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LIPID METABOLISM IN THE LIVER, ADIPOSE, AND MUSCLE WITH
GLUCOCORTICOIDS AND HOW THESE ORGANS CAN REGULATE EACH OTHER

By

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A Thesis

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Abstract

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Lipid metabolism in the liver, adipose, and muscle with glucocorticoids and how these organs can regulate each other.

Major Professor: Melissa, Puppa, PhD

Background: The present Western diet is characterized by high n-6 PUFA and deficient in n-3 PUFA.

Due to the structural difference of composing fatty acid, Omega-3 PUFAs have beneficial effects while omega-6 PUFAs elicit adverse effects on lipid metabolism, building the foundation of metabolic

syndrome and various diseases. *Objective:* Determine whether fat composition in an HFD affects GC-induced alterations in lipid handling by the liver, adipose tissue, and skeletal muscle. *Methods:* Male

wild-type C57BL/6 mice were randomized into two groups: n-6 (45% fat 177.5 g lard) and n-3 (45% fat 177.5 g Menhaden oil). After 4 weeks on their diets, groups were divided to receive either daily

injections of dexamethasone (3 mg/kg/day) or sterile PBS for 1 week while continuing diets. *Results:*

omega-3 HFD diet ameliorate adipocyte hypertrophy and hepatic fatty accumulation by involving associated lipid metabolism markers (CD36 and FABP). *Conclusion:* the present study's result

demonstrated that the change of fat composition in HFD could beneficially alter the fatty acid accumulation, adipocyte size, and associated lipid metabolism markers

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List of Abbreviations

AA – arachidonic acid
ALA – alpha-linolenic acid
ATGL – adipose triglyceride lipase
CD36 – cluster of differentiation 36
Crat – carnitine acyltransferase
DAG – diacylglycerol
DHA – docosahexaenoic acid
DNL – de novo lipogenesis
EPA – eicosapentaenoic acid
ER – endoplasmic reticulum
eWAT – epididymal white adipose tissue
FA – fatty acid
FASN – fatty acid synthase
FABPs – fatty acid-binding proteins
FFA – free fatty acid
GC – glucocorticoid
GLUT4 – glucose transporter type 4
HFD – high-fat diet
HSL – hormone-sensitive lipase
H&E – hematoxylin, and eosin
IGF – insulin-like growth factor
IMCL – intracellular lipid
LA – linoleic acid
LD – lipid droplet
MGL – monoglyceride lipase
MUFA – monounsaturated fatty acid
TG – triglyceride

PDH – pyruvate dehydrogenase

PUFA – polyunsaturated fatty acid

SFA – saturated fatty acid

UPR – unfolded protein response

VAT – visceral adipose tissue

WAT – white adipose tissue

11 β -HSD1 - 11 β -hydroxysteroid dehydrogenase type1

1. Introduction

In recent decades, the prevalence of the Western diet has grown and dramatically increased obesity, which is a serious and growing health problem in the world (1). The present "Western" diet or high-fat diet (HFD) is characterized by high levels of n-6 polyunsaturated fatty acid (PUFA) and deficient in n-3 PUFA (20:1 n-6/n-3), while the ideal ratio of n-6/n-3 is 2:1 (2). Since signaling molecules generated from n-6 PUFA exhibit pro-inflammatory properties (3), the accumulation of n-6 PUFAs can lead to the lipid metabolism disorder and metabolic syndrome (4), building the foundation of various diseases such as coronary heart disease, stroke, diabetes, and some types of cancer (5, 6). Therefore, what types of dietary fat we consume are a vital factor influencing whole-body metabolism as much as how many fats we consume.

Lipid is another word for fat and is essential for living cells because it forms fundamental structures in cells and tissues (7). In the postprandial state, once lipids are used, the rest are stored mainly in white adipose tissue (WAT) and the liver in the form of triglyceride (TG) (8, 9). In chronic western diet conditions, TG accumulation exceeds the storage limitation, which leads to hypertrophy and dysfunction in WAT and liver cells due to the characteristic of western diet composition with high levels of n-6 PUFA (Figure 7) (8, 9). The inflammation activity caused by the excessive TG accumulation and HFD rich in n-6 PUFA is involved in various cell internal system dysfunction such as macrophage infiltration, mitochondria, and transcription mRNA expression (Figure 8) (10). These changes disturb the insulin and glucose uptake, which accelerate lipolysis and fatty acid spillage into circulation. Therefore, the glucose and insulin that cannot get in the cell and move around in circulation build the foundation for the development of metabolic syndromes (11).

Glucocorticoid (GC) is a steroid hormone and one of the most widely prescribed therapies for treating numerous inflammatory diseases and multiple cancer types (12). GC

regulates various physiologic processes for life and exerts anti-inflammatory and immunosuppressive actions (13). However, GC's therapeutic benefits are concurrent with the deleterious side effects of high dose and chronic treatment, including lipid metabolism disruption and metabolic syndromes (14). Anna et al. found that a combination of HFD with GC resulted in increased hepatic lipid, collagen content, and hepatic steatosis than GC and HFD alone (15). These findings clarified that the chronic GC could contribute to the development of lipid metabolism dysfunction and metabolic syndrome factors, which resemble the phenotype associated with HFD. The effects are more aggravated when GCs are combined with HFD.

In contrast with n-6 PUFA, the signaling molecules generated from n-3 PUFA exhibit anti-inflammatory properties, preventing and reversing downregulation by lipid metabolism disorder adiposity, inflammatory cytokines, and insulin-glucose homeostasis (16). Therefore, n-3 PUFA has become one of the dietary interventions studied actively to improve lipid metabolism. A recent study by Sakamuri et al. demonstrated that a low n-6: n-3 PUFA ratio prevented high fructose-induced dyslipidemia, hepatic oxidative stress, and inflammation with the reduction of adiposity and circulatory TGs (17). Besides, Hill et al. found that the mice fed HFD-n3 high with GC showed decreased epididymal white adipose tissue (eWAT) weight, adipose size, and glucose concentration compared to the mice fed HFD-n6 high with GC (18). In light of these findings, it is becoming clear that high n-3 PUFAs intervention positively affects lipid metabolism dysfunction induced by GCs and HFD.

Hypertrophied WAT and fatty liver induced by the chronic HFD state release excessive lipid into circulation (19). The released lipid flux is deposited into other organs such as skeletal muscle that is not well-equipped to store lipids, causing lipotoxic stress and cell dysfunction, called lipotoxicity (19). The deposited fatty acid in skeletal muscle activates serine/threonine

kinases that impair the insulin receptor's ability to stimulate downstream pathways, decreasing the translocation of glucose transporter type 4 (GLUT4) and, therefore, reducing glucose uptake into skeletal muscle cells (20). Skeletal muscle is involved in the clearance of 25% of circulating glucose in a basal, fasting state and up to nearly 70–85% in a postprandial state (21). Therefore, the increased lipid deposition in skeletal muscle can be the main contributor to hyperglycemia in circulation (7), promoting lipid metabolism dysfunction in adipose tissue and the liver (14). However, the research on HFD composition and lipid metabolism induced by HFD with GC in skeletal muscle is limited. Therefore, the purpose of this study was to determine whether fat composition in an HFD affects GC-induced alterations in lipid handling by the liver, adipose tissue, and skeletal muscle.

Omega-3 and 6 Polyunsaturated Fatty Acid

Fatty acid (FA) species are classified by their varying degrees of saturation into 1 of 3 major categories: saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). PUFA is additionally classified into omega-3 and omega-6 groups, based on the first double bond from the fatty acid's methyl end. While the human body cannot synthesize omega-3 and omega-6 PUFA, it does have the capability to further metabolize these FAs through stages of elongation and desaturation (22) (Figure 9).

There are three types of omega-3 PUFAs: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA). EPA and DHA are primarily found in marine-based sources such as fish and krill, while ALA is typically found in plant-based sources such as flaxseed. These long-chain fatty acids act as signaling molecules and regulate enzyme activity, transcription of genes, and membrane fluidity as cell membranes' structural components (23). Besides, they regulate inflammatory and immune responses, with ALA having a pro-inflammatory effect and EPA and DHA exerting anti-inflammatory responses (24). These effects

of n-3 PUFA are associated with PPARs or GPR 120 and transcription factors such as NF- κ B. Additionally, n-3 PUFA was shown to counteract the high-fat-diet-induced expression of pro-inflammatory cytokine-like IL-6, MCP1, TNF α , and TLR4 (17). Apart from those, the effects of n-3 PUFAs have been demonstrated through many pathways such as macrophage infiltration, transcription mRNA, and insulin signaling. However, the conversion of ALA to EPA and DHA is comparatively limited in humans (25), with reports explaining the transformation from ALA to EPA and DHA is less than 10%. Therefore, increasing intake of marine fish and oils and novel foods with long-chain n-3 PUFA is recommended to obtain these fats directly from the diet.

The Western diet contains excessive saturated and trans fatty acids and has too few omega-3 PUFAs than omega-6 PUFAs (2). The n-6 fatty acids are the predominant PUFA in western diets, as they are widely distributed in most plant oils (e.g., corn, safflower, sunflower), margarine, and animal fat. There are two types of omega-6 PUFAs: arachidonic acid (AA) and linoleic acid (LA). Dietary LA has been observed to suppress the conversion of ALA to DHA and change into AA, which leads to pro-inflammatory actions (3). Since pro-inflammation is a central contributor to metabolic syndrome, the intake of these diets rich in omega-6 has been associated with an increase in various disease risks by involving in metabolic function such as glucose-insulin homeostasis, adipose tissue properties, and cytokines secretion (2, 14, 26). Therefore, the current body of research on the effect of a high-fat diet supports the notion that the absolute amount of fat may be less critical than is the type of fat when it comes to the risk of many diseases induced by the western diet.

Taken together, the structural dissimilarities of these FAs bring about functional differences such as their actions on inflammation and metabolism even though both the n-6 and

n-3 PUFA are essential in the human diet. Therefore, well-balanced dietary fat consumption is necessary to prevent the development of the metabolic disorder.

Lipid Metabolism in White Adipose Tissue, Liver and Skeletal Muscle

Lipid is another word for fat, including many different cells and organic substances insoluble in water. Lipid can be used for energy, depending on the metabolic needs of organisms. Also, lipids are essential for living cells because it is involved in forming fundamental structures in cells and tissues, regulating several homeostatic processes within and outside of cells, including organelle homeostasis, immune function, inter-organ communication, energy metabolism, and cell survival (7). In the postprandial state, lipids are stored mainly in adipose tissue and the liver. Besides, lipids can be stored within muscle cells and, to some extent, in the blood. TG constitutes the vast majority of the lipid consumed in the diet and stored within the body in the form of storage (8). In chronic HFD and decreased physical activity conditions, lipid accumulation exceeds the lipid storage limit in adipose and other organs. The over-accumulation of lipids contributes to the beginning of organelle dysfunction, chronic inflammation, disturbance in the endocrine and metabolic system (19). Therefore, the combination with an inadequate capacity to store lipids and HFD can become critical components of pathophysiological cascades, generating the basis for various diseases associated with metabolic syndrome.

White Adipose Tissue

WAT is a specialized storage organ for TG, packaged into a large lipid droplet (LD). The majority of dietary fatty acids were initially stored in WAT depots before their hydrolysis and release back into the circulation, the balance of which is critical to maintaining healthy energy homeostasis. In addition to its lipid-storing capacity, WAT has been described as an essential endocrine organ controlling the systemic handling of energy substrates (7). We now appreciate

that WAT secretes adipokines such as leptin and adiponectin. Many of these adipokines help to coordinate the systemic metabolic state.

During times of excess nutrient availability, WAT can primarily store excess lipids in one of two ways. First is through differentiation of resident tissue precursors and creating new adipocytes to store additional lipids into LDs (Figure 10). It is called "healthy obesity" because the new adipocyte can maintain the systemic metabolic state, levels of insulin-sensitizing, anti-inflammatory hormone adiponectin, and store excessive lipids with proper vascularization (8). As such, the new adipocytes can store overflowed nutrients safely and without adipocyte's metabolic disorder. The second is through the enlargement of existing adipocytes (Figure 11). Chronic HFD requires enlargement of LDs and adipocyte hypertrophy, culminating in larger fat depots and body weight (27). So, hypertrophy means that adipose tissue's capacity is becoming overwhelmed, causing stress, injury, and abnormalities in function. The hypertrophied adipocyte needs enough vascularization to maintain its massively expanded size. However, most cases do not satisfy the condition if HFD continues with adverse metabolic conditions (27). The insufficient vascularization induces an adipose tissue hypoxic response, which increases the expression of pro-fibrotic genes and leads to tissue fibrosis (11). The hypertrophied adipocyte becomes dysfunctional and induces lipolysis, breaking down TG into glycerol and fatty acids. The increased fatty acid in adipocytes contributes to the degradation of tissue function itself and the delivery of fatty acids to other tissue such as the liver and muscle, causing toxic lipid deposition (19).

As mentioned earlier, adipose tissue or lipid is responsible for several significant functions such as endocrine role, forming fundamental structure in cells and tissue, and energy metabolism. Thus, the hypertrophied adipocyte may be the foundation of the metabolic

syndrome state by cleaving excess TGs in LDs and moving fatty acids into circulation, and depositing them into other organs that are not well-equipped store lipids (19). The other organs, such as the liver and muscle, lose their function due to deposited fatty acid and aggravate the state's metabolic syndrome. Even though WAT is the specialized site for store fatty acids, the capacity is not infinite. These cells are not impervious to excess lipid accumulation, driving the deleterious environment locally and in distant tissues (10, 19). It appears more likely that WAT expansion's sufficient capacity is essential to prevent a spillover of fatty acids and lipotoxic damage in other tissues and hypertrophy in the adipocyte.

Liver

The liver is a central player in the whole-body energy homeostasis due to its ability to metabolize nutrients. In a normal state, the liver is also adapted to use lipid as a fuel and convert lipids from dietary fat into various lipid-containing products, including lipoproteins. In the setting of chronic nutrient stress, the liver takes additional fatty acid from adipose tissue (lipotoxicity) as well as three major nutrients (glucose, proteins, and fat) through diet consumption for converting into fatty acids and TGs (9). The HFD or excess energy consumption promotes fatty acid to be deposited in the liver. As fatty acid is accumulated in the liver, the liver is becoming enlarging, called hepatic steatosis. The increased lipid (TGs) content in hepatic steatosis involves; 1) excess dietary TGs by overeating; 2) increased de novo lipogenesis (DNL) for TGs synthesis; 3) excess fatty acid from lipolysis of adipose tissue; 4) decreased oxidation function of mitochondria (28). Consistent with adipose tissue hypertrophy, hepatic steatosis indicates the liver's dysfunction and contributor to metabolic syndrome.

The liver's lipid accumulation leads to activation of the unfolded protein response (UPR)/Endoplasmic reticulum (ER) stress, oxidative stress, and pro-inflammatory signaling. In the liver, the ER is a pivotal node at the crossroads of inflammation and lipid metabolism. ER

dysfunction may contribute to the liver's metabolic dysregulation through iNOS-mediated tyrosine nitrosylation of IRE1 or engaging inflammatory signaling cascades and the inflammasome (29, 30). A recent mass spectrometry approach identified 564 hepatocytic proteins, with 30% of those classified as classically secreted and 20% of them affected by excessive lipid accumulation/lipotoxicity (31). Plus, in mouse models of fatty liver disease, the mitochondria exhibit dysfunctional β -oxidation. This might be partially due to increased ER-mitochondria connections and associated calcium overload in mitochondria in obesity (32).

Of interest, the secreted factors from fatty hepatocytes induced inflammation and insulin resistance in other cell types, supporting the premise that lipotoxicity impacts secretion to influence metabolic tissue cross talk. Many hepatokines that are dysregulated in steatosis/lipotoxicity induce detrimental effects on other peripheral tissues (31). On top of that, the fatty acid by DNL in the liver would supply ~20% of newly accumulated TGs in adipose tissues (33), which means the source of lipids in adipose tissues can be derived from lipotoxicity from the liver as well as dietary fat. One emerging aspect of hepatic lipotoxicity is free cholesterol accumulation due to cholesterol homeostasis disturbances and transport (34). Excess free cholesterol causes damage in the liver through JNK-1- and TLR4-dependent mechanisms (30). As such, perturbations affecting fatty acid influx into the liver, such as de novo synthesis, conversion to TG, and oxidation stress, contribute to disturbances in hepatic lipid homeostasis. The aberrant lipid metabolism in the liver and concomitant excess circulating lipids directly affect insulin sensitivity, increasing the liver's glucose output, which is an essential contributor to impaired glucose tolerance and metabolic syndrome.

Taken together, the liver is one of the most highly explored organs in the context of lipotoxicity and plays an essential role in energy homeostasis by utilizing nutrients, including fat,

glucose, and protein. For this reason, hepatic steatosis is closely associated with a cluster of metabolic diseases such as obesity, insulin resistance, and diabetes. Thus, understanding hepatic fatty acid metabolism and lipotoxicity are of paramount importance.

Skeletal Muscle

Skeletal muscle plays a role in the body's energy expenditure, participating in thermogenic functions, glucose and lipid uptake, and other metabolic processes. Fatty acid uptake in the muscle is dependent on metabolic demands and lipid availability. Once fatty acids enter the skeletal fiber, they have different fates depending on the cells' metabolic status (35). In the chronic HFD state, an excessive lipid flux into the skeletal muscle influences lipid intermediates' accumulation, producing lipotoxic stress. More importantly, lipid accumulation in skeletal muscle leads to organelles' damage controlling for intracellular metabolism because of an excessive accumulation of lipid intermediates such as lipid-derived diacylglycerol (DAG) and ceramides (36). Fatty acid intermediates activate serine/threonine kinases that impair the insulin receptor's ability to stimulate downstream pathway, as IRS-1. This decreases the translocation of GLUT4 and therefore reduces glucose uptake into skeletal muscle cells (20).

The increased lipid deposition in skeletal muscle can be the main contributor to hyperglycemia and hyperinsulinemia because skeletal muscle is the central organ responsible for massive glucose uptake. Skeletal muscle is involved in the clearance of 25% of circulating glucose in a basal, fasting state and up to nearly 70–85% in a postprandial state (21). This is associated with the fact that chronic exposure to a high concentration of saturated fatty acids leads to inhibition of insulin receptor signaling through inhibition of IRS-1 via decreased tyrosine phosphorylation in muscle cells (20). Furthermore, saturated fatty acids also induce stress and inflammatory signaling molecules, such as PKC, JNK, ERK, STAT3, and iNOS, and

increase IL-6, TNF α , and IL-1 β expression (37), which, in turn, impact metabolism.

Additionally, increased ceramide, intramyocellular lipid (IMCL), DAG, and long-chain fatty acyl-CoA levels have been negatively correlated with insulin action. This negative correlation indicates the importance of understanding the link between obesity and the lack of insulin response in skeletal muscle (36, 38).

Many myokines are affected by lipotoxicity, including pro-inflammatory cytokines and proteins from the insulin-like growth factor (IGF) pathways, which are similarly regulated in human obesity (38). IL-6, myostatin, and irisin are the most studied myokines. High IL-6 levels are closely associated with metabolic syndrome even though IL-6 is involved with enhanced glucose uptake and insulin sensitivity in the context of exercise-induced secretion (39). Myostatin expression and secretion from skeletal muscle are increased in obese individuals who most often have increased skeletal muscle lipid accumulation (40). This is suggested to contribute to insulin resistance pathogenesis and inhibition of peripheral glucose uptake.

Even though fatty acid accumulates in muscle slowly compared to adipose tissue and liver, skeletal muscle might play a critical role in maintaining or accelerating aberrant systemic insulin metabolism because of skeletal muscle's function of massive uptake processes as well as myokines. Therefore, fatty acid metabolism in skeletal muscle requires a tight balance between the uptake and usage processes to avoid detrimental lipid intermediaries' accumulation.

Glucocorticoid

Glucocorticoid (GC) (e.g., dexamethasone, prednisone, and hydrocortisone) is steroid hormones synthesized and released by the adrenal gland in a circadian manner in response to physiological cues and stress. GC regulates various physiologic processes for life, such as cellular functions, homeostasis, metabolism, cognition, and inflammation. Also, they exert anti-

inflammatory and immunosuppressive actions, as well (13). Due to these significant effects, GC is the most widely prescribed steroid therapy for treating numerous inflammatory bowel diseases and multiple cancers (12). However, GC's therapeutic benefits are concurrent with the deleterious side effects of high dose and chronic treatment. The side effect is involved with glucose intolerance, visceral adipose tissue (VAT) accumulation, skeletal muscle wasting, metabolic syndromes, leading to a variety of diseases (13). Many of the side effects of GC treatment resemble the phenotype associated with high-fat feeding and obesity.

GC is a vital hormone in the lipid metabolism among different tissues. In WAT, GC promotes either lipolysis or lipogenesis depending on the acute (hours or days) or chronic (weeks) exposure (41). Whereas acute exposure to GC promotes lipolysis in WAT, chronic overexposure increases adiposity, especially in the VAT, along with an upregulation of enzymes involved in DNL and fatty acid storage (42). Chronic GC excess promotes visceral obesity accompanied by macrophage infiltration and ectopic lipid accumulation in the liver and skeletal muscle, all of them associated with insulin resistance and cardiovascular disease.

A recent study by published Guillermo et al. observed that chronic GC excess changed visceral adipose fatty acid composition, DNL, and the inflammatory state. Besides, GC contributes to hyperinsulinemia, insulin resistance, as the obesity model did (23). From this result, we can acknowledge that GC and HFD independently affect lipid and insulin metabolism and morphological changes in several cells and the body. Interestingly, it is possible to assume that the combination of GC and HFD exert more significant effects than either one independently. Anna et al. experimented to determine the synergistic effect of GC and HFD on hepatic development. The results showed that HFD with GC group had more increased hepatic lipid, collagen content, and hepatic steatosis than only GC and only HFD. Likewise, plasma lipid

metabolites (TG, cholesterol, and aminotransferase) were higher in HFD with the GC group (15). As such, the induced-HFD alternations are much more pronounced when combined with GC treatment.

GC is used both acutely and chronically to treat a variety of inflammatory and autoimmune diseases. However, GC involves widespread side effects such as lipid malnutrition, muscle loss, and lipid metabolism, contributing to low patient tolerance. Therefore, new interventions to prevent GC-induced side effects are necessary to improve patient survival outcomes and quality of life.

Lipid Metabolism Markers

CD36

A cluster of differentiation 36 (CD36) is a protein that facilitates free fatty acid (FFA) transport into various tissue across the plasma membrane (43). CD36 mRNA and protein levels can change depending on metabolic conditions such as high-fat diet feeding, muscle contractions, and insulin or glucose levels. HFD with GC condition has been shown to increase CD36 protein expression, contributing to the elevation of epididymal adipose tissue mass and hepatic steatosis (15). Moreover, since CD36 is responsible for fatty acid transportation into skeletal muscle (44), it would be essential to determine lipid metabolism in adipose tissue, liver, and skeletal muscle.

FABPs

Fatty acid-binding proteins (FABPs) family are small and abundantly expressed in the cytoplasm. They can bind hydrophobic molecules such as saturated and unsaturated long-chain fatty acids, eicosanoids and influence lipid transport and lipid signaling processes (45). They regulate lipid fluxes and subcellular localization of fatty acids, allowing them to be stored or

metabolized effectively. Since FABPs can be highly expressed across tissues where lipid metabolism is active, we can investigate the intracellular lipid metabolism by FABPs examination.

HSL

Hormone-sensitive lipase (HSL) is one of the sequential lipases with adipose triglyceride lipase (ATGL) and monoglyceride lipase (MGL). HSL's role is breaking TGs into fatty acid and glycerol from the lipid droplets into circulation. Functional cells metabolize and store excessive fatty acid depending on various hormones such as glucose, glucagon, and insulin levels. In terms of dysfunctional cell environments such as HFD and chronic GCs, excessive accumulation of fatty acid inhibits the hormone function. So constantly activating HSL levels and releasing non-esterified fatty acid and glycerol into circulation leads to systemic effects such as ectopic lipid, lipotoxicity, and insulin resistance (11).

FASN

Fatty acid synthase (FASN) is the enzyme involved in DNL. FAs derived from dietary fat are the primary source of TGs synthesis. However, DNL is also the metabolic route to synthesize lipids from dietary sources, contributing to lipid accumulation as TGs (46). Even though DNL has been considered no outstanding contribution to the synthesis of TGs, recent studies demonstrated that up to 20% of new TGs could be generated through DNL. Besides, FASN may increase intracellular inflammation such as ceramides, potentially toxic by-products of lipid accumulation.

Crat

Carnitine acyltransferase (Crat) is widely distributed in the overall intracellular environment and responsible for cell energy metabolic flexibility. The intracellular distribution

of Crat is involved in the requirement of every cell to regulate carbohydrate and fat metabolism. Moreover, Crat plays a role in combating nutrient stress and enhances insulin action by inhibiting acetyl-CoA's overaccumulation and promoting pyruvate dehydrogenase (PDH) (47). Dysfunction in Crat detracts from the cell energy system's proper function by decreasing the rates of conversion Acetyl-CoA, inhibiting delivery of activated fatty acids for oxidation (48).

2. Methods

Animals and Experimental Design

All experimental and housing protocols were approved by the Institutional Animal Care and Use Committee of the University of Memphis.

C57BL/6 male mice, 7 weeks of age, were purchased from Envigo. All animals were kept on a 12:12-h light-dark cycle and provided ad libitum access to food and water during the study. After 3 days of acclimation, animals underwent baseline testing, including MRI after being fasted for 5h. Mice were then randomized into two groups initially to receive either a high-fat diet rich in omega-6 (n-6, 45% fat (177.5 g lard), 35% carbohydrate, and 20% protein, n-6:n-3 PUFA, 13:1), n=16, or a high-fat diet rich in omega-3 (n-3, 45% fat (177.5 g Menhaden oil), 35% carbohydrate, and 20% protein, n-6:n-3 PUFA, 1:3, diet composition listed in Table 1), n=16. After 4 weeks on their respective diets, both groups were divided with half of the mice receiving either a subcutaneous injection of dexamethasone (3 mg/kg body weight) or sterile PBS while continuing their current diet throughout the 5th and last week— 45% n-6 + dexamethasone (n-6 + dex) n=8 and 45% n-3 + dexamethasone (n-3 + dex) n=8. We closely monitored the daily body weight, food intake, and grooming in all mice for the experiment's duration. The remaining food (from previous day consumption) and body weight were measured 3 times a week during the first four weeks and daily following dexamethasone injections. MRI testing was performed on mice at 8, 12, and 13 weeks of age to assess body composition (ECO

MRI-100, Houston, TX). Blood was collected on all animals at 8, 12, and 13 weeks of age via the facial vein. Approximately 50-100ul of blood was collected into an EDTA-containing tube for plasma collection. At the end of the study, animals were fasted for 5h before harvesting tissue. Tissue collection was completed with mice anesthetized by isoflurane and euthanized by cervical dislocation.

MRI Test

At 8, 12, and 13 weeks of age, mice underwent MRI testing to determine body composition of fat mass and fat-free mass (ECO MRI-100, Houston, TX).

Non-Survival Surgery

At the end of the study (14 weeks of age), all animals were fasted for 5 hours before harvesting tissue. Puromycin (0.04 μ mol/g) was injected intraperitoneally 30min before tissue collection to measure protein synthesis rates. Tissue collection was completed with the mouse anesthetized by isoflurane (2-5%). Mice were euthanized by cervical dislocation while anesthetized. Hindlimb skeletal muscles (soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitorum longus), epididymal fat pad, heart, and spleen were excised and snap-frozen in liquid nitrogen for further analysis. Before being frozen, the gastrocnemius was divided into red and white portions representing portions that are high in oxidative (red) fibers and glycolytic (white) fibers, respectively. Tibias were also removed and weighed as a correction factor for body size.

TG Assay

Triglyceride assay was performed on a serum to determine differences in TGs concentration. Free Glycerol Reagent and the Triglyceride Reagent were set up according to procedures. A series of labeled cuvetts for blank, standard, and sample were prepared for the pipette. 2ul of water or sample lipid was added to blank, standard, and sample plate cuvet in

triplicate. 160ul of Free Glycerol Standard was added to each cuvet. An initial absorbance of blank, standard, and sample was measured using a spectrophotometer at 540 nm (BioTek, Synergy2, ??) and Gen5™ software. 40ul of the Triglyceride Reagent was mixed with each covets and incubated at room temperature for 5 minutes. The second absorbance of blank, standard, and sample was measured at 540 nm. TGs concentration was calculated using the purified standard to construct a standard curve.

RNA Isolation and qPCR

The following genes were analyzed for expression: CD36, FABPs, HSL, Fasn, and Crat. To isolate RNA from mouse liver and white and red gastrocnemius muscle, tissues were homogenized in 3-5 mL RNA STAT-60. Total RNA was extracted from STAT-60 solution by the addition of chloroform: isoamyl alcohol (24:1). Extracted RNA was dissolved in water, reprecipitated using sodium acetate and isopropanol, washed with 75% ethanol, and quantified using a Nanodrop instrumentation (ThermoFisher Scientific, Waltham, MA). For qPCR measurement of RNA transcripts, 1 µg of RNA was reverse transcribed to cDNA. The cDNA was mixed with forward and reverse primers for the intended gene target and ABsolute Blue SYBR Green qPCR master mix. Change in gene expression was performed using the QuantStudio™ 6 flex system Real-Time PCR software. Real-time PCR was carried out with comparative 2^{-ΔΔCT} and SYBR® Green Reagents (Thermo Fisher Scientific, Waltham, MA). PCR reaction was as follows: 50 °C for 2min, 95 °C for 10min followed by 40 cycles of 95°C for 15s, 60°C for 1 min and 95 °C for 15s. See Table 2 for primer sequences.

Histology

A representative sample of eWAT was fixed in a 10% neutral buffered formalin solution (Fisher Scientific Co. LLC, Dallas, TX) for 48-72 h. These samples were dehydrated in a series of graded ethanol solutions, cleared with xylene, and then embedded in paraffin. Five µm

sections were stained with hematoxylin and eosin (H&E). Histological analysis was performed using an Imager M7000 microscope (Invitrogen™, EVOS™, M7000 Imaging system, ??). Individual adipocyte sizes were determined using Axiovision r4.8.2 software. Two to three fields at 20X magnification, with twenty to forty cells per field, were quantified for each mouse.

Statistical Analysis

All data are represented as means \pm SE. A two-way ANOVA was used to determine the effects of diet and dexamethasone treatment using GraphPad Prism 8. Bonferroni post hoc analysis was used to examine interactions. Significance was set at $p \leq 0.05$.

3. Results

Body compositions, MRI_Fat and MRI_Lean

From baseline (day 0) to end of study (day 37), body weight did not significantly differ between any groups even after dexamethasone injection. HFD composition difference (omega-6 vs. 3) did not alter body weight (Figure 1). N-6 dex significantly increased fat weight compared to that of n-3 dex and n-3 PBS groups (Figure 1). Likewise, n-6 diet groups significantly increased fat mass when compared to that of n-3 diet groups. The lean mass showed no significant difference by diet composition (omega 6 and 3) and dexamethasone injection (Figure 1).

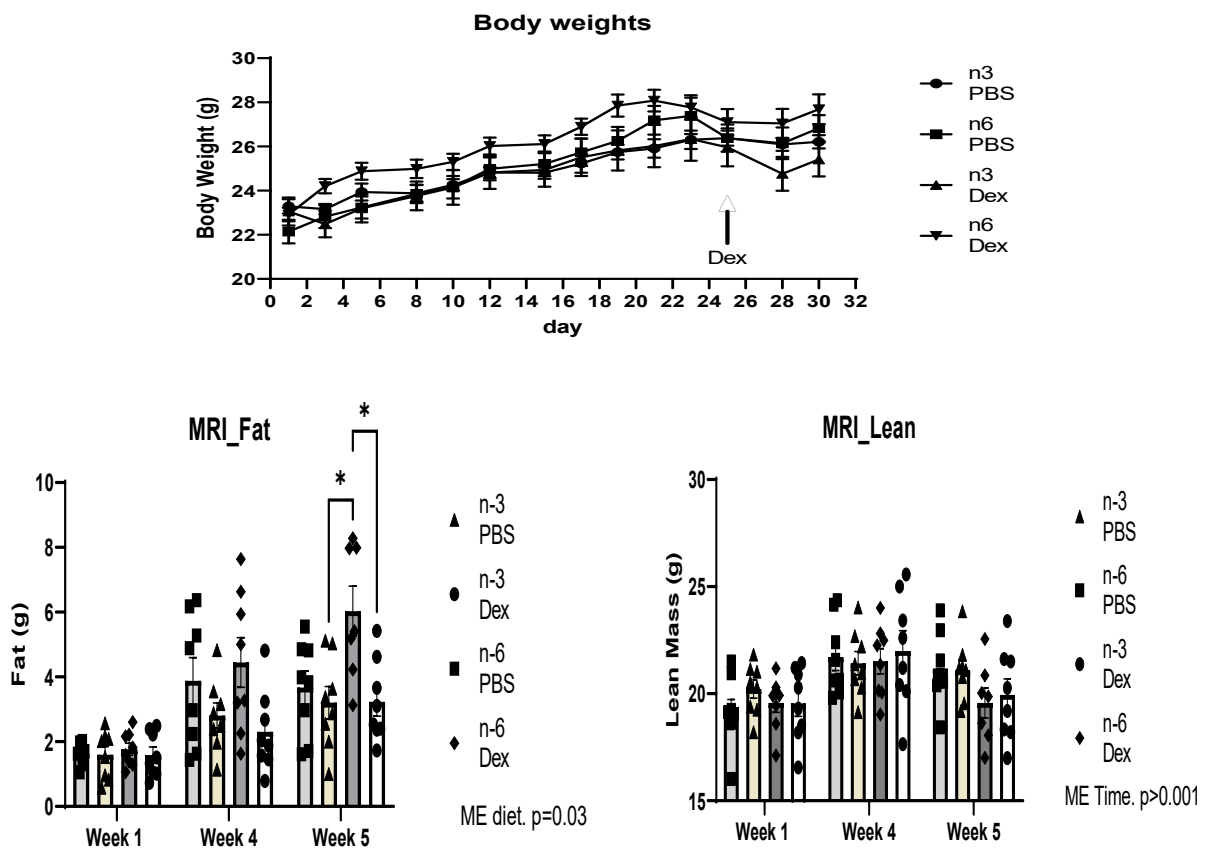


Figure 1. Body weight, fat, and lean mass in mice fed either an HFD rich in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean \pm SEM. Significance was set at $p < 0.05$.

Epididymal white adipose tissue (eWAT) weight and adipocyte size

Epididymal fat mass was significantly elevated with the n-6 diet (Figure 2). Likewise, within the dexamethasone treatment condition, the n-6 diet group had significantly increased Epididymal fat mass compared to that of the n-3 diet group (Figure 2). N-3 diet groups showed comparatively smaller adipocyte size than n-6 diet groups (Figure 2).

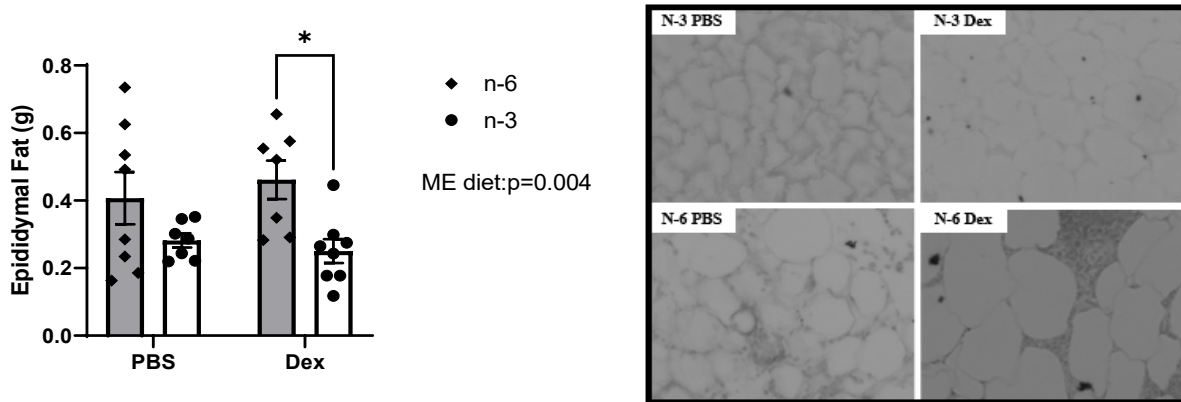


Figure 2. Epididymal fat and adipocyte size in mice fed either an HFD rich in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean \pm SEM. Significance was set at $p < 0.05$.

Liver Weight and Liver Triglycerides

N-6 dex significantly increased liver weight compared to that of n-6 PBS, while liver weight in n-3 PBS and dex groups showed no significant difference. N-6 diet groups increased the liver TG levels compared to that of n-3 diet groups (Figure 3).

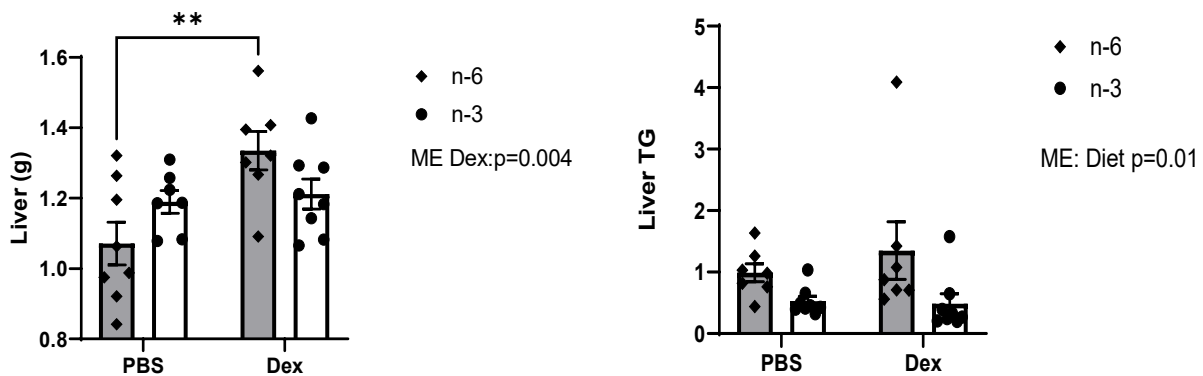


Figure 3. Liver weight and TGs in mice fed either an HFD rich in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean \pm SEM. Significance was set at $p < 0.05$.

Lipid Metabolism Markers in Liver

N-6 dex significantly increased CD36 and FABP mRNA levels when compared to that of all groups. There was an effect of the n-3 diet group to significantly increase HSL and Crat mRNA levels compared to that of n-6 diet groups (Figure 4). There is no significant difference in liver Fasn mRNA levels (Figure 4).

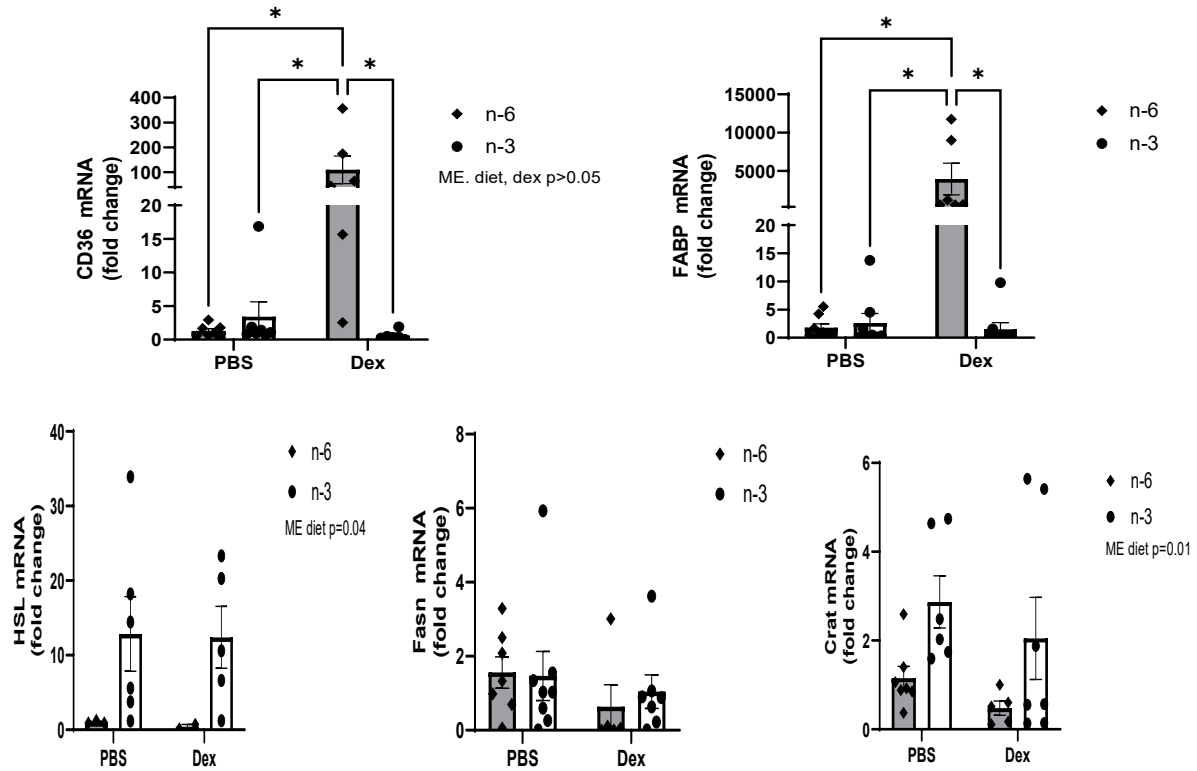


Figure 4. Effects of high-fat diet and glucocorticoid on lipogenesis, fat transporter, and lipolysis markers in the liver with mice fed either an HFD rich in lard (n-6) or Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean \pm SEM. Significance was set at $p < 0.05$.

Lipid Metabolism Markers in Glycolytic Muscle

We next looked at the effects of omega-3 diet and dexamethasone treatment on markers of lipid metabolism in the white portion of the gastrocnemius, a glycolytic portion of the muscle. N-3 diet groups significantly increased FABP mRNA levels when compared to that of n-6 diet groups. Dex groups significantly increased HSL mRNA levels when compared to that of PBS groups (Figure 5). There is no significant difference in CD36, Fasn, and CrAT mRNA levels between all groups (Figure 5).

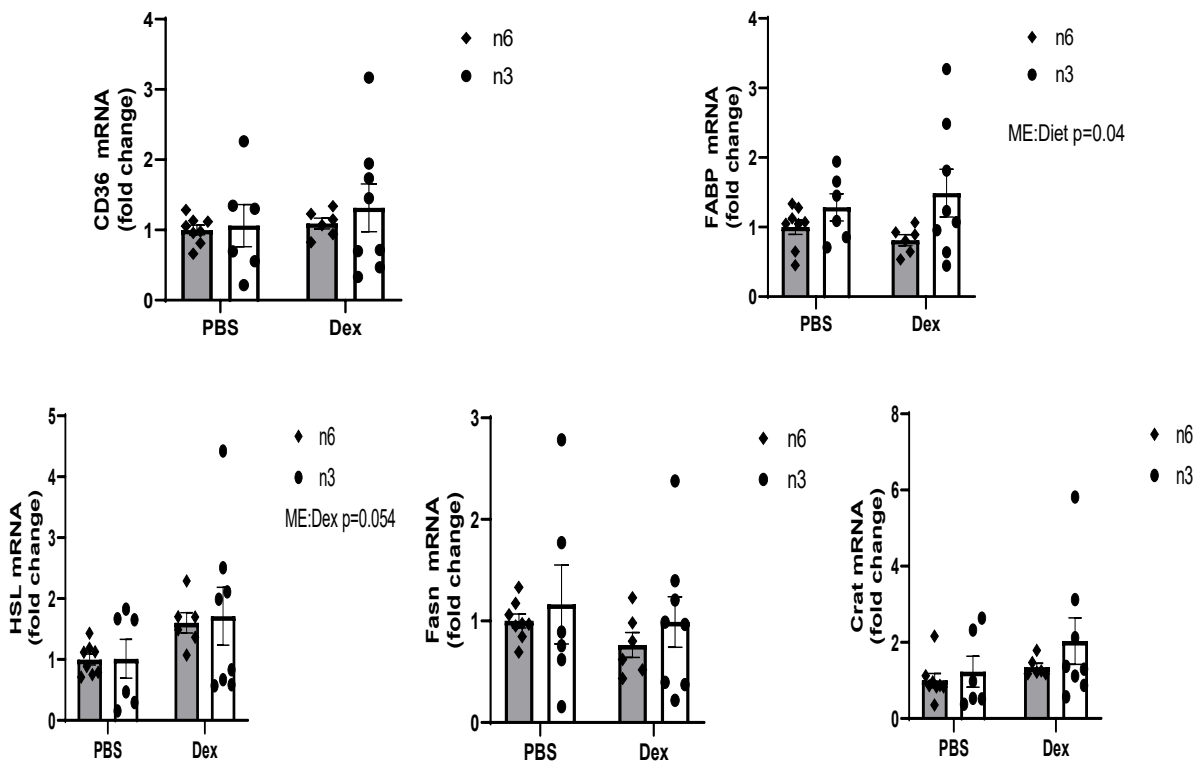


Figure 5. Effects of high-fat diet and glucocorticoid on lipogenesis, fat transporter, and lipolysis markers in glycolytic muscle with mice fed either an HFD rich in lard (n-6) Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean ± SEM. Significance was set at $p < 0.05$.

Lipid Metabolism Markers in Oxidative Muscle

We next examined an oxidative portion of the gastrocnemius muscle. Dexamethasone treatment significantly increased FABP, HSL, and Crat mRNA levels compared to PBS groups (Figure 6). There was no significant difference in CD36 and Fasn mRNA levels between all groups (Figure 6).

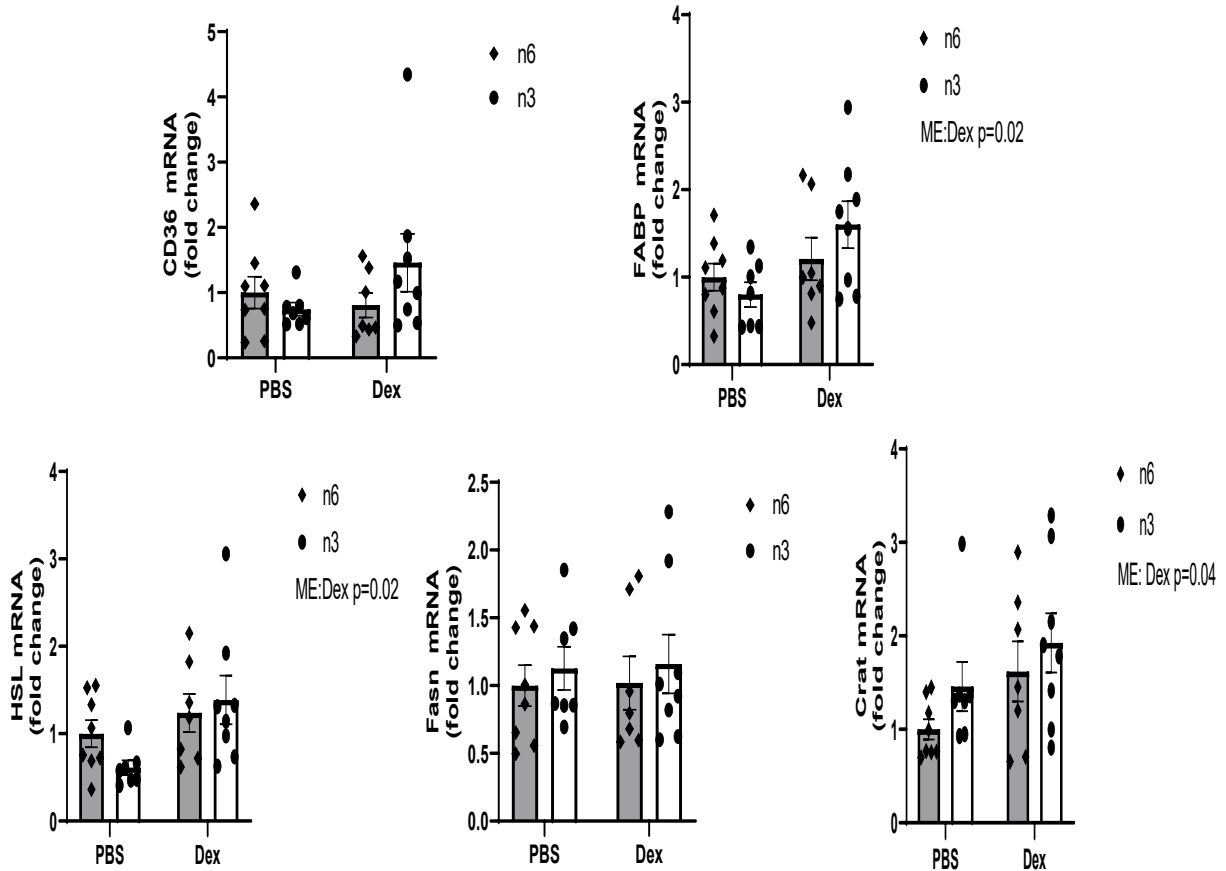


Figure 6. Effects of high-fat diet and glucocorticoid on lipogenesis, fat transporter, and lipolysis markers in oxidative muscle with mice fed either an HFD rich in lard (n-6) Menhaden oil (n-3) with dexamethasone injection. All data are presented as mean \pm SEM. Significance was set at $p < 0.05$.

4. Discussion

In the present study, we aimed to determine whether fat composition in HFD affects GC-induced alteration in lipid handling by the liver, adipose tissue, and skeletal muscle. We observed that omega-3 HFD improved adipose tissue and liver fat accumulation and the associated lipid metabolism markers. We also report that one week of dexamethasone treatment exacerbates the lipid condition induced by omega-6 HFD. Feeding mice with diets rich in omega-3 supplements menhaden oil protected mice from adipose tissue hypertrophy and hepatic steatosis by decreasing fat accumulation. Further, omega-3 HFD also reversed the lipid transport markers (CD36 and FABP), which play a critical role in fatty acid inflow into adipose tissue and the liver.

There were no significant differences throughout the study (Figure 1). Considering that body weight change has been confirmed in long experiments such as over five weeks (14, 15, 17, 49, 50), the short-term lipid dysfunction induced by HFD and GC in our study do not seem to have reached that point. Also, since the side effect of GC treatment involves skeletal muscle loss, we need to consider the body weight complementary action between fat increase and muscle loss induced even though statistical significance of MRI_lean mass in our study was not reached (Figure 1). As expected, the omega-6 diet with dexamethasone treatment increased significantly fat mass (Figure 1), which indicates the synergetic effects. Chronic omega-6 HFD diet leads to fatty acid over-accumulation in adipose tissue and liver by fibrosis, hypoxia, and inflammation. Therefore, the cells start to become hypertrophied, and the storage ability becomes dysfunctional, finally contributing to the unhealthy fat mass increase (8).

On the other hand, previous studies have demonstrated that omega-3 HFD diets improve fat mass by stabilizing lipid metabolism due to their anti-inflammatory action (49, 51). Likewise, in our study, the omega-3 HFD diet maintained fat mass even with dexamethasone treatment.

This data shows that fat composition in HFD is essential to ameliorate GC-induced fat mass increase.

To further investigate the effect of omega-3 HFD diet on fat mass and size, we examined epididymal white adipose tissue (eWAT) weight and adipocyte size (Figure 2). As MRI_fat weight data, the omega-3 HFD diet showed a lower eWAT weight than omega-6 diet groups, which indicates that omega-3 protects white adipose tissue from becoming hypertrophied and dysfunctional. This indication was demonstrated through an adipocyte size picture by microscopy (Figure 2). This picture exhibited that the omega-3 HFD diet had comparatively smaller adipocytes than the omega-6 HFD diet and dexamethasone treatment. It is possible to assume that omega-3 helps white adipose tissue store excessive fatty acid by differentiation of resident tissue precursors and creating new adipocytes. Simultaneously, omega-6 and dexamethasone treatment promote white adipose tissue to store excessive fatty acid through enlargement of existing adipocytes because of the decreased adipocyte function (8, 27). This process would be associated with insufficient vascularization, hypoxic response, and tissue fibrosis.

We measured liver weight and TG to determine the effect of fat composition in HFD on lipid metabolism in the liver. These findings align with eWAT data that omega-3 HFD diet showed little change of comparatively liver weight and TG while omega-6 and dexamethasone treatment increased those (Figure 3). The liver has a close relationship with adipocytes regarding lipid metabolism because it is a central player in the whole-body energy homeostasis. In the setting of chronic HFD, the liver conveys fatty acid by de novo lipogenesis to adipocytes, which is responsible for 20% of newly accumulated TGs in adipose tissue (33) and liver additional fatty acid from adipose tissue (9). Due to these reasons, the liver is sensitive to lipid over-

accumulation and the damage induced by lipid metabolism dysfunction. In the present study, the omega-3 HFD diet inhibited the increase of liver weight and TG (Figure 3). The omega-3 effect can be explained by a recent study published by Sakamuri et al. demonstrated that HFD rich in omega-3 improved hepatic tissue metabolic dysfunction by involving hepatic antioxidant enzyme, lipogenic, inflammatory, and oxidative ER stress gene expression as well as pre-receptor amplification of GC (17).

To advance our understanding of lipid metabolism, we examined lipid metabolism markers in the liver. CD36 and FABPs are proteins that facilitate FFA transport into various tissue across the plasma membrane by binding saturated and long unsaturated chain fatty acids (43, 45). The present data showed that the omega-6 HFD diet with dexamethasone treatment significantly increased CD36 and FABPs (Figure 4). The results indicate that omega-6 HFD diet with dexamethasone leads to over-lipid inflow into the liver, while omega-3 HFD diet groups did not show any elevation of CD36 FABPs. It is possible that the omega-3 HFD diet would prevent the liver from receiving excessive fatty acid from adipose tissue because the omega-3 HFD diet improves angiogenesis and storage ability. Therefore, CD36 and FABPs in the liver may not have to increase. In terms of HSL and Crat, they are both significantly higher in the omega-3 HFD diet group compared to omega-6 HFD (Figure 4). The liver constantly utilizes energy metabolism by oxidation and is released into circulation in normal conditions. Also, HSL is responsible for breaking TG for releasing fatty acid into circulation, and Crat plays a role in cell energy metabolic flexibility (11, 47). Therefore, HSL and Crat's significant increase in the omega-3 HFD group may indicate that they work correctly, which is supported by the beneficial effect of omega-3 on inflammatory, oxidative stress hepatic antioxidant enzyme we mentioned earlier (17). Considering that highly activated HSL is one of the aspects of cell dysfunction

induced by excessive lipid accumulation (9), the current study design does not seem to have reached that point where cells become dysfunctional and release fatty acid into circulation excess lipid accumulation exists.

Little research on the effect of fat composition on lipid metabolism in skeletal muscle has been covered even though skeletal muscle can be the main contributor to hyperglycemia, insulin resistance, and lipid metabolism dysfunction. The present lipid metabolism marker data in glycolytic and oxidative muscle showed that some markers (FABP and HSL: glycolytic / FABP, HSL and Crat: oxidative) have a significant difference only induced by dexamethasone treatment (Figure 5, 6). Given HSL mRNA in the liver, it is possible that lipotoxicity induced by lipid metabolism dysfunction does not reach skeletal muscle during short-duration glucocorticoid treatment. On the other hand, dexamethasone treatment affected a few lipid metabolism markers (Figure 5, 6). Based on previous research results (52, 53), GCs can be involved in lipid mobilization and insulin resistance in skeletal muscle. These effects may be regulated at the 11 β -hydroxysteroid dehydrogenase type1 (11 β -HSD1), which is involved in generating active glucocorticoids in tissues. With these results, however, we cannot explain GC's effect on lipid metabolism in skeletal muscle. Future research should consider the difference of GC between acute and chronic effects on skeletal muscle with the proteins or genes related to specific fatty metabolism that work in skeletal muscle, unlike liver and adipose tissue.

5. Conclusion

In summary, the present study's result demonstrated that the change of fat composition in HFD could beneficially alter the fatty acid accumulation, adipocyte size, and associated lipid metabolism markers. The western diet contains high levels of omega 6, which contribute to developing metabolic syndrome. Also, the side effect of GC treatment increases the anti-

metabolic effect induced by HFD rich in omega 6. Hence, consuming a well-balanced fat diet would be beneficial for preventing metabolic syndrome and associated diseases.

6. Limitations and Future Directions

One limitation to our study may be the short-term experiment period. Even though 4 weeks HFD and 1-week dexamethasone treatment ($<0.5\text{mg/kg}$) is proper to generate lipid metabolism dysfunction, the long-term experiment would be able to see the experimental effect on skeletal muscle. To keep track of lipid metabolism, additional lipid signaling and composition in adipose tissue, serum, liver, and skeletal muscle would help future research.

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Appendix

Tables and Figures

Table 1. Composition of high-fat lard (n-6) and high-fat Menhaden oil (n-3) diets.

| | n-6 | n-3 |
|-----------------------------|--------------|--------------|
| Ingredient | g | g |
| Lard | 177.5 | |
| Menhaden Oil, ARBP-F | | 177.5 |
| Soybean Oil | 25 | 25 |
| Total | 202.5 | 202.5 |
| Saturated (g) | 60.2 | 59.8 |
| Monounsaturated (g) | 67.7 | 41.3 |
| Polyunsaturated (g) | 62.8 | 88.5 |
| Saturated (%) | 31.6 | 31.5 |
| Monounsaturated (%) | 35.5 | 21.8 |
| Polyunsaturated (%) | 32.9 | 46.7 |
| C16, Palmitic | 36.8 | 35.2 |
| C18, Stearic | 19.8 | 6.6 |
| C18:1, Oleic | 64.1 | 22.8 |
| C18:2, Linoleic | 56.2 | 16.1 |
| C18:3, Linolenic, n3 | 4.2 | 4.3 |
| C20, Arachidic | 0.4 | 0.3 |
| C20:4, Arachidonic, n6 | 0.5 | 0.0 |
| C20:5, Eicosapentaenoic, n3 | 0.0 | 23.3 |
| C22:6, Docosahexaenoic, n3 | 0.0 | 29.0 |
| n6 (g) | 57.0 | 17.9 |
| n3 (g) | 4.4 | 66.6 |
| n6/n3 ratio | 13.1 | 0.3 |

Table 2. Gene primers for qPCR analysis.

| gene | Primers | |
|-------|---------------------------|----------------------------|
| | fwd | rev |
| Crat | 5'CTGTGGGATGGTGTATGAGC3' | 5'CTGAGGTTCTGTTTGGCTTTC3' |
| HSL | 5'CACAGACCTCTAAATCCCACG3' | 5'ATATCCGCTCTCCAGTTGAAC3' |
| FABP3 | 5'GCTGGGAATAGAGTTCGACG3' | 5'CTTCTCATAAGTCCGAGTGCTC3' |
| CD36 | 5'GATGTGCAAACCCAGATGAC3' | 5'ACAGTGAAGGCTCAAAGATGG3' |
| Fasn | 5'GATGACAGGAGATGGAAGGC3' | 5'GAGTGAGGCTGGGTTGATAC3' |

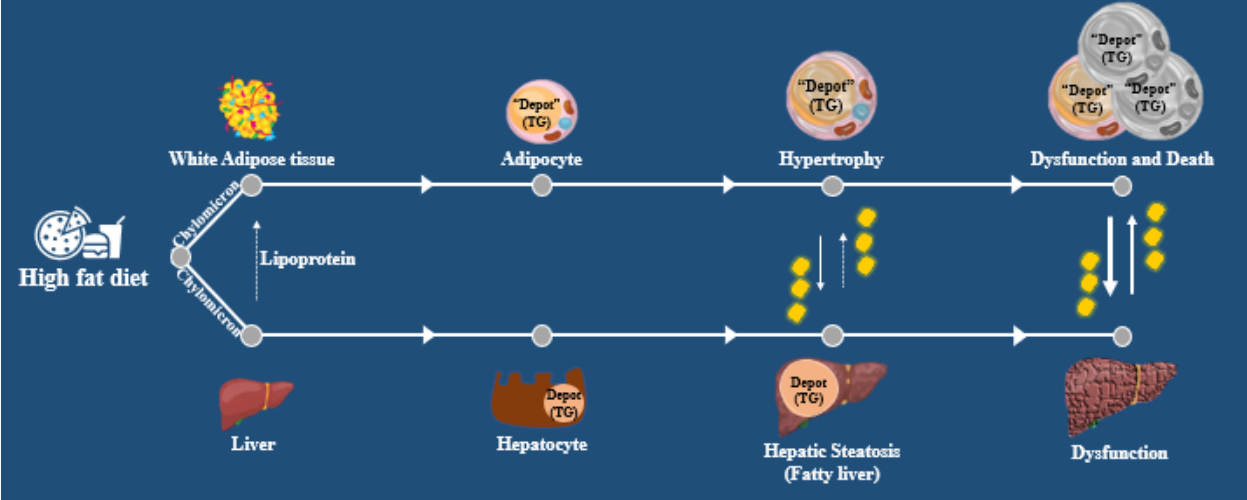


Figure 7. The process of storing lipids in adipose tissue and liver.

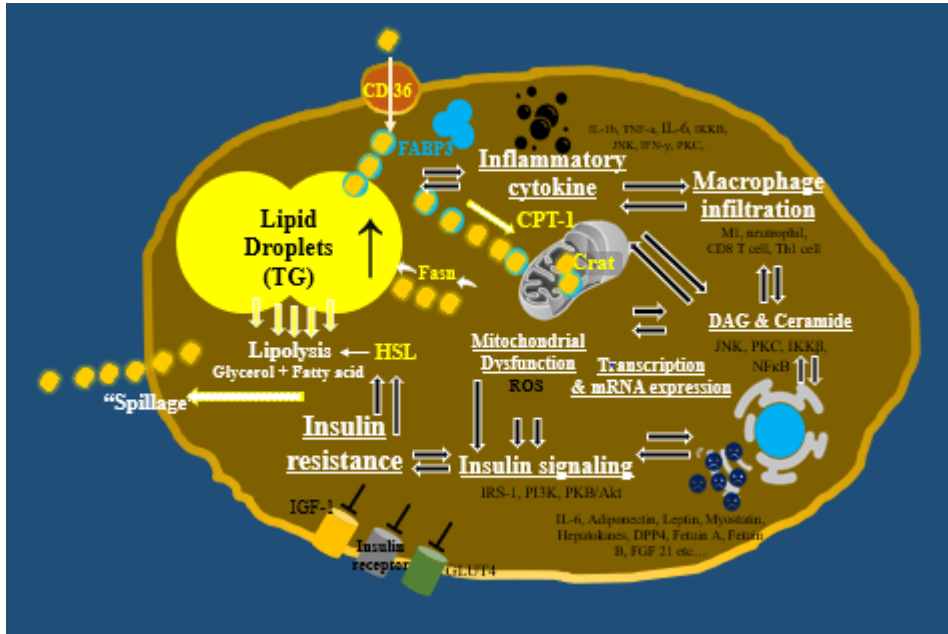


Figure 8. Common intracellular dysfunctional environment induced by HFD.

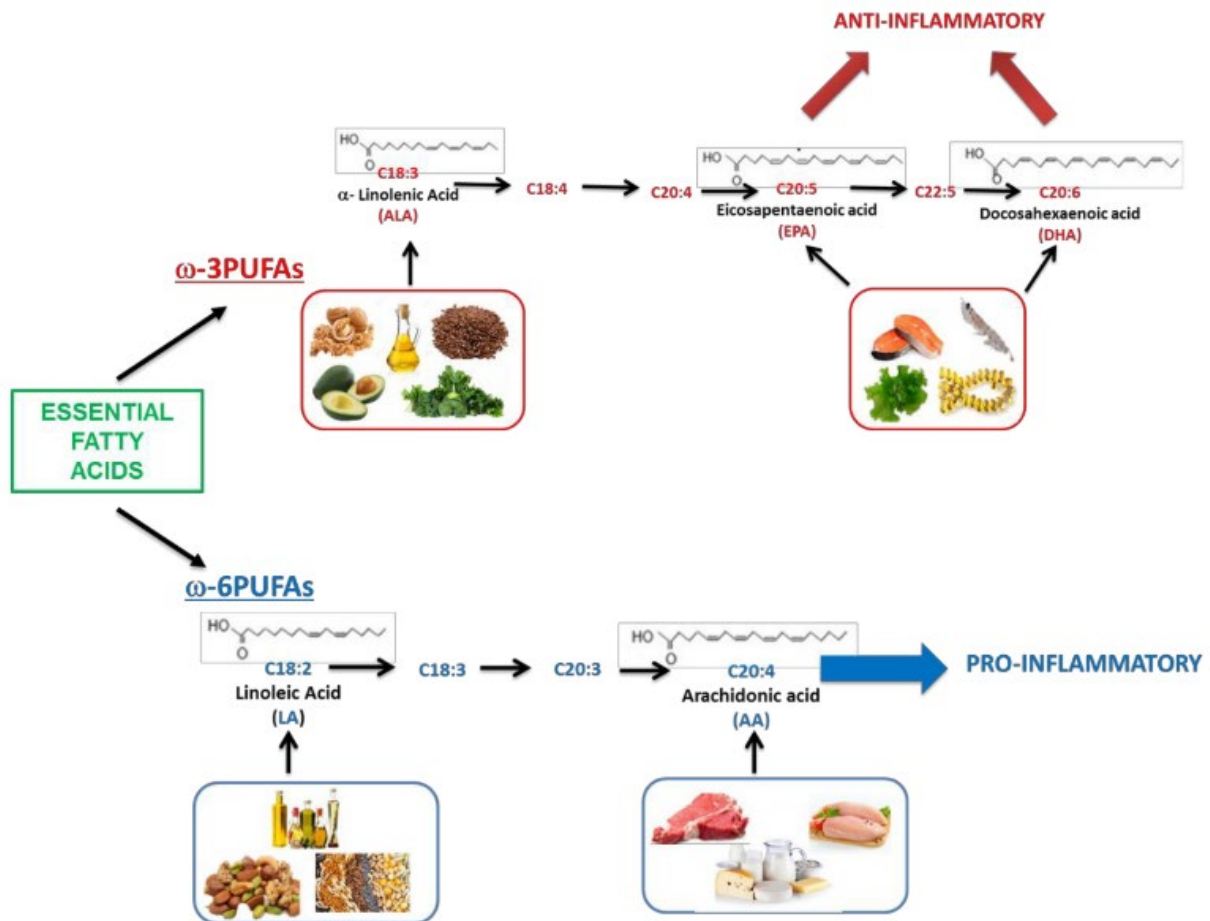


Figure 9. Compositions of omega-3 and 6 polyunsaturated fatty acid

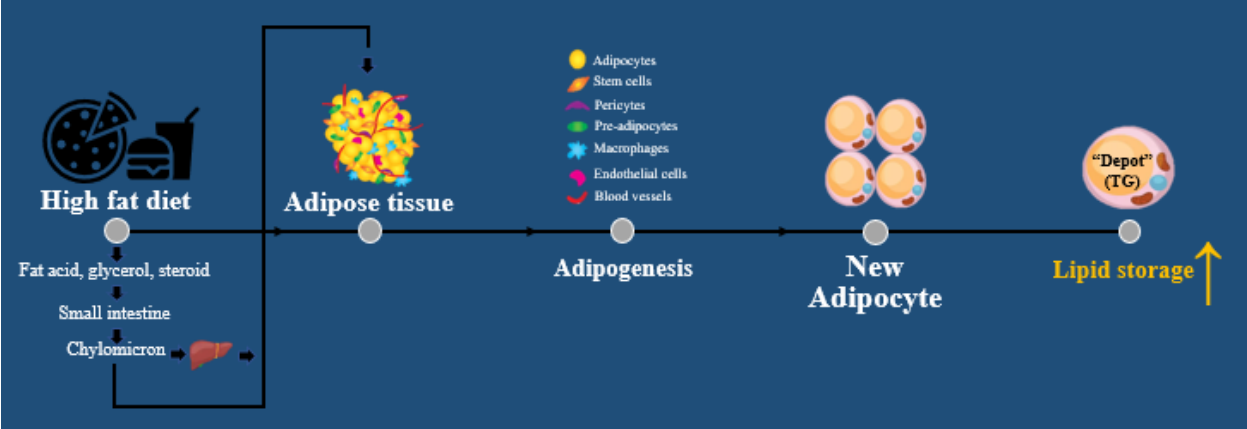


Figure 10. Differentiation and creating new adipocyte to store excessive lipids.

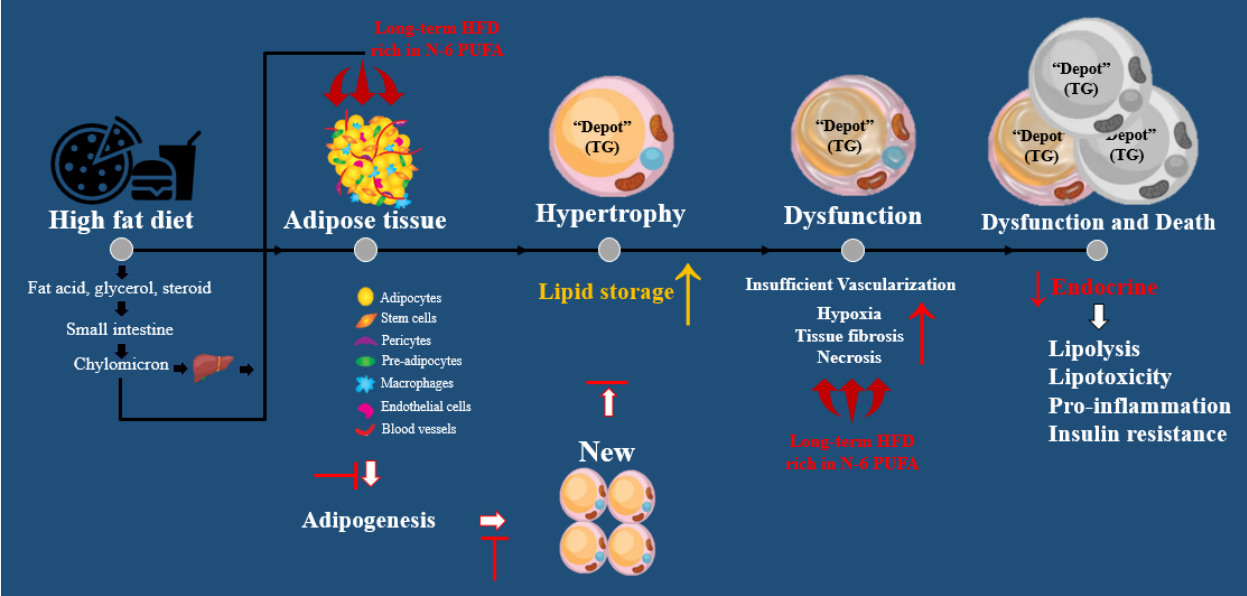


Figure 11. Enlargement of existing adipocytes to store excessive lipids.