cellulose from Ceolus PH-102, Asahi Kasei Chemicals Corp., (Tokyo, Japan), AIN93G Mineral mix from MP Biomedicals (USA), AIN-93VX Vitamin mix from CLEA Japan Inc., (Tokyo, Japan), Choline bitartrate from Wako Pure Chemical Industries Ltd., (Osaka, Japan), Lard from Bell Shokuhin Co., Ltd., (Sapporo, Japan) and All other reagents of analytical grade were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) unless otherwise specified.

**Plasma samples and extraction of total lipids**
Male 3-week-old WKAH/HkmSlc rats were housed in an air-conditioned room at 22 ± 2°C with 55 ± 5% humidity, and the light period was set from 8:00 AM to 20:00 PM. The rats were housed individually in standard wire-bottomed cages and allowed ad libitum access to food and water. During the 2-week acclimation period, the rats were fed a normal diet based on the AIN-93G formulation (Table 1). The fatty acid composition of the diet analyzed by LC/MS were given in Table 2. For long-term experiments, the acclimatized rats (n = 13) were divided into two groups and were fed either the normal diet (ND) (n = 6) or high fat diet (HFD) (n = 7) for 8 weeks. To prepare the HFD about 230 g of lard was added to the normal diet per kg in place of the dextrin.
The same animal samples were used for another part of the study, in finding the association between bile acid and hepatic steatosis (Hori S et al., unpublished data). Portal and aortic blood were collected from rats under anesthesia using sodium pentobarbital (Somnopentyl, 50 mg/kg body weight, Kyoritsu Seiyaku Corporation, Tokyo, Japan) at week 8 without food deprivation. An anticoagulant agent (heparin, 50 U/ml blood, Nacalai Tesque, inc., Kyoto, Japan) and a protease inhibitor (aprotinin, 500 KIU/ml blood, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were added to blood samples for preparation of plasma. Thereafter, the rats were killed by exsanguination. Experimental procedures were in accordance with Japanese laws on the use of animals for experimentation and were approved by the Hokkaido University Ethics Review Committee on Animal Experiments with approval number 17-0050.

Total lipids were extracted from the rat plasma the method reported by Folch et al. In brief, 50 µl of each plasma samples were extracted with 200 µl of ice-cold methanol having pre-mixed internal standards and were vortexed for 30 sec at 3500 rpm. Followed by 200 µl of chloroform (vortex, 5 min) and 50 µl of milli-Q (vortex, 30 sec) were added. The extracts were centrifuged at 4°C, 252 g for 10 min and the residue was re-extracted with 200 µl of chloroform. The combined extracts were dried under vacuum and redissolved in 100 µl of methanol and were transferred to the LC/MS vials. The lipids from liver and epididymal adipose tissue were extracted by the same protocol as described above after homogenization with methanol using beads for 15 sec (Bead Mill 4, Fisher Scientific).

**Untargeted lipidomic analysis on UHPLC-LTQ-Orbitrap MS**

Chromatographic separation was performed by using a Shimadzu Prominance UHPLC system (Shimadzu Corp., Kyoto, Japan) with a binary solvent delivery system and a standard autosampler. An Atlantic T3 C18 column (2.1 × 150 mm, 3 µm, Waters, Milford, MA) was utilized for separation, with a flow rate of 200 µL/min. The mobile phase consists of (A) Milli-Q (5 mM CH₃COONH₄), (B) Isopropanol and (C) Methanol. A linear gradient was optimized as follows, Negative mode: 0-1 min, 30% B and 35% C, 1-14 min, 80% B and 10% C, 14-27 min, 85% B and 10% C, 27-28 min, 30% B and 35% C, 28-30 min, 30% B and 35% C. Positive mode: 0-1 min, 6% B and 90% C, 1-10 min, 83% B and 15% C, 10-19 min, 83% B and 15% C, 19-19.5 min, 6% B and 90% C, 19.5-22 min, 6% B and 90% C. The injection volume was 10 µl and the column
temperature was maintained at 40 °C for each run.

The untargeted lipidomic analyses were conducted on a Shimadzu Prominence UHPLC system (Shimadzu Corp., Kyoto, Japan) connected to an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific Inc.). Both ESI-positive and ESI-negative ionization mode acquisitions have the identical MS conditions: Capillary voltage (3.0 kV), nitrogen-sheath gas flow (50 units,) and nitrogen-auxiliary gas (5 units). The MS1 data were acquired in Fourier Transform mode with a resolving power of 60,000 and a scan speed of 2 Hz. The tandem mass data were obtained using collision-induced dissociation in ion-trap mode and data-dependent acquisition with MS2 and MS3 scans at a collision energy 35 V and 45 V respectively. MS1 scan range were set at m/z 150–1700 and was m/z 250–1600 for positive and negative modes, respectively. The alignment, peak extraction, and extracted ion chromatogram (EIC) peak area integration and identification of lipid molecules from the raw data was performed by the MS DIAL software version 3.9 (prime.psc.riken.jp). The lipids were relatively quantified by choosing a suitable internal standard for each class. Data were plotted in Excel 2016 and multivariate hierarchical cluster analysis was performed by ClustVis web-based software tool (biit.cs.ut.ee). Student’s t test was conducted and p<0.05 was considered statistically significant. Data were presented as the mean ± standard deviation.

Results

Body weight, adipose and liver tissue weight

The seven rats on a high fat diet (HFD) had a body weight of 348.35 (312.8–392.6) g, which was considerably higher compared to the six mice on a normal diet (ND) with 316.6 (308.2–329) g (Fig. 2A). Food intake by weight in the HFD group was markedly lower (p<0.05) than the ND group given by 847.9±14.4, 721.6±57 respectively. The total tissue weight of liver and adipose tissue was significantly elevated in the rats fed with fat (Fig. 2B).

Untargeted analysis of the rat plasma lipids and statistical analysis

The high-resolution MS data was acquired by an LTQ Orbitrap mass spectrometer in both positive and negative ionization modes. The total ion chromatogram (TIC) of the lipid profile of rat plasma acquired in negative mode (Fig. 2C) mainly shows the presence of free fatty acyls (FAs),
lysophospholipids such as lysophosphatidylserine (LPS), lysophosphatidylethanolamine (LPE), and lysophosphatidyl choline (LPC). Phospholipids include phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylserine (PG), and phosphatidylethanolamine (PE). In the data acquired in positive mode (Fig. 2D), TIC shows the abundance of triacylglycerols (TAG) followed by diacylglycerols (DAG), acyl alkyl glycerols (AAG), sphingomyelin (SM) and cholesterol esters (CE).

The principal component analysis (PCA) results of 94 identified lipid metabolites are shown in Fig. 3A. The plots of lipidomic profile show a clear separation between HFD and ND-groups, indicating the distinct effect of diet on the plasma lipid profile. On the other hand, volcano plot (Fig. 3B) shows the significantly altered metabolites between the two groups. In particular, several saturated fatty acid (SFA) or monounsaturated fatty acid (MUFA) containing TAGs such as TAG 52:0, TAG 45:1, TAG 54:1 and PIs, PI 36:1, PI 34:1 were increased under HFD and positively associated with obesity. In contrast, PCs such as PC 32:1, PC 34:4, PC 32:4, PC:36:5, polyunsaturated fatty acid (PUFA) containing TAGs, TAG 52:6, TAG 54:7, FA 20:5 and PI 36:4 were significantly decreased under HFD and are negatively associated with obesity.

Concentrations and hierarchical cluster analysis of FA and TAG
The concentrations of free FAs identified in rat plasma is shown in Fig. 4A. The PUFAs such as FA 18:3, FA 20:5, FA 22:5 and MUFA s, FA 24:1 and FA 26:1 are significantly decreased in the rats fed with HFD. Hierarchical cluster analysis (HCA) shows the distinct clustering between the ND and HFD groups. The red color indicates the positive association with high levels of FAs, whereas blue color indicates the negative association with their low levels. The significantly altered TAGs (p<0.05) were plotted in Fig. 4B. TAGs with poly PUFA shown to be decreased in the rats fed with high fat, whereas TAGs having MUFA or SFA were increased drastically with the HFD. The HCA reveals the well-defined clustering between the ND and HFD groups. The red color indicates the positive association with high levels of TAGs, whereas blue color indicates the negative association with their low levels. As shown their SFA and MUFA containing TAGs were diminished in rats under ND conversely PUFA TAGs were increased.

Concentrations and hierarchical cluster analysis of PC, LPC, and PI
The concentrations of PC, LPC, and PI identified, and their HCA results were shown in Fig. 5A
and 5B respectively. Among, PCs, PC 32:1, PC 31:2, PC 16:0-18:3 and FA 20:4 containing PC 14:0-20:4, PC 16:0-20:4, PC 16:1-20:4 were significantly decreased in HFD group. However, an ethereal type PC 15:1e-15:1 is drastically decreased under HFD. Also, LPCs were unchanged to the diet. PI molecular species show contradictory results, PIs having FA 18:1, PI 16:0-18:1, PI 18:0-18:1, shows a positive association with obesity, whereas, PIs having FAs 20:4 and 18:3, PI 16:0-20:4, PI 18:1-18:3 and PI 16:0-16:1 were negatively associated. Also, an oxidized PI namely, OxPI 18:0-20:4+1O is identified and is decreased significantly in the plasma of rats fed with HFD.

Concentrations and hierarchical cluster analysis of PS, PG, PE, LPE and other altered metabolites

The concentrations PS, PG, PE, and LPE molecular species identified and their HCA results were shown in Fig. 6A and 6B respectively. And the normalized signal intensities of the other unquantified lipid class were shown in Fig. 6C. Among the identified PS, PS 14:0-24:2, PS 20:0-20:4, and PS 18:0-22:4 were considerably reduced under HFD and shows a negative association with obesity. Whereas PGs such as PG 13:0-20:0, PG 21:0-13:1, and PG16:4e-16:4 are positively associated with the HFD. PE and LPE molecular species were unaltered by the diet. Interestingly, the cholesterol ester (CE 22:6) (Fig. 6C) was greater than two folds higher in ND group compare to HFD group. CE having FA 22:6 could be a docosahexaenoic acid (DHA), an essential ω-3 FA with diverse biological benefits. As per our knowledge CE 22:6 is identified for the first time in plasma of HFD rats. Another new class of lipids such as alkyl acyl glycerol’s (AAG), AAG 18:5e-24:1 and a sphingomyelin, SM d18:1-16:0 were also significantly increased in the plasma of rats fed with HFD.

Concentrations of free fatty acids in Epididymal adipose tissue and Liver

In the epididymal adipose tissue, concentrations of SFA and MUFA were not significantly altered between the ND and HFD groups except FA 18:0, which shows positive association with obesity, whereas PUFAs such as FA 22:2, FA 22:4, and FA 22:5 were significantly decreased under HFD shown Fig. 7A. In the liver (Fig. 7B), SFA and MUFA such as FA 18:0, FA 24:0, and FA 20:1 shows positive association with obesity whereas PUFA such as FA 22:5 is unaltered by the diet. Surprisingly FA 22:4 shows the increasing trend in the rats fed with HFD.
Discussion

Diet is a prominent factor to control various metabolic diseases. Particularly, high fat diet and physical idleness cause overweight or obesity, which is a foremost factor in the pathogenesis of metabolic disorders. The concurrence of obesity, insulin resistance, hypertension and dyslipidemia is generally referred to as the ‘metabolic syndrome’. This condition affects about 20–40% of the population in the industrialized nations, and its prevalence is expected to rise further in the next decades. Also, HFD induced obesity is lead to a harmful activation of the immune system, with the increasing risk of heart disease, diabetes, and cancer. Lipids are a very distinct group of molecules concerning their structure and function and it is known that impaired lipid metabolism plays a significant role in obesity related disorders. In clinical chemistry, blood-based lipid biomarkers for obesity are crucial for the diagnosis of the disease. Moreover advanced LC/MS techniques were widely applied for lipid biomarker discovery in various models. In the present study, a rodent rat model was used, and rats were fed with ND and HFD (fat source: HF-L-lard) for 8 weeks. Then, the plasma samples were collected and extracted for total lipids by Folch method and comprehensive lipidomics studies was performed by using LTQ-MS coupled to UHPLC.

The body weight measurement showed a significant difference between the ND and HFD groups soon after the 4 weeks of diet (Fig 2A). Rats fed with HFD have significantly higher body mass, liver and adipose tissue weights (Fig 2B) but lesser food intake compared to rats on ND. These are the key marker of the model and the results were consistent with previous studies. The TIC profile of rat plasma in negative and positive mode (Fig 2C, 2D) was clearly illustrate the acquired classes of lipid metabolites in each mode. In positive mode measurement TAGs are abundant whereas in negative mode fatty acids and phospholipids. The statistical analysis of all identified lipid metabolites in both the modes was performed. The Fig. 3A showed the PCA analysis results of 94 identified lipid metabolites in plasma samples of ND and HFD groups. The data show that, both the groups have significantly different lipid levels, indicated by the well-defined separation between ND and HFD groups in the PCA plot. On the other hand, the volcano plot (Fig. 3B) of –log10 (p-value) versus log2 (ND/HFD) showed the significantly altered lipid metabolites.
SFA and MUFA containing TAGs such as TAG 52:0, TAG 45:1, TAG 54:1 and PIs, PI 36:1, PI 34:1 were significantly increased under HFD and are positively associated with obesity. The relative TAG composition varies with the obesity model, for example, in a diet-induced weight loss model, weight reduction in mice was associated with decreased levels of TAG containing SFA and are consistent with our results. Whereas in plasma samples of rats fed with HFD for 3 weeks shown decreased SFA and MUFA TAGs levels while developing obesity. Earlier studies show MUFA PIs (PI 34:1, PI 36:1) were significantly higher in serum of obese mice, and the results are coherent with our results. In contrast, several MUFA and PUFA containing PCs such as PC 32:1, PC 34:4, PC 32:4, PC:36:5, polyunsaturated fatty acid containing TAGs; TAG 52:6, TAG 54:7, an unsaturated fatty acid FA 20:5 and PI 36:4 were significantly decreased under HFD and negatively associated with obesity. These results are consistent with previous reports which shows decreased SFA and MUFA PCs levels in the plasma and liver samples of obese mice. The increased PUFA TAG with HFD were observed in rats having developing obesity, whereas decreased PUFA TAG were seen in plasma samples of human under HFD. Decreased PUFA TAG under high fat diet in our study is coherent with human studies. PI 36:4 was significantly decreased in plasma of mice under HFD and is identical with our results.

The PUFAs such as FA18:3, FA 20:5, FA 22:5 and MUFAs, FA 24:1 and FA 26:1 were negatively associated with obesity (Fig. 4A). And the hierarchical cluster analysis also illustrates the distinct clustering between the ND and HFD groups with significant negative association of PUFAs and MUFAs with obesity. In a previous human study under HFD, the plasma ω-3 PUFAs was decreased, but increased in low fat diet and also in the liver samples of obese mice shows the same trend with decreased ω-3 PUFAs. In contrast, PUFAs was increased under HFD in obesity prone rats. Our experimental results showed increased PUFA, FA 22:4 level in liver but decreased at epididymal adipose tissue of rats fed with HFD. In addition, other PUFAs such as FA 22:2 and FA 22:5 were also decreased in adipose tissue, indicating strong correlation with the PUFAs in plasma and suggesting PUFAs as potential biomarkers for obesity. The increased PUFA, FA 22:4 in liver is consistent with previous study with obese prone rats. Moreover, the high fat diet itself as high levels of SFA and MUFA compare to normal diet as demonstrated in Table 2, indicating the direct effect of diet on the plasma and tissue lipid profile. The outcomes of our
analysis are highly interrelated with human and mouse studies and demonstrates their significance in diet to control obesity-associated disorders.

The total TAG levels in both liver and plasma largely increased in rodent models and human obesity. Nevertheless, the relative composition of TAG varied with the type of obesity model. Most importantly, the effect of unsaturation is clearly observed in several lipid classes including TAG, PC, PI and PE. TAGs with PUFAs were decreased and TAGs with SFAs were increased under HFD condition (Fig. 4B). Hierarchical cluster analysis also shows the good clustering between the ND and HFD groups with a significant negative association of PUFA TAGs with obesity. These results were consistent with several other models, as for examples SFA containing TAGs were decreased in diet induced weight loss model and increased in serum of individuals with nonalcoholic fatty-liver disease (NAFLD). In contrast, PUFA containing TAGs were decreased by 40% in ob/ob mice and negatively associated with NAFLD. Phosphatidylcholine’s such as PC 32:1, PC 31:2, PC 15:1e-15:1 and FA 20:4, FA 18:3 containing PC, PI and oxidized PI were significantly decreased under HFD but LPCs were unchanged to the diet (Fig. 5A,5B). These are the results consistent with previous reports on the liver tissue lipid profile in NALFD model and serum of obese mice, where the several PUFA PC, PIs were negatively associated with obesity. However, some of the PIs having FA 18:1 shows significant increase in our study and are similar to the results obtained in serum lipid profile of obese mice where MUFA PIs such as PI 34:1, PI 36:1 were significantly higher in HFD. To our knowledge OxPIs have not measured in rodent models of obesity so far. The OxPI 18:0-20:4+1O is decreased significantly in the plasma of rats fed with HFD, this could be due to the decreased levels of PIs having FA 20:4.

The other phospholipids such as PS, PG, PE, and LPE profile were described in Fig. 6A and 6B, along with their HCA results. There is a significant clustering between ND and HFD groups for PS and PG. But the levels of PE and LPE were not so significant and unaltered in our study. Several MUFA and PUFA derived PS were determined for the first time in rat plasma and are negatively associated with obesity. These results are consistent with liver tissue PS profiles of obese mice. Moreover, it is known that the nutritional supply of PS can protect brain against oxidative stress in HFD rats, signifies the potential need of PS enriched diet to overcome obesity.
related disorders. Additionally, in this study, we measured several PG for the first time in rat plasma and which are positively associated with the obesity and shows a completely different trend from other type of phospholipids. These results are also consistent with a previous report, where PS levels were positively allied with obesity on plasma lipidomic analysis in a rodent model of obesity by HFD\textsuperscript{35}. Among, other detected molecular species, cholesterol ester CE 22:6 (Fig. 6C) is determined for the first time in rat plasma, which shows a significant difference between the ND and HFD groups. And negatively associated with obesity, which is consistent with previous studies where several ω-3 FA derived CE were decreased under HFD\textsuperscript{17,19,30}. An ether type lipid AAG 18:5e-24:1 was also determined in rat plasma for first time and is significantly elevated in HFD group. Previous study shows the increased level of sphingomyelin molecular species under HFD\textsuperscript{17} and is consistent with our data, SM d118:1-16:0 showed a positive relation with obesity.

In conclusion, the plasma of ND and HFD rat models were compared and characterized using lipid profiles to provide new intuitions to lipid metabolism in obesity-associated disorders. Substantial differences in the relative concentration of lipid species were observed between ND and HFD rats. PUFAs and its derived plasma lipids, were significantly decreased and negatively associated with obesity demonstrating the importance of PUFAs enriched diet to devastate obesity-associated diseases. Hence, decreased levels of PUFAs in plasma could be the novel biomarkers of obesity. This study disclosed that UHPLC/LTQMS-based untargeted lipid profiling is an effective analytical tool for the depiction of global lipid profile in ND and HFD fed rats.

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**Conflict of Interest**

The authors declare no conflict of interest.
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Tables:

**Table 1.** Composition of normal diet (ND) and high fat diet (HFD) rats group

| Diet Composition | g/Kg of Diet | Normal diet | High fat diet |
|------------------|-------------|------------|--------------|
| Dextran          | 529.5       | 299.5      |              |
| Casein           | 200.0       | 200.0      |              |
| Sucrose          | 100.0       | 100.0      |              |
| Soybean oil      | 70.0        | 70.0       |              |
| Cellulose        | 50.0        | 50.0       |              |
| Mineral Mixture  | 35.0        | 35.0       |              |
| Vitamin mixture  | 10.0        | 10.0       |              |
| Choline bitartrate| 2.5         | 2.5        |              |
| L-Cystine        | 3.0         | 3.0        |              |
| Lard             | -           | 230.0      |              |

**Table 2.** Amount of free fatty acids in normal diet and high fat diet samples

| Fatty acids | Normal diet (Conc. in pmoles / mg ±SD) | High fat diet (Conc. in pmoles / mg ±SD) |
|------------|---------------------------------------|----------------------------------------|
| FA 16:0    | 1.82 ± 0.17                           | 2.23 ± 0.04                           |
| FA 18:0    | 2.25 ± 0.23                           | 2.94 ± 0.14                           |
| FA 18:1    | 1.48 ± 0.22                           | 2.12 ± 0.05                           |
| FA 18:2    | 1.10 ± 0.12                           | 1.38 ± 0.07                           |
| FA 19:1    | 0.17 ± 0.04                           | 0.18 ± 0.007                          |
| FA 20:2    | 0.01 ± 0.001                          | 0.04 ± 0.002                          |
| FA 22:1    | 0.02 ± 0.003                          | 0.05 ± 0.001                          |
| FA 23:0    | 0.07 ± 0.01                           | 0.05 ± 0.007                          |
| FA 24:0    | 0.32 ± 0.11                           | 0.24 ± 0.018                          |
Figure Captions

Figure 1. Representative structures of the lipid metabolites measured in the study. (FA: Fatty acids, TAG: Triacylglycerol, DAG: Diacylglycerol, AAG: Alkylacylglycerol, CE: Cholesterol ester, PS: Phosphatidylserine, PI: Phosphatidylinositol, PC: Phosphatidylcholine, PG: Phosphatidylglycerol, SM: Sphingomyelin)

Figure 2. Growth parameters of rats under normal diet (ND) and High fat diet (HFD): A. Change in body weight B. Changes in the weight of liver and adipose tissue. [ND(n=6), HFD (n=7), the data shown as average ±SD] and plasma lipidomic profile of rats by untargeted analysis: C. Total ion chromatogram (TIC) acquired in negative ionization mode B. TIC acquired in positive ionization mode.

Figure 3 A. *Principal Component Analysis (PCA) plots of lipidomic profile showing clear separation between HFD-group and ND-diet group. B. Volcano plot representation of significantly altered lipid metabolites between ND and HFD groups. The red dots indicate lipids which are significantly decreased, and blue dots represent those increased in HFD group (**p<0.05). *Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y-axis show principal component 1 and principal component 2 that explain 51.8% and 16.2% of the total variance, respectively. N = 13 data points.

Figure 4. Plasma lipidomics profile of rats under normal diet (ND) and High fat diet (HFD) and their hierarchical cluster analysis A. Fatty Acyls (FAs) B. Triacylglycerols (TAGs) (*Statistically significant TAGs were shown with p<0.05)

Figure 5. Plasma lipidomics profile of rats under normal diet (ND) and High fat diet (HFD) and their hierarchical cluster analysis A. Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) B. Phosphatidylinositol (PI) (**p<0.05)

Figure 6. Plasma lipidomics profile of rats under normal diet (ND) and High fat diet (HFD) A. Phosphatidylserine (PC), Phosphatidylglycerol (PG) Phosphatidylethanolamine (PE) and their hierarchical cluster analysis B. Lysophosphatidylethanolamine (LPE) C. Normalized signal intensities of Cholesterol esters (CE), Diacylglycerols (DAG), Sphingomyelin (SM). (**p<0.05, **p<0.1)
Figure 7. Concentrations of free fatty acids of rats under normal diet (ND) and High fat diet (HFD) A. Epididymal adipose tissue, B. Liver

Figure 1:

Figure 2:
Figure 3:

A. PCA Analysis

B. Volcano Plot

Figure 4:

A. FA

B. TAG*
Figure 5:

A. PC and LPC

B. PI

Figure 6:

A. PS PG and PE

B. LPE

C.
Figure 7:

A. Epididymal adipose tissue

B. Liver

Graphical Index

Rodent model

Normal Diet
High Fat Diet
Untargeted Lipidomics

Data analysis

Biomarker discovery

Neg
Pos