COMPARISON OF ACUTE AND CHRONIC EXERCISE EFFECTS IN THE LIPID DROPLETS TOPOGRAPHY SKELETAL MUSCLE, FOLLOWING HIGH AND LOW-FAT DIET IN MICE

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ABSTRACT

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In the modern world, diet patterns high on lipids, sedentary lifestyle and obesity contribute all in the development of metabolic syndrome, which in turn can lead to type 2 diabetes. All the excess fats, that the majority of the population consumes nowadays, are stored as ectopic fat, particularly in the skeletal muscle, a tissue beyond doubt insulin-sensitive. Physical activity has been proposed according to various studies to be the most drastic factor, which can induce changes in skeletal muscle lipid metabolism, as well as different diet models. We hypothesized that different exercise patterns and diet-changes can alter the spatial arrangement in lipid droplets (LDs) in different skeletal muscle fiber types of C57BL/6J mice. Muscle samples from *gastrocnemius* were collected from 8 groups: sedentary controls fed with a high-fat (HFD, n=10), or a low-fat diet (LFD, n=10), chronic exercise group fed with a high-fat (HFDR, n=10) or a low-fat diet (LFDR, n=10) and acute exercise group that either killed immediately after exercise or 6 hours later following a high-fat (HFD0, n=7 or HFDR6, n=7) or a low-fat diet (LFDR0, n=7 or LFDR6, n=7). Then immunohistochemistry and laser confocal microscopy were used, followed by image analysis. Fiber type LD size showed a significant difference between all groups, apart from the chronic exercise group fed with the low-fat diet and the acute exercise group fed with the high-fat diet and sacrificed 6 hours after exhaustion. Fiber type LD density showed a very significant difference ($P<0.001$) between all groups. It seems that physical activity with or without the high-fat diet, induced changes in size and density of LDs, meaning that those two factors exercise and diet play a critical role in the prevention of metabolic syndrome later on. A precise comprehension of metabolic pathways participating in obesity and metabolic syndrome reinforces the worldwide effort of diminishing or totally eliminate them.

Keywords: lipid metabolism, lipid droplets, physical activity, insulin resistance, obesity, mice, diet, fiber typing

ACKNOWLEDGEMENTS

LIST OF TERMS AND ABBREVIATIONS

Adipocyte	Adipose tissue cell
ADP	Adenosine Di Phosphate
ADRP	Adipophilin, a protein of PAT family
Amphipathic	With hydrophobic and hydrophilic properties
Antibody	Protein produced by an immune system to destroy a specific antigen
Antigen	Substance recognized by an immune system as foreign to the organism
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine Tri Phosphate
Caveolins	Group of proteins involved in endocytosis
CE	Cholesterol ester
Cytosol	Intracellular liquid surrounding organelles
DAG	Diacylglycerol
Enterocytes	Intestinal absorptive cells
ER	Endoplasmatic reticulum
Esterification	Formation of an ester as the reaction product
FA	Fatty acids
FFA	Free Fatty Acid
FIT	Fat-inducing transcript proteins
FLD1	Seipin protein gene ortholog
GLUT4	Glucose transporter type 4
HCV	Hepatic C Virus
HDL	High Density Lipoprotein
Hepatocytes	Liver basic cell
HFD	High-fat diet, sedentary
HFD0	High-fat diet, acute exercise, killed immediately after maximal test
HFD6	High-fat diet acute exercise, killed 6 hours after maximal test
HFDR	High-fat diet, voluntary running

HSL	Hormone Sensitive Lipase
Hydrolysis	Chemical separation of water molecules
Hydrophilic	Not binding to or repelling water molecules
Hydrophobic	Having the capacity for binding to water molecules
IMCL	Intramyocellular lipids, also known as IMTG
IMCL	Intramyocellular lipids
IMF	Intermyofibrillar mitochondria
Immunohistochemistry	Biochemical procedure for marking specific cellular sites with antibodies
IMTG	Intramyocellular triglyceride
Intramyocellular	Inside muscle cell
IR	Insulin resistance or Insulin resistant
IS	Insulin sensitivity
LCFA	Long-chained fatty acids
LD	Lipid droplet
LDL	Low Density Lipoprotein
LFD	Low-fat diet, sedentary
LFD0	Low-fat diet acute exercise, killed immediately after maximal test
LFD6	Low-fat diet acute exercise, killed 6 hours after maximal test
LFDR	Low-fat diet, voluntary running
Lipase	Enzyme responsible for catalyzing lipid hydrolysis
Lipolysis	Breakdown of TAG into FAs
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
MGL	Monoacylglycerol Lipase
Micrograph	A photograph taken by means of a microscope
Mitochondrion	Organelle of great importance in cell's function, especially in ATP production
Mrna	Messenger RNA
MS	Metabolic Syndrome
Myocyte	Skeletal muscle cell

NEFA	Non esterified fatty acids
OB	Obesity
OXPAT	Protein belonging to the PLIN family
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDK4	Pyruvate dehydrogenase kinase 4
PE	Phosphatidylethanolamine
Perilipin	Perilipin, a protein belonging to the PLIN family
Peroxisome	Organelle responsible for metabolic process
PKC	Protein Kinase C
PLIN proteins	Group of specific proteins coating LD, standing for "Perilipin; Adipophilin, TIP47, S3-12 and OXPAT
RAAS	Renin-Angiotensin-Aldosterone System
S3-12	Protein belonging to the PLIN family
Sarcolemma	Cellular membrane of muscle fibers
Sarcomere	Skeletal muscle basic unit
Slide	Piece of thin glass in order to place and examine biological material specimen
SS	Subsarcolemmal thin intramyocellular region between sarcolemma and IMF region
T2D	Type 2 Diabetes
TAG	Triacylglycerol
TG	Triglyceride
TGH	Triglyceride Hydrolase
TIP47	A protein belonging to the PAT family
VO2max	Maximal Oxygen consumption or aerobic capacity
Wavelength	Light spectrum in microscopy
β-oxidation	FA breakdown into Acetyl-CoA in mitochondria for ATP production

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1   INTRODUCTION

Dysfunctional lipid metabolism, represents a major threat in universal public health because it is affiliated with several and expensive diseases across the world, to mention some of the most common: obesity and diabetes. Therefore, Lipid Metabolic Disorder is considered to be one of the most critical risk factors for the metabolic syndrome. A balance between energy demand and energy supply is critical for maintaining one's health (Randle 1998). The gradual gathering of fat in adipose tissue, liver and muscles, blemishes their ability to metabolize fatty acids, which results in the resistance of insulin (IR). This precipitates an early pathogenesis of Type 2 Diabetes (T2D) (Robert et al. 2012). As far as mammals are concerned, lipid metabolism involves several organs, such as adipose tissue, brain, muscles, liver and gut. These organs communicate through hormones, neurons and metabolites and are a part of complex homeostatic system. When energy homeostasis is disrupted and the duration of leisure-time activities are diminished, then excess fat is stored in adipose tissue and liver fat stores are increased as well, which can lead to obesity and diabetes. Elevated blood lipids are a characteristic feature of insulin resistant (IR) populations, like obesity (OB) and T2D, as mentioned before. Moreover, Van Loon & Goodpaster 2006, have introduced the research community to a phenomenon known as a "metabolic paradox", in which high insulin sensitive (IS) individuals like endurance athletes, have equal or elevated intramyocellular lipid reservoirs. Therefore, it is important to better understand and investigate skeletal muscle morphology and its connection to health implications. Forasmuch as future research needs to be done and be focused upon understanding these and other potential mechanisms, in order to pinpoint therapeutic targets for reducing metabolic syndrome risks, such as sufficient levels of physical activity and maintaining a healthy body weight, or even concrete pharmacologic interventions.

2 LIPID METABOLISM OVERVIEW

2.1 Lipids in Mammals

Lipids are naturally occurring compounds present in almost all living cells (Maughan et al. 1997) and they are part of both body composition and nutrition. They are long chained, branched or non-branched, hydrophobic or amphipathic biomolecules, which in nature are mainly hydrocarbons. Lipids contribute to cellular metabolism as structural components, energy source, biological intermediates, part of the transport system across cell membrane and as essential mediators in inflammation (Garrett & Grisham 2010).

Lipids are a diverse group of molecules and have been classified into different classes based on their differences in synthesis, structure and properties (Fahy et al. 2009). The major lipid storage form of energy for mammals are the triglycerides or triacylglycerol (TAG), commonly named as fats and can be obtained through diet. TAG is present in blood, facilitates bidirectional transfer from the liver of adipose fat and blood glucose, and derived from glycerol and three fatty acids (Maughan et al. 1997, Parks 2002). Diglycerides (DAG) belong to lipids as well, consisting of two fatty acids bonded to a glycerol molecule and they main role is to activate PKC (protein kinase C) (Blumerg 1988). Apart from TAG, lipids include cholesterol, phospholipids and long-chained fatty acids (LCFA), also supplied by food (Silverthorn 2007). Those last ones LCFA, are of high importance by handling the fatty acid composition of adipose tissue (Yost & Eckel 1989).

Lipids can be found through the whole body in mammals, as free fatty acids, derived from TAG breakdown or lipoproteins, which due to their hydrophilic, spherical structures are serving as a way of transportation for lipids in blood, while those being hydrophobic. On the other hand, lipids can be found as cholesterol or phospholipids in cell membranes and signaling molecules. Nevertheless, the adipocytes are the cells specialized in storing energy as lipids, the main TAG pool. In addition to adipocytes, the liver and the skeletal muscle notably behave as a great deposit for lipid stores (Maughan et al. 1997).

Lipids are the ideal cellular fuel and virtually endless (McArdle et al. 2010). Lipids also act as energy sources and transporters of dietary fat, they are of great importance in metabolism as they contain more than twice the energy of (approximately 9 kcal/g or 38 kJ/g) carbohydrates (approximately 4 kcal/g or 17 kJ/g) (Parks 2002), providing enough chemical energy for biological work, like muscle contraction (Maughan et al. 1997). Last but not least, they are also serving hormonal regulations (Mooren & Völker 2005).

2.2 Lipid Metabolism Basics

Lipid metabolism involves synthesis of lipid stores and its degradation upon requirement to generate energy and/or intermediates for use in other anabolic processes of the cell (Maughan et al. 1997). Most of the energy required by mammals to function properly is produced through the process of oxidation of lipids and carbohydrates. Though carbohydrates offer an immediate source of energy, lipids act majorly as energy reservoirs, while the FA in TAG are broken down as a backup energy source (McArdle et al. 2010). The energy stored in the form of lipids is more as compared to the energy stored in the form of glycogen due to the fact that mammalians organisms do not have the capability of storing more glycogen in relation to lipids.

In lipid metabolism different lipids such as bile salts, cholesterols, eicosanoids, glycolipids, ketone bodies, FA, phospholipids, sphingolipids, steroids and triacyglycerols (TAG) are differently digested, absorbed and transported within the mammalian organisms. The major aspects of lipid metabolism include fatty acid oxidation to produce energy, the synthesis of lipids, a process known as lipogenesis and everything is connected but glycolysis *per se* does not produce lipids and all of them are the most important procedures that are involved in generating fats in all living cells via blood glucose (Kersten 2001).

The process of fatty acid synthesis is known as lipogenesis and is catalyzed by the multienzyme fatty acid synthase (White et al. 2005). The fatty acid synthase enzyme catalyzes all the reactions required for the fatty acid synthesis. The first step of

lipogenesis is covalent linking of acetyl-Co-A or malonyl-Co-A units, which are the precursors of fatty acids.

TAG, as we saw before, is made up of three FA chains bound to a hydrogen-containing compound called a glycerol. FA then can be liberated when the body requires these substrates for energy.

The digestion of lipids taken in as food takes place in the small intestine. The bile salts are utilized for the process of emulsification, the lipids are hydrolyzed into fatty acids, soaps, monoglycerides, diglycerides or glycerol by the pancreatic lipase (Silverthorn 2007). Though it is not yet clear in what form the lipids pass through the intestinal walls but it is known that, TAG exists in the blood and lymph system. Due to the fact that lipids cannot dissolve in water, they are transferred in the form of lipoproteins through the blood after combining with proteins that are soluble in water in the blood as it is the case for FA too. The supply of lipids in the blood is fairly constant, though their concentration rises after digestion. The liver cells absorb blood lipids and thus produce energy for the functioning of cells. The liver is also responsible for maintaining the required amounts of lipids in blood. Also the lipids are utilized by the brain cells to help synthesize nerve and brain tissues (Mooren & Völker 2005). The extra lipids in the blood are usually transformed to adipose tissues. In the event that lipid concentration in blood becomes too low, lipids are synthesized from other foods or removed from storage by the body. The body also disposes some forms of lipids such as soaps, FA or fats as part of the solid excretion of human beings. It is also known that when the body levels of cholesterol and triglycerides are quite high, they contribute to the hardening of arteries, also called atherosclerosis, which is due to the fact that lipids cannot dissolve in blood thus end up being deposited in the arteries walls, forming hard structures named plaques (Li et al. 2014).

2.3 Skeletal Muscle Lipid Metabolism

The average male bodyweight owes 40% of its weight to skeletal muscle; this organ is responsible not only for movement but for processing large amounts of energy even during resting conditions. Skeletal muscle comprises the greater insulin-sensitive tissue

in the body and is the primary position for insulin-stimulated glucose utilization. Skeletal muscle resistance to insulin is underlying to the metabolic dysregulation related to obesity and physical inactivity, leading to the development of the Metabolic Syndrome (MS). The inability of efficiently taking up and storing fuel, during times of scarcity (low insulin) or caloric abundance (high insulin) contributes to a whole body metabolic impairment, cardiovascular risk and finally to increased risk of metabolic disorder (Stump et al. 2006, Welch et al. 2014). A potential mechanism to reduce insulin signaling and action in skeletal muscle, is adipose tissue expansion and increased inflammatory adipokines, as well as increased Renin-Angiotensin-Aldosterone System (RAAS) activity, and decreases in muscle mitochondrial oxidative capacity. Last but not least increased intramuscular lipid accumulation, and increased reactive oxygen species (Stump et al. 2006).

More recent research in muscle lipid metabolism, has been started using the consideration of IMTG turnover, in which concept there is a balance between lipolysis and lipid synthesis (Moro et al. 2008). Sarcolemmal fatty acid transport and binding proteins can increase skeletal muscle fatty acid uptake (Bonen et al. 2007). Fatty acids are coupled to a co-enzyme A (coA) group upon their way into muscle cells. Thus, they are shifted against either anabolic processes, including incorporation into neutral lipids and storage as lipid droplets for later use, or catabolic processes, by being the central place to be broken down into acetyl-CoA in β-oxidation (Kanaley et al. 2009). The first step according the biochemical pathway of human myocyte lipid metabolism is the transportation of fatty acids into the mitochondria. The carrier substance that is used here is carnitine (Figure 1). Once inside the mitochondria, the fatty acids divides away from the carnitine and are degraded in β-oxidation and oxidized finally in the citric acid cycle (Nelson & Cox 2000).
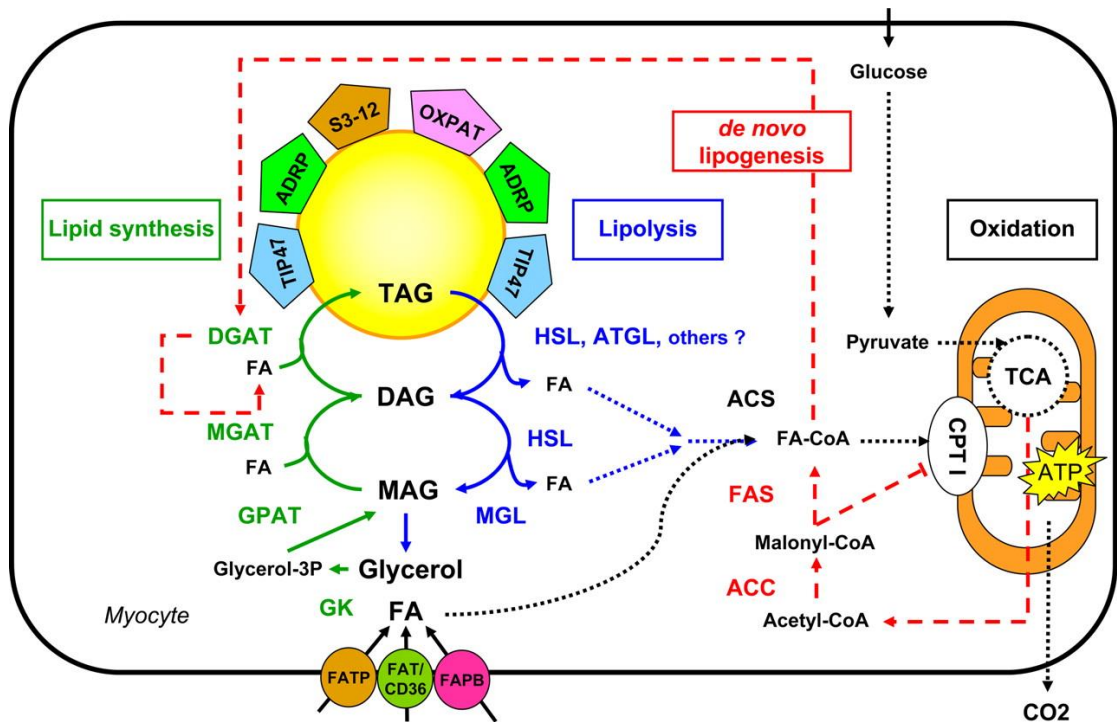
FIGURE 1. Biochemical pathways of human myocyte lipid metabolism. Fatty acids are entering human myocyte through fatty acid binding protein (FAPB), fatty acid translocase (FAT/CD36) and fatty acid transport protein (FATP). Mitochondrial β-oxidation is taking place by delivery of free fatty acids into the mitochondria either by esterification or/and oxidation. In green is represented the lipid synthesis pathway. In degradation of TAGs, adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), those two crucial enzymes are taking part. In blue, lypolysis is represented, de novo lipogenesis in red, mitochondrial oxidation in black. (GK=glycerol, GPAT=glycerol-3-phospate acyltransferase, MGL=monoglyceride lipase, MGAT=acyl-coenzyme;monoacylglycerol acyltransferase, ACS=acyl-coenzyme A synthase, ACC=acetyl-CoA carboxylase, FAS=fatty acid synthase, TCA=tricarboxylic acid cycle, CPT I=carnitine palmitoyltransferase I). Adjusted from Moro et al. 2008.

3 ANIMAL MODEL AND RELEVANCE TO HUMANS

In physiology, rodents are the most commonly animal model used because of their genetic background and their physiological resemblance to humans. Mice are the model that is similar to humans and someone can easily standardize the factors in order to study specific parameters. As Kim et al. 2004, has shown in studies using mice in order to study about metabolic implications placing rodents in a high diet usually induces weight gain. The animal model studied in our experiment (C57BL/6J) was used because of its ability to develop T2D, hyperinsulinemia and hyperglycemia (Toye et al. 2005) and because it is a model affected by a high-fat diet. Moreover Carr et al. 2012 showed that this specific mice strain of C57BL/6J can store excess TAG as ectopic deposits in muscle and in the liver and not only in adipose tissue as expected.

4 INTRAMYOCELLULAR LIPID DROPLETS

Animal cells produce a large amount of neutral fats (sterol esters and TAG) and these, along with others, are stored as cytosolic reserves in the cell. Proper storage of lipids, since its synthesis is crucial for its efficient metabolic activity, both for prompt energy and structural demands of the cell, and in the majority of mammals, TAG are stored in the form of lipid droplets (LD) (Brasaemle 2007). In the skeletal muscle, after glucose is exhausted, energy is supplied by stored lipid reserves which have been observed as intramyocellular triglycerides (IMTG). It has been found that after absorption, due to fatty acid metabolic pathway defects, there is a decrease in uptake of fatty acids, and an increase in esterification and subsequent storage of lipids as intramyocellular TAG (Kelley & Goodpaster 2001). IMTG have long been known to be an important source of energy in skeletal muscles, but have been recently implicated in many metabolic diseases, including obesity and T2D. These intramyocellular (muscle) lipids are stored in the form of LDs and act as energy reserves in rest and during high energy expenditure status, like exercise (Kiens 2006; van Loon 2004) but in excess, they have been successfully linked to muscle IR (Kelley & Goodpaster 2001; Guo 2007).

High levels of IMTGs have been found to activate a surge of intracellular FA, leading to inhibition of insulin signaling (Guo 2007). The back and forth mechanism of its action and its overproduction or underutilization is the main reason for its over-accumulation in skeletal muscle, and for subsequent metabolic disorders. A link has been identified between accumulation of TAG in skeletal muscle and IR (Goodpaster et al. 2001, van Loon et al. 2004). Moreover, regulated diet and exercise can augment metabolism of glucose and FA in patients with clinical conditions, like T2D and lifestyle disorders like obesity (Kelley & Goodpaster, 2001).

4.1 Biogenesis

Lipid droplets, unlike other organelles, do not self-replicate and it appears that are formed *de novo* by fat aggregation and aided by specific proteins. The precise mechanism of their formation has not yet been proven due to their dynamic arrangement, mobility alterations, low extraction efficiency and narrow structural data from electron microscopy. Many studies propose the fact that the enzymes that catalyze the last synthesis steps of TAG and cholesterol ester (CE) (*diacylglycerolacyltransferases* and *acyl-CoA:cholesterolacyltranserases*), reside in the endoplasmic reticulum (ER) (Jacquier et al. 2011).

When the newly-synthesized lipid esters in the ER accumulate above the basal level, required for phospholipid layer formation, they start forming a lipid mass between the ER membranes and falls off like a bud, or hatches out, once it reaches a certain limit (Fujimoto et al. 2008). Those lipids, such as TAG, are insoluble in water and their hydrophobic core is covered by a single layer of phospholipids, embedded with a number of coat proteins (Figure 2). These structures, the LDs have been studied mostly in adipocytes and is not until recently that they were recognized as highly dynamic organelles (Ohsaki et al. 2008; Farese & Walther 2009). LD size and number varies remarkably by cell type, and they can change their size expeditiously rely upon changes in metabolic status.
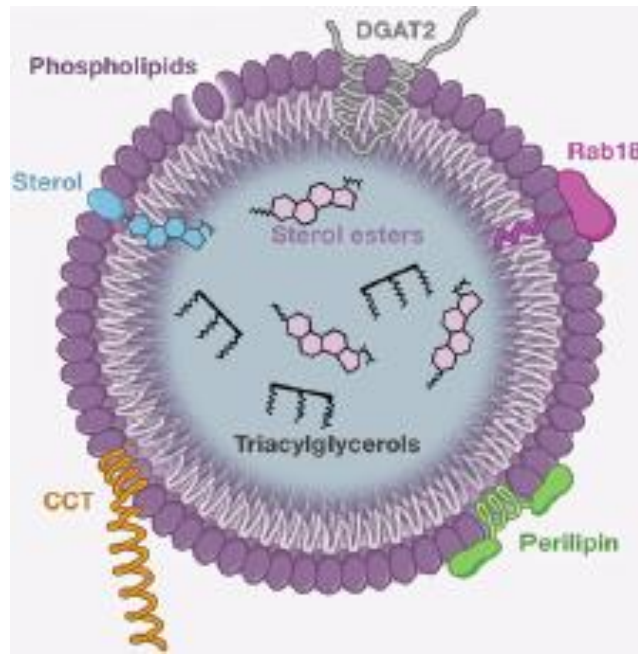
FIGURE 2. The Lipid Droplets structure and components in adipocyte. (Adapted from Krahmer et al. 2009)

There are also hypotheses for the potential of LD expansion, by taking up TAG by a nascent lipid droplet and stabilizing it against coalescence by phospholipids, like phosphatidylcholine (Walther & Farese 2012; Bulankina et al. 2009; Soni et al. 2009).

There are mechanisms other than mutual fusion/fission cycles that allow for LDs to grow in size. They can do so by taking up more lipid esters by infusing nascent lipid esters that are newly formed from the ER-LD structural connection or by producing new lipids *de novo,* inside themselves (Fujimoto et al. 2008). LDs can accumulate in various sites. They are mostly stored in adipose tissue, and in some insects and most vertebrates, they are stored in highly specialized white adipocytes and adipose tissue. After their formation, such droplets need necessary proteins for maintenance, modification, lipid storage and proper involution in order to remain stable. For such functions, there are different proteins linked to lipid droplets, depending upon their cell type and role, such as PLIN and heat shock proteins (Yamaguchi et al. 2006; Jiang et al. 2006). There have been studies on genes that regulate LD formation, and some genes that have been identified by knockdown studies, such as fat-inducing transcript proteins (FIT), seipin protein gene ortholog (FLD1), have been linked to LD formation and maintenance.

These proteins have been found to be critical in LD function and LD formation, and growth got diminished once these proteins were inactivated.

4.2 Biochemistry and Functions

LDs are cytoplasmic fat aggregates that sequester neutral fats (TAG and cholesterol esters) in their core, covered by a single phospholipid layer around them. They are unique in architecture, are found ubiquitously across organisms and have been linked to many vital cellular functions, such as energy production, cell membrane formation and protein degradation (Thiele & Spandl 2008; Sato et al. 2002). They are microscopic round structures of diameter of 0.1 - 5 mm in non-adipocytic cells (but can go up to100 mm in white adipocytes cells) and its phospholipid layer might be composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (Fujimoto & Parton 2011, Fujimoto et al. 2008).

Due to their different structure and small size, they have been studied using various techniques, like light microscopy, phase contrast microscopy and electron microscopy. Recently, it has been revealed by conventional and cryo-electron microscopy that the composition and distribution of the cholesterol esters and TAGs in the core differs, depending upon the type of cell and the method of sample preparation (Fujimoto et al. 2008). Outside the phospholipid layer, proteins had been postulated to be embedded or adhered to the LD surface; but some recent studies have also suggested the presence of some proteins in its core (Robenek et al. 2004; Cermelli et al. 2006). These organelles have dynamic architecture and have been proven as organizing centers for synthesis of specific lipids, such as TAG, found to be linked to protein storage and recently found to be involved in Hepatitis C Virus (HCV) assembly (Athenstaedt et al. 1999; Kuerschner et al. 2008; Herker & Ott, 2011).

Lipids of LDs can be fragmented via sequential lipolysis in adipocytes, by enzymes like ATGL (adipose triglyceride lipase), HSL (Hormone-Sensitive Lipase), MGL (monoacylglycerol lipase) etc., to generate energy and membrane lipids. Alternatively, LD lipids can also be degraded within lysosomes by lysosomal lipases, by a process called as macroautophagy. This process has been noticed being induced by nutrient

deprivation and hormonal signals. These enzymes are recruited in a regulated manner to efficiently release the pool of FFA, to be utilized for energy release, membrane formation, used as cofactors for signaling or to be exported out of the cell.

LDs have been assigned many functions. The canonical function of LDs is the storage of neutral lipid esters, a mechanism by which it ensures proper energy release upon requirement and also prevents lipotoxicity in the cell (Schaffer 2003). The lipids stored inside LDs are protected from lipases and also promotes TAG production, which helps mammalian cells to bypass toxicity posed by saturated fatty acids. Some enzymes such as triglyceride hydrolase (TGH), required in lipid metabolism, have been shown to be linked to the incorporation of TAGs to the lipid droplet; they have also been implicated in the better mobilization of lipids during cellular needs. Enzymes involved in lipid metabolism (fatty acid, steroid, eicosanoid synthesis and activation) have been found in LDs and thus suggest LDs as a site for formation and metabolism of signaling lipids.

LDs, contrary to earlier belief, are not just depots of lipid storage. Rather, they have been recently found to have other vital functions too, such as protein sequestration, providing template for proteasome degradation, intracellular molecule trafficking. As seen in hepatocytes, lipid apolipoprotein B-100 (ApoB) accumulates in LDs when either the proteasome or autophagy is inhibited (Ohsaki et al. 2008). This suggests LDs as a temporary accumulation site to store the proteins by hydrophobic interactions, and aid in their proteasome mediated degradation.

In contrast, there are some proteins, like histones in *Drosophila melanogaster* that are sequestered inside LDs, probably via electrostatic interactions, and are released at a later stage of development when they are an actually requisite to the cell. They have been found to remain associated with organelles like ER, and a proximity to mitochondria and peroxisome has also been observed. These findings lead to speculations of LD to be associated with molecular trafficking of ER products, lipid metabolism after beta-oxidation in mitochondria and peroxisomes and fluorescence resonance energy transfer (FRET) between the intermediary molecules, to aid molecular energy transitions after Krebs cycle (Fujimoto et al. 2008). Though, more research is needed about these highly dynamic organelles LD's and still many of their functions are not fully understood, after considering that a great number of proteins are in association with them.

4.3   Distribution

LDs are dynamic entities and have interactions with different organelles inside the cell. Due to their unique structure and unpredictable nature, their distribution in the cell is also not completely clear. Not only have they been found close to ER, mitochondria or peroxisomes, but also, found dispersed randomly inside the cell. In mammalian cells, their distribution has been found to follow a pattern, sometimes organized into lipid superstructures. Their distribution in the cell may be partially determined by active transport via microtubules (Guo et al. 2008; Savage, Goldberg & Schacher 1987). Here, it is vital to mention once more, that most of the research has been carried out in adipocytes and not in skeletal muscle until very recently (Shaw et al. 2009), where the adipose tissue seems to have one or few droplets in the center whereas the small droplets disposed towards more peripheral locations (Murphy 2001).

Their varied distribution patterns and involvement in vital metabolic activities, along with recent advancements in microscopy and cell biology techniques, has given LDs profound attention for research, in the hopes of using them as a loading and delivery agent for efficient intramolecular and intermolecular trafficking of active lipids and proteins (Bosma et al. 2012a).

4.4   Muscle cell characteristics

As it is already mentioned, myocyte arrangement is totally different than the adipocyte one as well as other cells. In skeletal muscle cells, LD's are commonly located in the vicinity of Z-lines adjacent to intermyofibrillar (IMF) mitochondria (Hoppeler et al. 1973; Tarnopolsky et al. 2007; Shaw et al. 2008). It has been observed that there is an interaction between those two organelles, LDs and mitochondria. They do exist, both organelles in two different regions of the cell, in subrarcolemmal (SS) region close to membrane and in IMF region far from the membrane (Shaw et al. 2008). As it has been pointed out most of the times LDs only sporadically surpass 1 micrometer of diameter, being present either in cells close to IMF mitochondria or SS in muscle, allowing

matching of stored lipids with decisive completion of lipids for fuel (Hoppeler et al. 1973; Tarnopolsky et al. 2007; Shaw et al. 2008).

Muscle fibers are categorized in specific fiber types. Slow-twitch muscle fibers (type I) are oxidative and on the grounds that they have large amounts of mitochondria, they have high rates of oxidative metabolism. These oxidative type I muscle fibers have been proved to have more LDs than the glycolytic ones, fast-twitch muscle fibers (type II) (Walther & Farese 2012). In addition to this, it has been shown that there is a greater aggregation of IMTG in obese individuals in type I oxidative muscle fibers and most likely this is linked with IR (Walther & Farese 2012). Besides that, is already known from Malenfant et al. 2001 that type I fibers tend to obtain more fat than the type II muscle fibers.

4.5   Lipid Droplet Coat Proteins

The regulation of the formation of LDs is adjusted by a family of proteins which are greatly phosphorylated and located at the surface of the LD. Perilipins (PLINS) are the best characterized and the most important proteins that coat lipid droplets (Bosma et al. 2012a). PLINS are not limited to the surface of the LD but also have access to the lipid core and thus can associate with other cellular components necessary for the biogenesis of LDs (Wolins et al. 2006a). Following activation by protein kinase A, they move from the LD allowing HSL enzyme together with ATGL and MGL that hydrolyzes adipocyte triglycerides to produce non esterified fatty acids (NEFA). Moreover, they allow glycerol to be utilized in the body metabolism and hormone regulated lipolysis in adipocytes. The PLIN genes in the human body have been mentioned to be related with the difference in body weight maintenance and may be influencing obesity and may be associated with T2D (Wolins et al. 2006a, Wolins et al. 2003).

PLIN1, known as Perilipin was the first member of perilipin family and is an adipocyte protein, found in adipose tissue (Bickel et al. 2009). PLIN2, also known as Adipophilin, ADPR or ADPF is ubiquitously expressed and found to be constitutively bound to lipid

droplets (Bickel et al. 2009). PLIN3, also known as Tip47, M6PRBP1 and PP17, is known to be found throughout the cytosol and is associated with the intracellular transport of mannose-6-phosphate receptors (Brasaemle et al. 1997). The PLIN protein PLIN4, also known as S3-12 has a sequence similar to others perilipins and it can be found mostly in white adipose tissue. It is worth noting that for the formation of S3-12 on the surface of the LDs, fatty acids and glucose are required and that they can be included in the TAG (Wolins et al. 2003).

Lipid droplet proteins like PLIN1, PLIN2, and PLIN3 control cellular neutral lipid stock. PLIN5, the most recently discovered of PLIN proteins is expressed in exceedingly high oxidative tissues. This protein is referred to as OXPAT, Isdp-5, MLDP and PAT-1 (Kimmel et al. 2010). PLIN5 is found on lipid droplets with the PAT protein adipophilin in primary cardiomyocytes. FA induced TAG accumulation is promoted by ectopic expression of OXPAT in addition to long chain fatty acid oxidation and mRNAs related to oxidative metabolism. From studies carried out before, they show that OXPAT helps in creating the positive responses to the burden of fatty acids that is accompanied by insulin deficiency, over nutrition, and fasting though these responses are not effective with obese populations (Wolins et al. 2006a). It is worth noting here, that there is evidence that in insulin-resistant populations, LDs are bigger in size where at the same time the location and the total amount of PLIN proteins is different from a common setting in those subjects (Meex et al. 2009).

5 INTRAMYOCELLULAR LIPIDS AND INSULIN RESISTANCE

5.1 General

A state in which responsiveness to normal, circulating, levels of insulin is reduced can be defined as insulin resistance (IR) (Savage et al. 2007). Furthermore IR is one of the trademarks of T2D and MS. The pre-mentioned condition, is usually associated with body malfunctions such as obesity, as demonstrated by below normal rates of whole-body glucose uptake during normal level circumstances of glucose in the blood – euglycemic- or excess levels of insulin circulating –hyperinsulinemic clinch (Felber et al. 1987). Moreover, IR is correlated with prolonged physical inactivity, and/ or ectopic lipid accumulation. It has been demonstrated that especially weight loss promote insulin-mediated glucose disposal by strengthening both oxidation and glucose storage in skeletal muscle (Henry et al. 1986). Time after time has been proved that excess lipids might explain the molecular underpinnings of IR, as a result of a hypercaloric environment combined together with physical inactivity (Koves et al. 2008; Booth et al. 2012; Thyfault 2007).

Several mechanisms have been claimed to be accountable in the development of IR. To name one of them, ectopic lipid augmentation (Samuel & Shulman 2012). Already Randle et al. (1963) have shown in studies carried out in rodent heart that muscle's fatty acid impaired insulin-mediated glucose uptake by restriction of pyruvate dehydrogenase, leads to reduced glucose oxidation and accumulation of glycolytic intermediates (Randle et al. 1963). Moreover, there are indications that not only the circulating fatty acids are a decent predictor for muscle insulin resistance but the IMTG content is a stronger one, suggesting that elevated intramyocellular lipids (IMCL) may cause muscle IR (Krssak et al. 1999). Because the principal site of insulin-stimulated glucose clearance is skeletal muscle, it is of high importance to comprehend the mechanisms of lipid-induced metabolic disorganization of skeletal muscle.

5.2 Skeletal Muscle

Glucose transporter type 4 (GLUT4) is a protein and is the insulin-regulated glucose transporter that can be found primarily in adipose tissue and skeletal muscle and can increase glucose-transport by its stimulation. During exercise, muscles utilize glucose in large amounts, even when insulin has no presence in blood and it has been shown that muscle sarcolemmal glucose-transport during exercise is primarily due to GLUT4 translocation (Richter & Hargreaves 2013).

In muscle, fatty acids are essentials for oxidation and energy production (Gyuton & Hall 2006). Lipids are placed ectopically into insulin-sensitive tissues, such as skeletal muscle and can be found in the liver as well, when average adipose tissue storage capacity surpasses the average one and this leads to the development of IR (Frayn et al. 2006). The dominant theory is that increased FA oxidation may be responsible for IR, shown by Randle et al (1963), the so-called Randle cycle. On the other hand, some studies are not fully satisfied with this theory of underlying the effects of FFA on glucose metabolism like Savage et al. (2007). Furthermore, some studies have reported that glucose, rather than FFA, can inhibit fat oxidation in skeletal muscle, known as the 'reverse' Randle cycle (Sidossis & Wolfe 1996).

Bruce et al. (2003), indicates the skeletal muscle oxidative capacity as a better predictor of insulin sensitivity than IMTG concentration or long-chain fatty acyl-CoA content. Moreover, there are other studies that have observed a negative correlation or no association between IMTG content and insulin sensitivity (Krssak et al. 1999; Goodpaster et al. 2001).

However, later studies from Borén et al. 2013 show that lipotoxicity in skeletal muscle and the liver, mainly ectopic tissues is highly associated with insulin resistance. Last but not least, someone must make allowance for the fact that impaired insulin-stimulated glucose uptake, is likely to happen barrens of alterations in insulin signaling pathways (Goodpaster 2013). At that point, it is important to take a closer look at elevated amounts of lipids in skeletal muscle, which could be served as the ground of IR or one of its origins, owing to high-fat diet and sedentary lifestyle (Koves et al. 2008).

6 EXERCISE AND DIET IN LD PHYSIOLOGY

Physical exercise is the body movement performed by an individual with the aim of increasing energy expenditure beyond normal level (Tarnopolsky et al. 2007). Exercise may involve repetitive movement of body parts or non-sportive activity and it is classified into three categories according to level of intensity: light, moderate and vigorous exercise (Ahlborg et al. 1974). According to the effort that is required in any physical activity, three main different metabolic pathways are followed. First one, is the ATP-PCr system or Phosphogen system, in which phosphocreatine (PCr) breaks down, releases a phosphate and energy in order to rebuild ATP, it is anaerobic and activated in more explosive exercise (Wallimann et al. 1992). Secondarily, there is the glycolytic system, which comes up when the previous one- ATP-PCr – runs out. During glycolysis, carbohydrates, either in blood glucose form or muscle glycogen, is broken down through some chemical reactions in order to form pyruvate and in turn to produce ATP. The final product of glycolysis can either be lactic acid, named as anaerobic glycolysis or can remain pyruvate named as aerobic glycolysis (Yin et al. 2007). The last metabolic pathway, the most complex one is the oxidative system, is dependent to oxygen and activated mostly for long termed low intensity exercise. Four processes are taking part in order to produce ATP in the oxidative system: aerobic glycolysis, Krebs cycle, electron transport chain and β-oxidation. It is worth mentioning that all these pathways do function simultaneously, although different physical activity types promote the use of some pathways over others (Gastin 2001).

Physical activity appears to increase IMTG. This increase is associated with decreases in size and function of mitochondria, as well as with total body fat (Goodpaster & Wolf 2004). Nevertheless Tarnopolsky et al. 2007, has shown that endurance training equitably increases not only IMTG number in total but the number of LDs, the size of mitochondria, their colocalization and finally the FA oxidation. Obese subjects seem to have increased size of LDs compared to controls and endurance exercise seems to decrease it (He et al. 2004). Intensive exercise decreases hyperglycemia and enhances insulin sensitivity, while increasing glucose transport in the skeletal muscle through stimulation of insulin and reversing the hepatic insulin resistance (Moro et al. 2009).

Roger et al. 2010 suggest that adipogenesis delays the MS induced by prolonged caloric surplus. Diet-induced weight loss alone or combined with exercise does not affect the muscle TAG depots though insulin sensitivity is improved considerably (Krssak et al. 1999). Sedentary lifestyle facilitates the accumulation of FFAs in the plasma and leads to ectopic deposition of lipids in other body tissues (Schrauwen-Hinderling et al. 2007).

Ectopic lipids usually accumulate in form of TAG. Reduction in the oxidation of lipids in the skeletal muscle can contribute to the accumulation of IMTG, IR and lipotoxicity. Sedentary subjects are exposed to lipotoxicity due to decreased IMTG (Russell 2004). Studies show that IMTG is related to total body fat in sedentary people (Hulver et al. 2003). Moro et al. 2009 suggest that high adiposity result in the accumulation of IMTG while intramyocellular DAG determines the extent of IR in sedentary people. Paradoxically, individuals who are engaged in physical exercise display higher total IMTG levels compared to IR populations, a phenomenon known as the athlete's paradox (Goodpaster et al. 2001; Van Loon & Goodpaster 2006). Van Loon et al 2004 suggest that elevated rates of IMTG in athletes is not entirely dependent on fiber type distribution. Type I fibers are more oxidative and have greater amount of IMTG in contrast to type II fibers. He et al 2001 also showed that elevated IMCL amounts found in obese populations in skeletal muscle are independent of fiber typing. Increased IMTG concentrations can be explained by increasing FFA delivery to the training muscles (Corcoran et al. 2007). Studies have shown that a single resistance exercise session can improve IS and, likely due to reductions in IMTG (Koopman et al. 2006). Likewise, many studies have shown that a single endurance session results in the reduction of IMTG concentrations (Roepstorff et al. 2002, Watt et al. 2002). An acute training session or a chronic or resistance exercise lead to decreased IMTG concentrations, being the lipids the main energy source in aerobic long duration exercise (Van Loon 2004).

Besides different types of exercise, different types of diet play an important role in LDs also. High fat diet in humans is correlated with body fat content (Miller et al. 1990). High fat diet impairs glucose tolerance, decreases IS and the insulin suppression of hepatic glucose production and disposal (Cha et al. 2001). The highly consumption of lipids affect glucose metabolism, IS and IR. High fat diet promotes obesity, which is a

high risk factor for developing T2D and cardiovascular diseases (Eckardt et al. 2011). Furthermore, hypocaloric low fat diet results to weight loss in obese people compared to high fat calorie-restricted diet. There is a major effect of dietary lipids on fat and weight loss when someone is subjected to calorie restrictions. Therefore, fat accretion in the adipose tissue is a result of increased transport of adipose glucose (Zierath 2002).

In summary, diet and exercise play an important role in LDs physiology, many studies have showed so. Tarnopolsky et al. 2007, showed that sometimes IMCL content can increase even after exercise, the same phenomenon that Goodpaster et al. 2001 named as "paradox". Endurance trained athletes, meaning high insulin –sensitive subjects have almost the same amount of IMCL content to those obese or T2D subjects, which are clearly insulin-resistant (Dubé et al. 2008). Diet-induced weight loss, decreased fat mass and decreased adiposity, may lead to decreased amount of IMCL, as well (Kallunki et al. 1992). This has been confirmed by Goodpaster et al. 2000, reduction of body weight can reduce IMCL content. Same authors discussed about elevated lipid accumulation in obesity regarding fiber types and decreased amounts of those lipids when weight loss is taken place. Then again, physical activity sessions can point out increased IMCL or even unaltered IMCL (He et al. 2004). For those reasons more research need to be carried out in order to specify the effects of diet and exercise in LDs and in which depth LDs are affected by different types of diet and physical activity.

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7 PURPOSE AND HYPOTHESES OF THE STUDY

Intramyocellular lipids seem to be affected by acute and chronic exercise, by the absence of physical activity and different patterns in diet. The present thesis aims to raise a research question and investigate about the effects of physical activity, sedentary lifestyle and diet change on intramyocellular lipid droplets in mice. Over and above, the aim was to better understand the lipid droplets topography on mouse skeletal muscle. Ultimately, this can conduce to the development of sufficient strategies about physical activity and diet that can diminish the risk factors that are affiliated with the IR, obesity, metabolic syndrome and other related disorders.

Hypotheses:

➢ The size and number of lipid droplets in mice skeletal muscle is altered with exercise.
- No exercise (sedentary lifestyle)
- Acute exercise (maximal test and mice sacrificing immediately after or 6hours later)
- Chronic exercise (voluntary running)

➢ The size and number of lipid droplets in mice skeletal muscle is altered with diet.
- Low-fat diet (control diet)
- High-fat diet

The purpose of this study is to assess intramyocellular LDs between different diet and exercise groups, and therefore understand in more depth the LDs and their behavior and contribution in the skeletal muscle lipid metabolism.

8 METHODS

8.1 Experimental setup

8.1.1 Animals

The 68 mice used in the study were male from the genetic background of C57BL/6J type. All mice were obtained from Taconic (Taconic Europe, Ejby, Denmark) at the age of six weeks. They were housed in individual cages with standard conditions (temperature 22 ℃, humidity 50 ± 10%, light from 8.00 am to 8.00 pm).

8.1.2 Ethics Statement

The treatment of the animals was in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocol was approved by the National Animal Experiment Board, in Finland. All efforts were made to minimize suffering.

8.1.3 Experimental Design

The mice were divided into eight different groups according to the different applied interventions. More specifically, the groups were divided by _A_. dietary consumption into: 1) low-fat diet (control) and 2) high-fat diet and divided further according to their _B_. exercise into: 1) control which were following a sedentary lifestyle, either adopting a low-fat diet (**LFD,** n=10) or a high-fat diet (**HFD,** n=10), 2) chronic (voluntary wheel running) either adopting a low-fat diet (**LFDR**, n=10) or a high-fat one (**HFDR,** n=10) and 3) acute (running exercise in a treadmill until exhaustion). In addition, the acute exercise groups were separated by the time of sacrificing, either immediately after exercise (0h) or 6 hours later (6h0) following low or high-fat diet as well (**LFDR0,** n=7 or **LFDR6,** n=7) and (**HFD0,** n=7 or **HFDR6,** n=7). They were housed in individual cages under standard conditions and they have been acclimated to their new

surroundings for 1 week before they have been divided into the 8 intervention groups, as following (Figure 3):
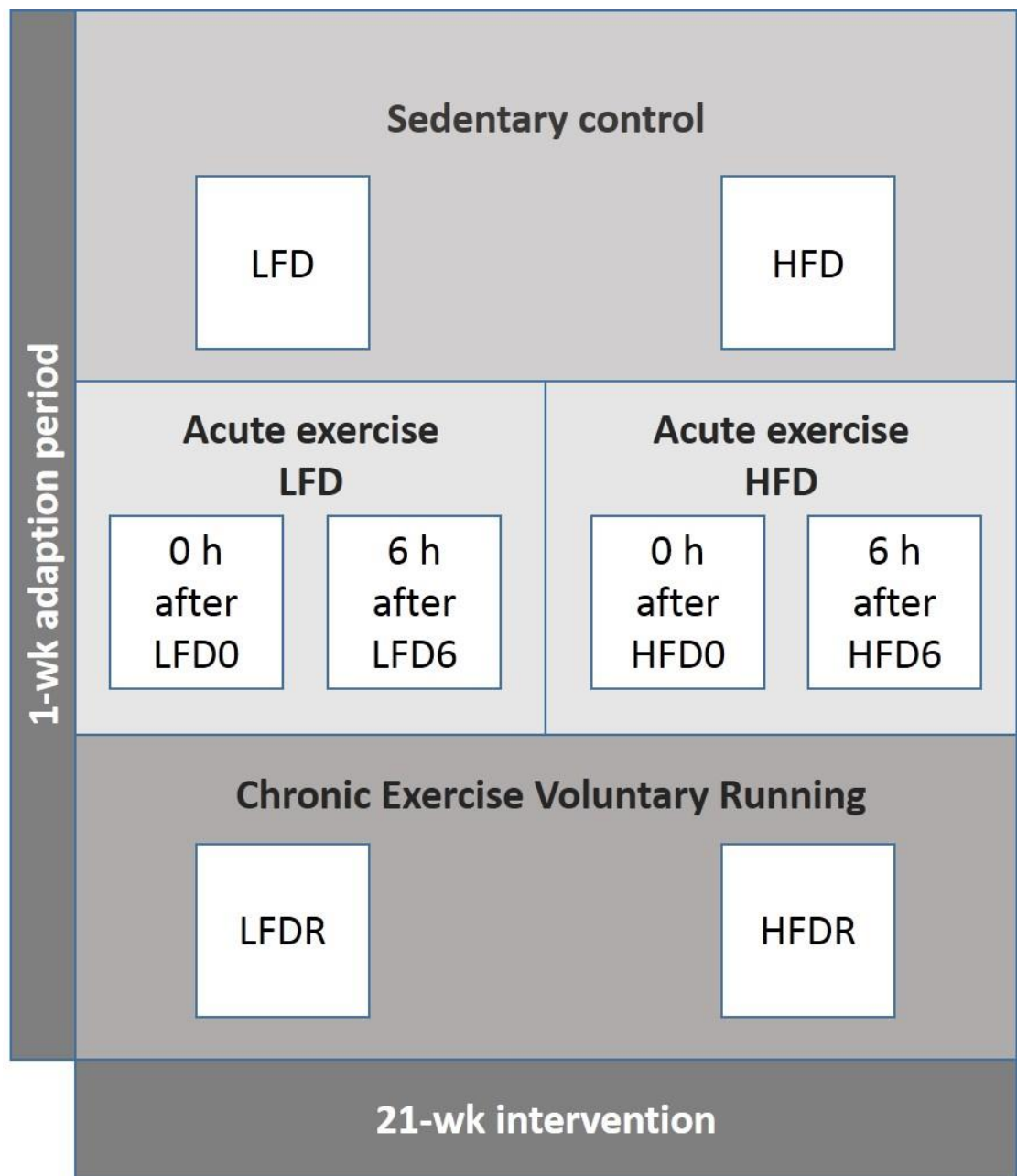

FIGURE 3.   Summary of study design. This diagram summarizes the experiment setup and the groups used in the study during the 21-weeks of the experiment.

8.1.4 Exercise modalities

The chosen exercise modalities were voluntary wheel running for the trained mice and a maximal run test to exhaustion for the mice performed acute exercise. The trained mice -chronic exercise group- were housed solitary in custom made cages with a free 24h/day access to a running wheel (12 cm diameter, 8 cm width) for 21 weeks. A magnetic switch was recording total wheel revolutions every day, with total exercise performed per day determined by multiplying the number of wheel rotations by the circumference of the wheel. Control mice were housed in a similar manner, without the running wheel.

During week 16 of intervention, all mice performed an enforced maximum running test on a motor-driven treadmill. An inclination of 0.8º was preserved during the test, and the starting speed was 10m/minute for 5 min and was gradually increased at 5-minute intervals by 1m/minute until exhaustion. During the whole test the total distance covered was recorded.

8.1.5 Diets

The mice had free access to tap water and food, either the standard rodent diet (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden) or the high-fat diet, a lard-based purified diet (D12492: 60% fat, 20% carbohydrate, 20% protein, 5.24 kcal/g; Research Diets, New Brunswick, NJ).

8.1.6 Muscle Dissection

After 21 weeks from the start of the experiment, all the mice were sacrificed by cervical dislocation. Immediately after, the proximal part of the right leg muscle gastrocnemius was surgically removed, weighted, snap-frozen in liquid nitrogen, and stored at -70 ºC until further analysis.

8.2   Immunohistochemistry

Sectioning and Fixing.

From the gastrocnemius muscle, 16μm cross sections were obtained in a cryostat at -25ºC (Leica CM 3000, Germany). Sections were collected in 13mm round coverslips and immediately fixed in 4% paraformaldehyde for 15 minutes in phosphate buffer saline (PBS).

Blocking and Primary Antibody incubation.

After 3x5 minutes PBS washing, the sections were blocked with 5% goat serum for 30 minutes (diluted in PBS-0,05%   saponin) and then   quick dip-washed in PBS. All primary antibody dilutions were made in 1% bovine serum albumin (BSA) as seen in Table 1, and incubated for 1 hour in dark/moisture at room temperature.

TABLE 1. Primary antibodies used and respective dilutions in PBS-0,05% saponin.

Purpose	a-gene	Species	Code	Manufacturer	Dilution
Type 1 fibers	Myosin heavy chain	Mouse	N2.261	DSHB	1:25
Membrane	cav-3	Rabbit	a cav-3	abcam	1:200

Secondary Antibody and BODIPY incubation.

Immediately after, each sample was washed 3x15 minutes with PBS-0,05% saponin before incubated with secondary antibodies in 1% BSA for 1 hour in room temperature dark/moisture accordingly to Table 2. A 3x10 minutes PBS washing followed, till this point all steps were still performed with 0.05% saponin. LDs were then stained with BODIPY 493/503 (Invitrogen) after neutral lipids, using a 1-500 dilution in PBS-0,05 saponin for 15 minutes in the dark.

TABLE2. Secondary antibodies used and respective dilutions in PBS-0,05% saponin.

Purpose	a-species	Species	Code/Wave-Length	Manufacturer	Dilution
Type 1 fibers	mouse	goat	DL405	Jackson ImmunoResearch	1:200
Membrane	rabbit	goat	546	Jackson ImmunoResearch	1:200

Negative Controls.

Negative controls were performed by verifying the absence of significant fluorescence signal in staining samples from the same muscle with either a) no antibodies whatsoever, b) only the primary antibody and c) only the secondary antibodies.

Mounting.

The mounting medium utilized was Mowiol with 2.5% DABCO, freshly added and no older than a week for anti-fading purposes. The medium was left to dry for at least 1 hour in the dark at 4C°.

For quantitative purposes all samples were observed under the microscope within 12 hours after mounting in a glass slide.

8.3 Laser confocal microscopy

Raw confocal microscopy data collection was performed with a Zeiss LSM 700 (Carl Zeiss AG, Germany). Images were collected using a 40x objective as shown in the following Table 3. As much as possible, similar exposure times were used when collecting images.

TABLE 3. Image acquisition parameters.

pixel size: 0.156μmx0.156μm

pixel dwell:0.64μs

pinhole for LDs/bodipy: 29μm (z thickness:0.9μm) 1Airy unit

lasers: 405/488/555nm

8.4   Image processing and analysis with TopoCell

General image processing.

After collection, all images were processed further with *ImageJ* (version 1.49; National Institutes of Health, Bethesda, MD, USA, (http://rsbweb.nih.gov/ij/download.html). With this software 8-bit images were displayed, edited, analyzed and processed for descriptive statistics purposes. All the images in this current thesis for quantification and visualization were treated and arranged respectively to each observation intention. In order to analyze further, a software package was created. TopoCell, identifies cells, identifies the particles within each cell, gives their coordinates, and allows to measure biophysical proprieties like size, distances, densities and distribution.

Image preparation for numerical data extraction.

In addition, for numerical analysis, all the processed images, followed a thorough and controlled methodology. Consequently, up to twelve 3-channel RGB images per subject were selected by sample and imaging quality. The RGB channels correspond to the imaging of Type1- myosin for fiber typing, caveolin for the membrane and neutral lipids for LDs.

The channel corresponding to the membrane staining was processed and binarized before segmented with the TopoCell segmentation tool, giving each cell a distinct

border from the background and ID value Figure 4A, 4B, 4C. After processed and binarized equally, the 2 remaining channels (LDs and fiber type) were then merged with the segmented cell channel Figure 4D.
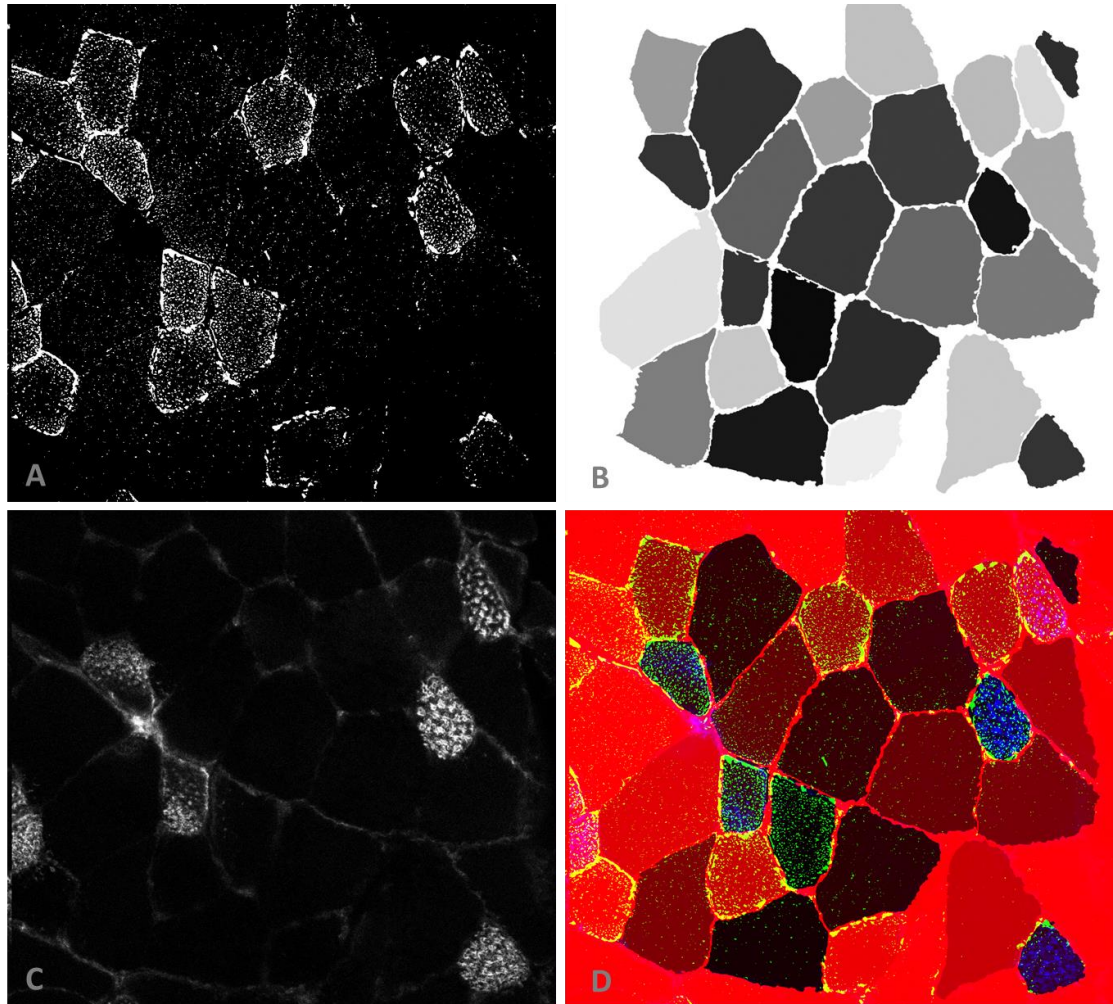
FIGURE 4.   Image preparation for analyses. (A) LD's. (B) Segmented cells. (C) Fast myosin (D) Merge of A, B, C.

Finally, a stack of these subject images was montaged into one mosaic image, including all cells from one subject, ready to proceed for quantitative analysis Figure 5.
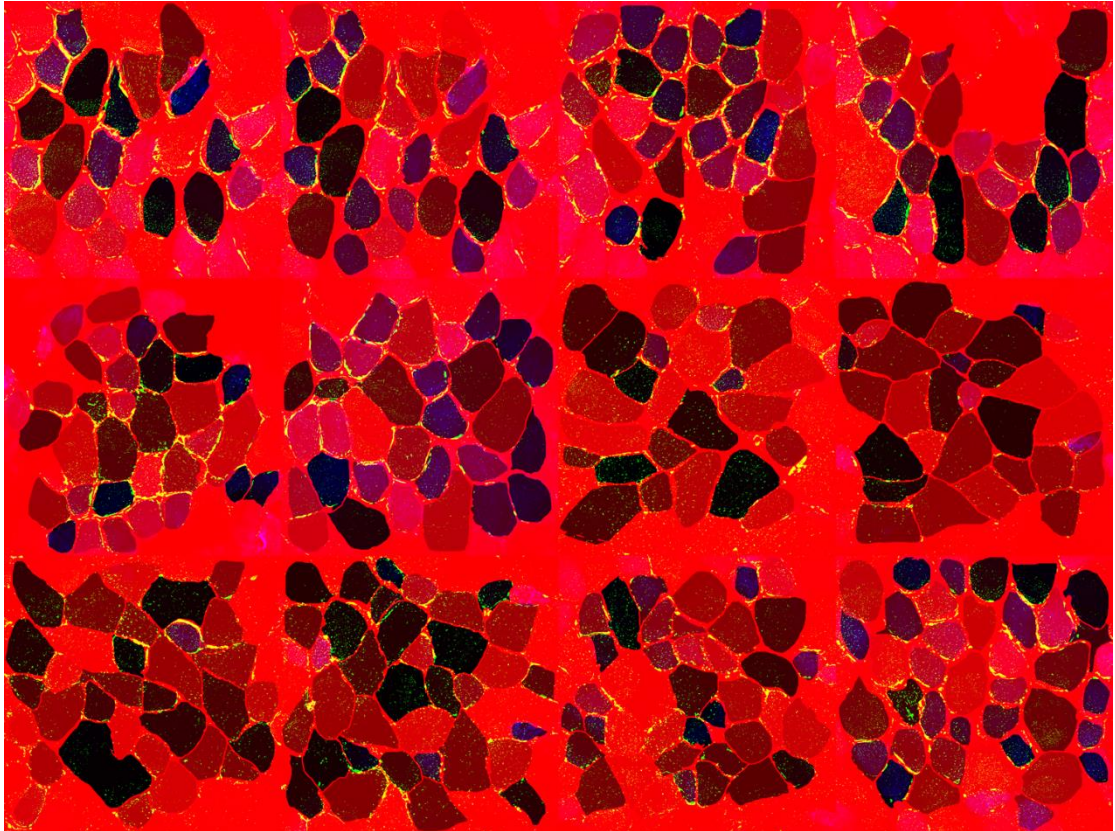
FIGURE 5.   Ready image for quantitative analyses, a montage image by all the cells of
one subject.

8.4.1   Image data extraction to numerical tables

Quantitative data, such as cell and particle size, number, intensity and coordinates, were assessed in order to determine intracellular topography of LDs in cells with different (positive or negative) myosin type 1 values. This was achieved with TopoCell, within ImageJ, by extracting image data into .tsv files (FIGURE 6).

```
Group    HFD
Subject 11
ParticleMap    A        lds     B       myosin1
#0        4650.000      50.438  228.072 0        9180.000      36.000  9134.000
3198.000
0       A      21.000  214.000 0.000   32.629  1.000   1.000   255.000 255.000 1.000
1       A      25.000  211.000 0.000   30.636  3.000   1.000   255.000 255.000 1.000
2       A      31.000  216.000 0.000   22.882  10.296  1.000   255.000 255.000 1.000
3       A      31.000  225.000 0.000   19.679  12.530  1.000   255.000 255.000 1.000
4       A      33.000  257.000 0.000   33.777  3.000   1.000   255.000 255.000 1.000
5       A      37.000  206.000 0.000   25.841  9.899   1.000   255.000 255.000 1.000
6       A      38.000  247.000 0.000   22.648  13.000  1.000   255.000 255.000 1.000
7       A      38.500  257.500 0.000   31.757  2.550   2.000   255.000 255.000 0.500
8       A      40.000  199.500 0.000   30.419  6.500   2.000   255.000 255.000 0.500
9       A      41.000  241.000 0.000   16.006  19.026  1.000   255.000 255.000 1.000
10      A      42.000  255.000 0.000   28.219  5.385   1.000   255.000 255.000 1.000
11      A      42.500  261.000 0.000   33.871  0.500   2.000   255.000 255.000 0.500
12      A      43.500  192.000 0.000   36.734  0.500   2.000   255.000 255.000 0.500
13      A      45.000  206.000 0.000   22.732  13.928  1.000   255.000 255.000 1.000
14      A      48.333  261.500 0.000   33.494  0.601   6.000   255.000 255.000 0.907
15      A      51.000  224.000 0.000   4.111   31.401  1.000   255.000 255.000 1.000
16      A      54.000  222.000 0.000   7.040   30.067  1.000   255.000 255.000 1.000
17      A      55.000  231.000 0.000   5.421   30.000  1.000   255.000 255.000 1.000
18      A      55.000  263.000 0.000   35.224  0.000   1.000   255.000 255.000 1.000
19      A      57.000  253.000 0.000   25.777  9.000   1.000   255.000 255.000 1.000
20      A      62.000  192.000 0.000   37.880  1.000   1.000   255.000 255.000 1.000
21      A      65.000  207.000 0.000   25.615  15.000  1.000   255.000 255.000 1.000
```

FIGURE 6. tsv data within ImageJ

8.4.2 Variable definition and data filtering

Treatment of the raw numerical data in order to first create - and then analyse –variables like size and density, defined by different fiber type and cell density was performed by TopoCell tools within Matlab.

8.4.3 Statistical analysis

All data are presented as mean $\pm SE$. All statistical analyses were performed using Matlab© (Mathworks, Natick, Massachusetts, U.S.A). Correlation significance levels were set at $P<0.05$ (represented as *) and $P<0.01$ (represented as **). All comparisons were performed between all the groups. Data variability is reported with standard deviations. At this point it is crucial to mention that due to the high "n" (statistical cases), despite a non-normal distribution, t-tests were appropriate to use.

9   RESULTS

9.1   Fiber type LD size

Lipid droplet size in Type I and II fibers. In the examined gastrocnemius muscle, regarding fiber type differences, LDs size appeared to be significantly bigger in type I when compared to type II fibers in most groups (P<0.05). The only exceptions were in HFD6 and LFDR where type I and type II fibers were not different (P>0.05) (Figure 7).
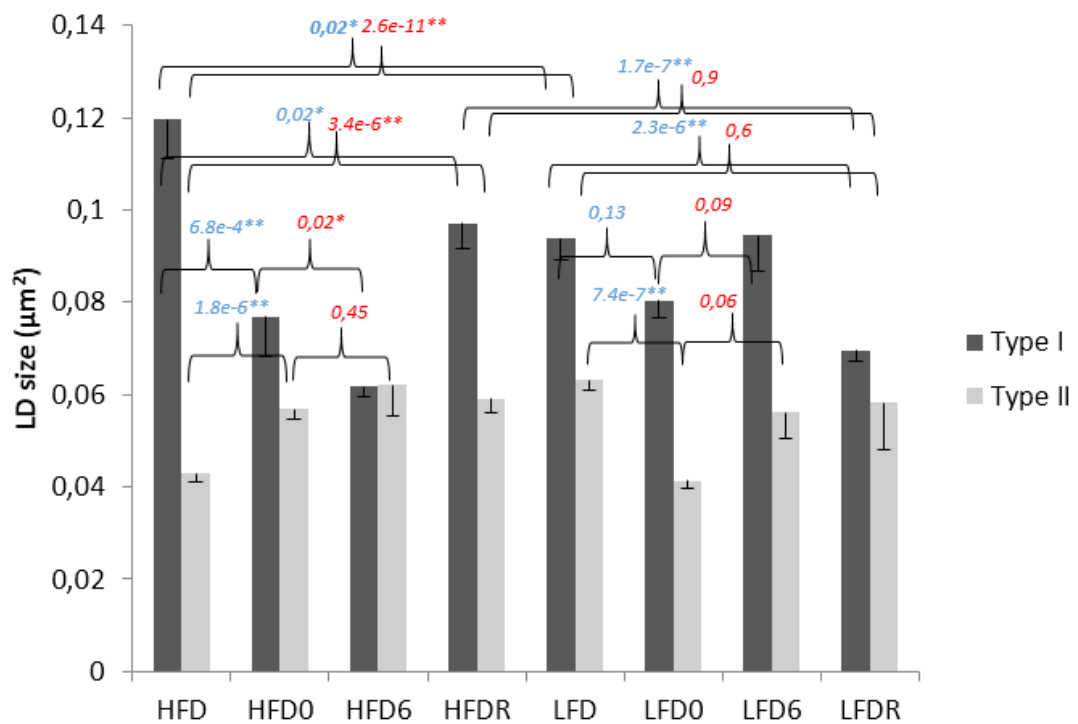
FIGURE 7. LD size in different gastrocnemius fiber types. Values are reported as mean$\pm$**SE**. Significance levels were set at P<0.05 (represented as *) and P<0.001 (represented as **).

Diet effect on LD size of sedentary subjects. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFD type I fibers are significantly bigger than in LFD type I (P<0.05). LDs in HFD type II fibers are significantly smaller than in LFD type II (P<0.001) (Figure 8).

FIGURE 8. Lipid droplet size in HFD and LFD sedentary control groups. Values are expressed as means$\pm$ *SE*. P<0.05 *, P<0.001 **.

Diet effects on LD size of chronically active subjects. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFDR type I fibers are significantly bigger than in LFDR type I (P<0.001). LDs in HFDR type II fibers do not have any significant difference from LFDR type II fibers (Figure 9).

FIGURE 9. Lipid droplet size in HFDR and LFDR, chronic voluntary running groups.
Values are expressed as means±**SE**. P<0.001 **

Effects of chronic exercise with different diet on LD size. In the examined
gastrocnemius muscle, regarding fiber type differences, LDs in HFD type I fibers are
significantly bigger than in LFD type I (P<0.05). LDs in HFD type II fibers are
significantly smaller than in LFD type II (P<0.001). LDs in HFDR type I fibers are
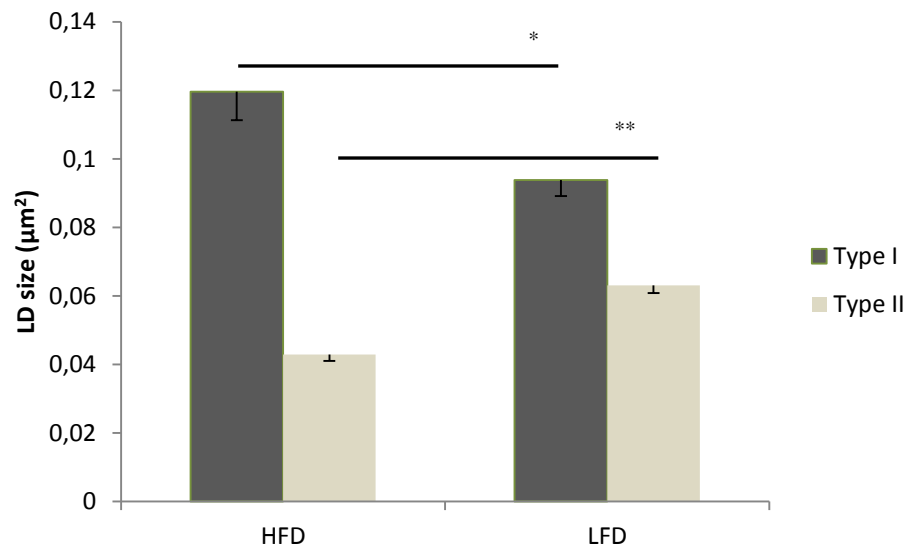significantly bigger than in LFDR type I (P<0.001). LDs in HFDR type II fibers do not
have any significant difference from LFDR type II fibers. LDs in HFD type I are
significantly bigger than in HFDR (P<0.05). LDs in HFD type II fibers are significantly
smaller than in HFDR type II (P<0.001). LDs in LFD type I fibers are significantly
bigger than in LFDR type I (P<0.001). LDs in LFD type II fibers do not have any
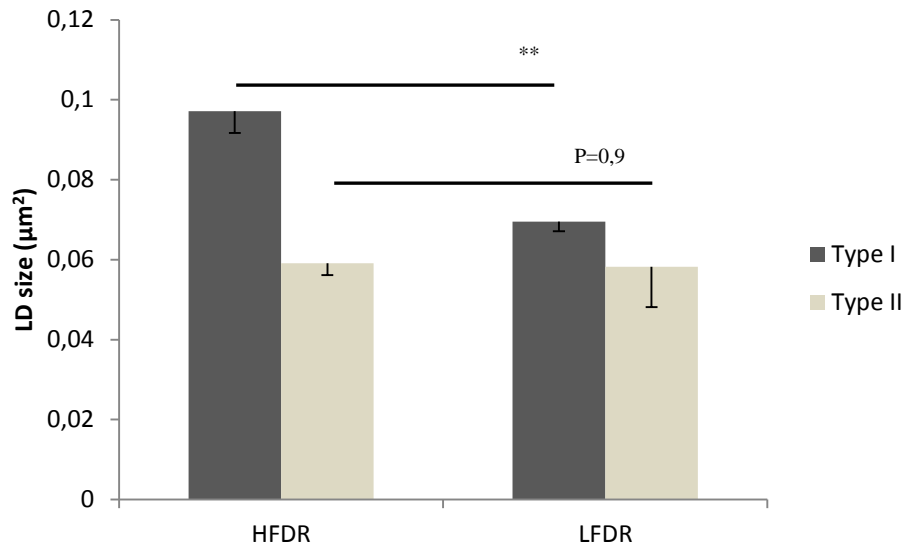significant difference from LFDR type II fibers (Figure 10).
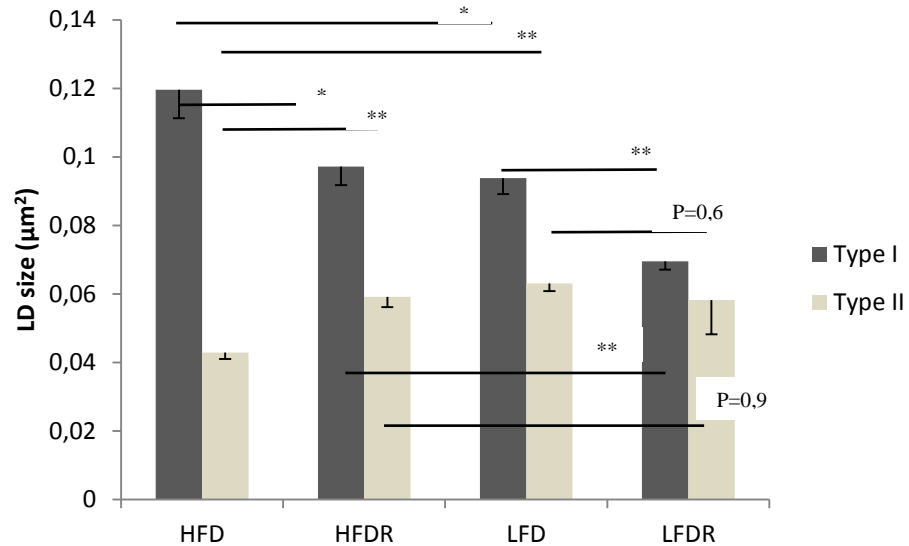
FIGURE 10. Lipid droplet size in HFD, HFDR, LFD and LFDR, sedentary control and chronic voluntary running groups. Values are expressed as means$\pm$ **SE**. P<0.05 *, P<0.001 **.

Effects of acute exercise with different diet on LD size. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFD0 type I fibers are significantly bigger than in HFD6 type I (P<0.05). LDs in HFD0 type II fibers do not have any significant difference HFD6 type II. LDs in LFD0 type I fibers do not have any significant difference from LFD6 type I fibers. LDs in LFD0 type II fibers do not have any significant difference from LFD6 type II fibers (Figure 11).
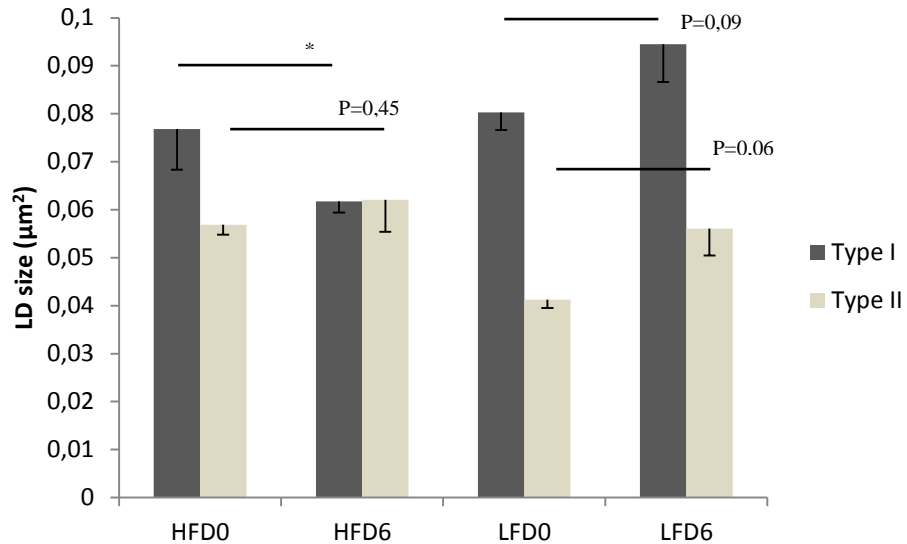
FIGURE 11. Lipid droplet size in HFD0, HFD6, LFD0 and LFD6, acute exercise, performing maximal test on a treadmill until exhaustion with some of the subjects to be sacrificed immediately after exhaustion and some others 6 hours later. Values are expressed as means$\pm$**SE**. P<0.05 *.

9.2   Fiber type LD density

Lipid droplet density in Type I and II fibers. In the examined gastrocnemius muscle, regarding fiber type differences, LDs density appeared to have very significant differences (P<0.001) between the more dense type I and the less dense type II fibers in all groups (Figure 12).
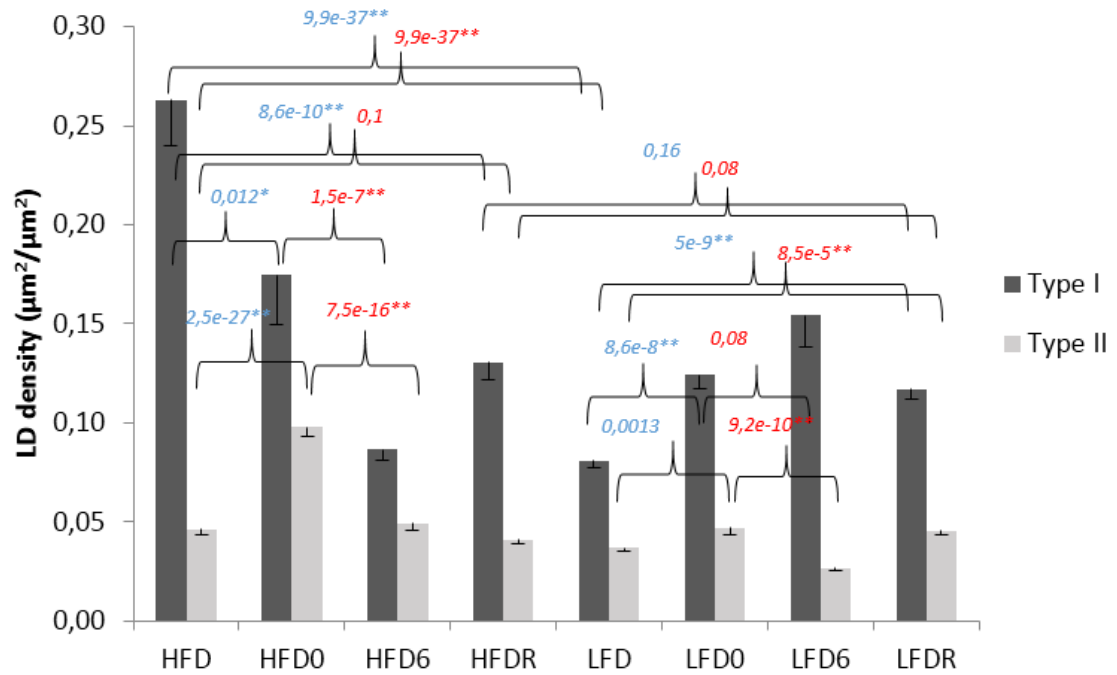
FIGURE 12. LD density in different gastrocnemius fiber types. Values are reported as mean±***SE***. Significance levels were set at P<0.05 (represented as *) and P<0.01 (represented as **).

Diet effect on LD density of sedentary subjects. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFD type I fibers are significantly more dense than in LFD type I fibers (P<0.001). LDs in HFD type II fibers are significantly more dense than in LFD type II fibers (P<0.001) (Figure 13).
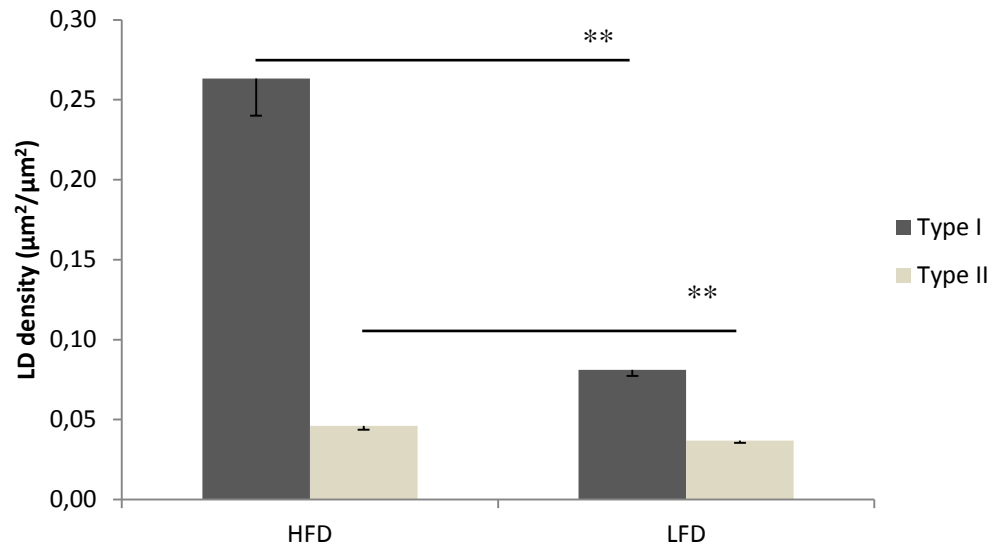
FIGURE 13. Lipid droplet density in HFD and LFD sedentary control groups. Values are expressed as means±**SE**. P<0.001 **.

Diet effects on LD density of chronically active subjects. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFDR type I fibers do not have significant differences from LFDR type I fibers. LDs in HFDR type II fibers do not have any significant differences from LFDR type II fibers (Figure 14).
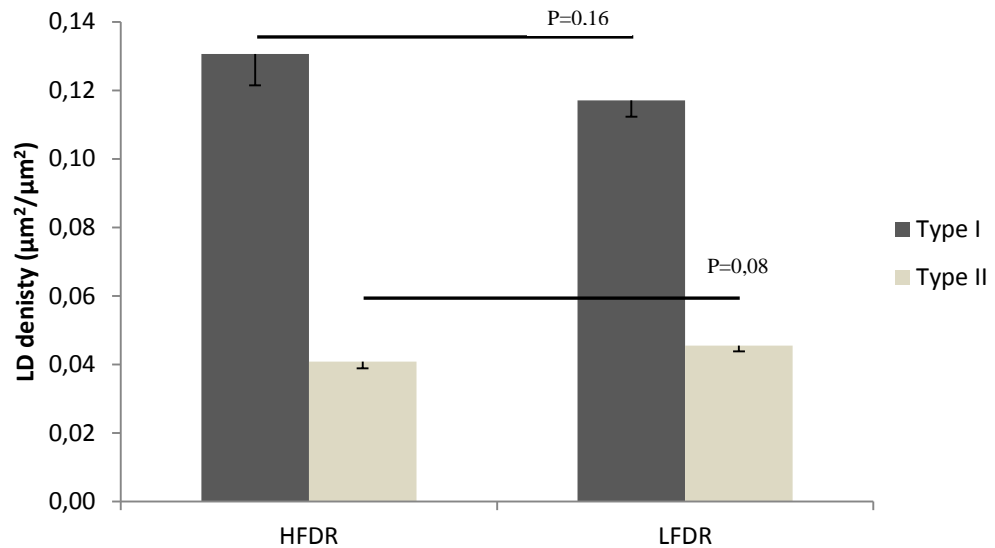
FIGURE 14. Lipid droplet density in HFDR and LFDR, chronic voluntary running
groups. Values are expressed as means±**SE**.

Effects of chronic exercise with different diet on LD density. In the examined
gastrocnemius muscle, regarding fiber type differences, LDs in HFD type I fibers are
significantly more dense than in LFD type I ($P<0.001$). LDs in HFD type II fibers are
significantly more dense than in LFD type II ($P<0.001$). LDs in HFDR type I fibers do
not have significant differences from LFDR type I fibers. LDs in HFDR type II fibers
do not have any significant differences from LFDR type II fibers. LDs in HFD type I
fibers are significantly more dense than in HFDR type I fibers ($P<0.001$). LDs in HFD
type II fibers do not have any significant difference from HFDR type II fibers. LDs in
LFD type I fibers are significantly less dense than in LFDR type I fibers ($P<0.001$). LDs
in LFD type II fibers are significantly less dense than in LFDR type II fibers ($P<0.001$)
(Figure 15).
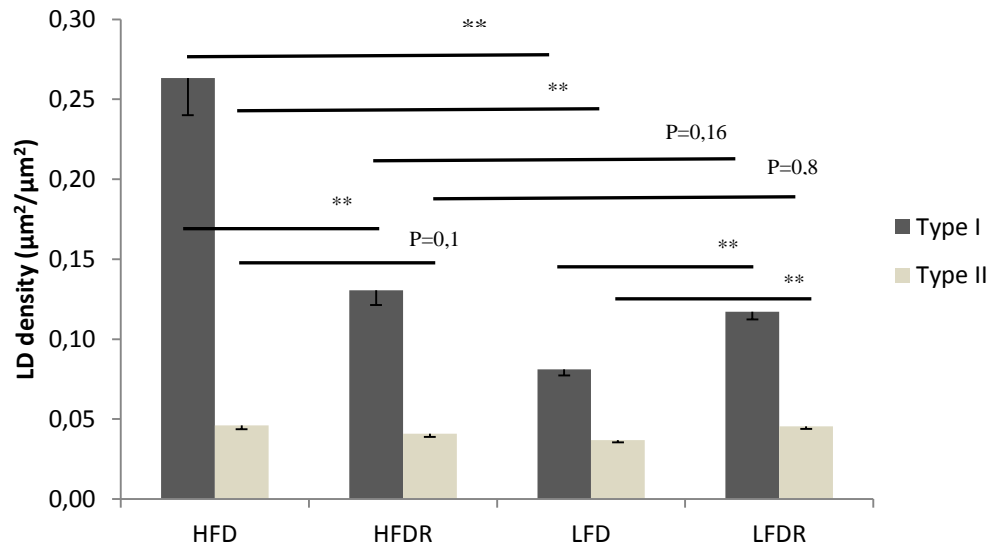
FIGURE 15.   Lipid droplet density in HFD, HFDR, LFD and LFDR, sedentary control and chronic voluntary running groups. Values are expressed as means$\pm$***SE***. P<0.001 **.

Effects of acute exercise with different diet on LD density. In the examined gastrocnemius muscle, regarding fiber type differences, LDs in HFD0 type I fibers are significantly more dense than in HFD6 type I (P<0.001). LDs in HFD0 type II fibers are more dense than in HFD6 type II (P<0.001). LDs in LFD0 type I fibers do not have any significant difference from LFD6 type I fibers. LDs in LFD0 type II fibers are significantly more dense than in LFD6 type II fibers (P<0.001) (Figure 16).
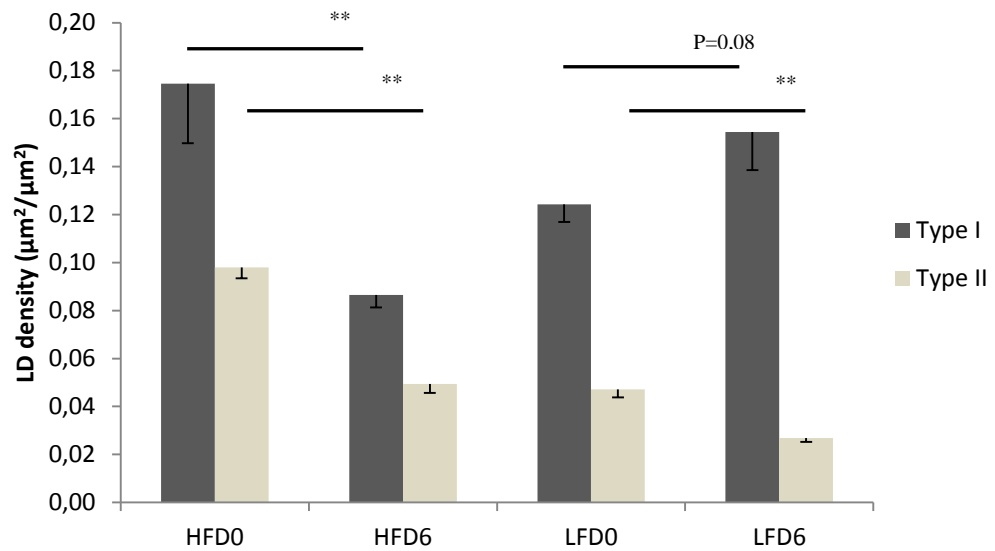
FIGURE 16. Lipid droplet density in HFD0, HFD6, LFD0 and LFD6, acute exercise, performing maximal test on a treadmill until exhaustion with some of the subjects to be sacrificed immediately after exhaustion and some others 6 hours later. Values are expressed as means±*SE*. P<0.001 **.

10 DISCUSSION

This study investigated the role and the significance of intramyocellular lipids after changes in exercise and diet patterns. The aim was to investigate the effects of high or low-fat diet, particularly of a diet change intervention accompanied by either a sedentary lifestyle, chronic exercise or single acute exercise bout. The main hypothesis of this study was that the size and density of LDs in skeletal muscle were altered with exercise and diet. This hypothesis was confirmed since size and density of LDs indeed changed by different patterns in exercise and diet.

10.1 Effect of exercise and diet

 A high-fat diet promotes obesity and reduces insulin sensitivity compared to a low-fat diet. Furthermore, a low-fat diet, exercise and the combination of these treatments have beneficial effects in reversing diet-induced obesity. Vieira et al. (2009), has shown that exercise and low-fat diet play an important role in the prevention of obesity-related metabolic disturbances. They also demonstrated that after six weeks on a high-fat diet, both low-fat diet and exercise seemed to result in reduction of body weight, but a low-fat diet by itself had a more clear effect. These results coincide further with another study done in humans, which demonstrates significant diet-induced weight loss, but not by exercise (Stefanick et al. 1998). Nevertheless, at least one study has shown that exercise have great advantages compared to diet alone (Tsai et al. 2003).

10.1.1 Fiber type LD size

It is worth noting that in fiber type I LD size was larger in HFD group that followed a high-fat diet and a sedentary lifestyle than in LFD. The volume of LDs in skeletal muscle is increased in OB indeed (Goodpaster et al. 2000). We have shown in this experiment that all the groups had significant differences in fiber type LD size, meaning that exercise alone, diet alone or the combination between exercise and diet had an impact in LD size. The size of LDs is significantly smaller after combined low-fat diet

and exercise, either acute or chronic in our experiment, a conclusion confirmed by He et al. (2004). The smaller droplets that were observed indicate a switch in the depots of fat within muscle fibers, maybe a setting of more droplets but smaller. Our results show that chronic exercise reduce muscle LDs regarding fiber types I and II compared to previously sedentary mice. Dube et al. (2008) has proposed that moderate exercise reduce IMCL. Maybe in our study this is a result of chronic exercise and maybe the intensity of it and the metabolic pathway that finally used for it.

Acute bout of exercise in mice fed with a low-fat diet attenuates smaller LDs in both fiber types compared to those feeding a high-fat diet. The sequence of diet and exercise reduced the size of LDs.

10.1.2 Fiber type LD density

In our experiment diet change intervention, chronic and acute exercise and their combination contributed to changes in LD density. In all groups very significant differences were observed regarding different fiber types. Thus, we hypothesize that there are differences in the spatial arrangement of LDs, their distribution Quite clearly, the LDs in type I fibers of sedentary mice feeding a high-fat diet were more dense compare to the other groups. Then the acute group fed with a high-fat diet and sacrificed immediately after exhaustion had more dense LDs as well. The IMCL content is significantly greater in muscle fibers type I, confirmed by van Loon et al. (2004).

In summary, both acute and chronic exercise lead to changes in LDs size and density but through different mechanisms. In those mechanisms can be accountable, the ectopic fat deposition and the changes occurred by training in total body fat (Bellou et al. 2013). Study results like this and future studies about LDs size and density, location of LDs in relation to mitochondria and PLIN proteins will enlighten us in a deeply insight of IMCL skeletal muscle metabolism to further outline efficient guidelines regarding lifestyle changes in the direction of treatment and prevention of obesity, IR and metabolic syndrome.

10.2 Strengths and Weaknesses

Its recorded by Surwit et al. (1998) that C57BL/6J mouse strain, which was used in this study, develop obesity and T2D after a high-fat feeding, a concept that follows a pattern same as in humans. So, the 21 weeks of this experiment was long enough for the mice to show metabolic changes concerning the above metabolic dysfunctions.

The mice in the chronic exercise group (LFDR and HFDR), were assigned a running wheel in their cage, meaning that they were performing voluntary running. The weakness here is that by voluntarily exercise it is more difficult to assess the physical activity level of each mouse, that is why most likely there will be some individual differences. However, a long-term effect of exercise was observed in the study.

Our results suggests that diet alone may play a predominant role in the metabolic dysfunction. But still here is arising a question mark about the high-fat diet, was it really a very high-fat diet, 60% of fat, that manage to conceal the exercise effects either from the voluntary running or the maximal test or not?

Notwithstanding that gastrocnemius muscle samples were collected and treated in the same way, it is very likely that the circumstances were different some times and this could have resulted in poorly shaped samples, inducing problems with the cells and the fibers that were analyzed. Here we can mention, that different samples and different groups have different numbers of cropped and analyzed cells, meaning that some of did not have the ideal number of cells for analysis. The total number of the samples, 68, was nevertheless quite satisfactory.

Immunohistochemistry sometimes, can incorporate some bias, depending on the sample handling (de Matos 2010). Here, we tried to eliminate all those factors, by being loyal to our protocol, discussed in the methods part.

10.3 Future Perspectives

It is plausible that additional studies are required in order to reveal or validate precise mechanisms into the research area of the lipid metabolism. It would be critical enough to seek if the findings of this study, have a strong linkage and could be applicable to humans and if this is happening to what extend a diet-change can cause alterations in human skeletal muscle.

In general, diet and physical activity level changes, lead to turnover in the metabolic profile of an individual. In our experiment we saw that indeed switches of diet and of exercise patterns, contribute to changes in LD's size and density in the cells. In future studies, it would be interesting to examine if exercise alone, without diet change interventions could be attributable to changes in skeletal muscle lipid metabolism and in its arrangement not only in LDs size and density, including SS and IMF regions of the cell and localization of LDs with mitochondria and in which extend.

Examination of LDs physiology and by extension a better understanding into lipid metabolism as a whole, is required. This could be done by investigating the relations between lipid droplets, lipid droplet coat proteins -PLIN family members- and skeletal muscle metabolism.

Lastly, it is important to be said that we need to know in extremely detailed way the regulation of lipid droplets in rodents, in order to disclose human lipid metabolism.

11 CONCLUSIONS

The present study presents the following findings concerning eight different examined groups of C57BL/6J mice:

➢ HFD, HFD0, HFDR, LFD, LFD0, LFD6 had significant differences in fiber type LD size, except HFD6 and LFDR group where type I and type II fibers were not different.

➢ All groups, HFD, HFD0, HFD6, HFDR, LFD, LFD0, LFD6, LFDR had a very significant ($P < 0.001$) difference concerning the LD density, between the fiber types.

➢ Our results regarding fiber type LD density confirm other studies that have been carried out and showed that oxidative type I fibers have more LDs than the glycolytic ones type II fibers.

➢ Combination of low-fat diet and chronic exercise, here voluntary running, reduces the size of LDs.

➢ Only diet alone accompanied by a sedentary lifestyle decreases the fiber type LD size, meaning that diet-induced weight loss reduces the lipid accumulation.

➢ Positive effect of acute exercise sessions combined with a low-fat diet compared to sedentary controls in fiber type LD size.

➢ Different exercise modalities and different diet patterns, indeed change the size and the density of LDs, regarding fiber type differences.

This study provides information on the impact and changes that engendered by acute and chronic exercise in skeletal muscle LDs size and density following high and low-fat diet in mice. Our findings coincide with other data published. Regular exercise and a consumption of a low-fat diet is the most appropriate combination for balancing the FA availability, depots and oxidation. Thus, future research needs to be done, concerning daily physical activity and a restricted diet, which indeed reduces IMCL content especially in type I fibers, in order to clarify the intramyocellular spatial arrangement of lipid metabolism and morphology of skeletal muscle.

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13 APPENDICES

SUBJECTS ID's

TABLE 4. Number of subjects and cells per group and per fiber type.

		Size		Density	
		Type I	*Type II*	*Type I*	*Type II*
HFD	n (cells)	71	1113	71	1200
	Subjects	6	7	6	7
LFD	n (cells)	362	1282	377	1408
	Subjects	7	10	7	10
HFD0	n (cells)	52	693	53	708
	Subjects	3	7	3	7
LFD0	n (cells)	105	356	105	356
	Subjects	4	6	4	6
HFD6	n (cells)	270	692	271	710
	Subjects	5	5	5	5
LFD6	n (cells)	93	907	93	704
	Subjects	3	6	3	6
HFDR	n (cells)	152	1043	158	1067
	Subjects	7	9	7	9
LFDR	n (cells)	407	1242	408	1275
	Subjects	7	9	7	9

ANALYTIC STATISTICS ABOUT LD SIZE

TABLE 5. Analytic statistics about the fiber type LD size.

	Sedentary Control				Chronic			
	LFD		HFD		LFDR		HFDR	
	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
Mean	0,09381	0,06312	0,1196	0,04291	0,06955	0,0582	0,09716	0,05914
Median	0,07636	0,04142	0,1107	0,02428	0,05775	0,03167	0,08617	0,03547
Stderr	0,004641	0,002262	0,008359	0,00192	0,002444	0,01002	0,005443	0,002958

	Acute Maximal Test							
	LFD				HFD			
	LFD0		LFD6		HFD0		HFD6	
	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
Mean	0,08029	0,04128	0,09449	0,05607	0,07682	0,05684	0,06175	0,06208
Median	0,07687	0,03071	0,09077	0,03133	0,05044	0,04104	0,05513	0,03469
Stderr	0,003693	0,001749	0,007876	0,005606	0,008476	0,002012	0,002339	0,006676

ANALYTIC STATISTICS ABOUT LD DENSITY

TABLE 6. Analytic statistics about fiber type LD density.

	Sedentary Control				Chronic			
	LFD		HFD		LFDR		HFDR	
	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
Mean	0,081	0,037	0,263	0,046	0,117	0,046	0,131	0,041
Median	0,061	0,019	0,199	0,010	0,092	0,021	0,114	0,014
Stderr	0,004	0,001	0,023	0,002	0,005	0,002	0,009	0,002

	Acute Maximal Test							
	LFD				HFD			
	LFD0		LFD6		HFD0		HFD6	
	Type I	Type II	Type I	Type II	Type I	Type II	Type I	Type II
Mean	0,124	0,047	0,154	0,027	0,175	0,098	0,087	0,049
Median	0,124	0,024	0,136	0,011	0,108	0,061	0,058	0,017
Stderr	0,007	0,003	0,016	0,002	0,025	0,005	0,005	0,004