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ROLE OF PEROXISOMES IN SKELETAL MUSCLES IN HIGH-FAT DIET-INDUCED OBESITY

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ABSTRACT

Skeletal muscles, the most prominent tissue in terms of mass and volume within the human body, play a pivotal role in regulating whole-body metabolism. Preserving an optimal skeletal muscle mass is associated with improved quality of life, healthy aging, and reduced risk of mortality. Peroxisomes are dynamic cellular organelles involved in essential metabolic pathways, including biosynthesis of ether lipids, ROS detoxification, and fatty acid oxidation. Emerging studies suggest that peroxisomes are important regulators of energy homeostasis and that disruption of peroxisomal functions influences the risk for obesity and the associated metabolic disorders, type 2 diabetes, and hepatic steatosis. However, further investigation is required to uncover the role of peroxisomes in skeletal muscles in basal conditions and in metabolic-induced stress, such as obesity.

In this study, muscle-specific Pex5 KO mice were fed with a standard diet (SD) and a high-fat diet (HFD) to induce obesity. Then, variations in body composition, glucose metabolism, and muscle performance were monitored to understand peroxisomal adaptations to basal and obesity-induced stress conditions in skeletal muscle tissue. Our results indicate that Pex5 KO mice on an HFD experience elevated body weight gain and increased fat accumulation. However, the size of organs and tissues does not appear to be affected by peroxisomal dysfunction in the context of obesity. Furthermore, glucose and insulin sensitivity, as well as energy metabolism, remained unaltered in skeletal muscles lacking Pex5 and the Pex5 KO mice did not differ from the controls. On the contrary, when skeletal muscle peroxisomes were dysfunctional, HFD seemed to exacerbate insulin levels, and muscle performance was compromised under metabolic stress conditions.

RIASSUNTO

I muscoli scheletrici, il tessuto più importante in termini di massa e volume all'interno del corpo umano, svolgono un ruolo fondamentale nella regolazione del metabolismo dell'intero organismo. La conservazione di una massa muscolare scheletrica ottimale è associata a una migliore qualità della vita, a un invecchiamento sano e a un ridotto rischio di mortalità. I perossisomi sono organelli cellulari dinamici coinvolti in vie metaboliche essenziali, tra cui la biosintesi dei lipidi eterici, la detossificazione dei ROS e l'ossidazione degli acidi grassi. Studi emergenti suggeriscono che i perossisomi sono importanti regolatori dell'omeostasi energetica e che l'interruzione delle funzioni perossisomiali influenza il rischio di obesità e dei disturbi metabolici associati, del diabete di tipo 2 e della steatosi epatica. Tuttavia, sono necessarie ulteriori indagini per scoprire il ruolo dei perossisomi nei muscoli scheletrici in condizioni basali e in condizioni di stress indotto dal metabolismo, come l'obesità.

In questo studio, topi Pex5 KO specifici per il muscolo sono stati alimentati con una dieta standard (SD) e una dieta ad alto contenuto di grassi (HFD) per indurre l'obesità. Quindi, sono state monitorate le variazioni della composizione corporea, del metabolismo del glucosio e delle prestazioni muscolari per comprendere gli adattamenti perossisomiali a condizioni di stress basale e indotto dall'obesità nel tessuto muscolare scheletrico. I nostri risultati indicano che i topi Pex5 KO sottoposti a HFD presentano un elevato aumento di peso corporeo e un maggiore accumulo di grasso. Tuttavia, le dimensioni di organi e tessuti non sembrano essere influenzate dalla disfunzione perossisomiale nel contesto dell'obesità. Inoltre, la sensibilità al glucosio e all'insulina, così come il metabolismo energetico, sono rimasti inalterati nei muscoli scheletrici privi di Pex5 e i topi Pex5 KO non differivano dai controlli. Al contrario, quando i perossisomi del muscolo scheletrico erano disfunzionali, l'HFD sembrava esacerbare i livelli di insulina e le prestazioni muscolari erano compromesse in condizioni di stress metabolico.

1. INTRODUCTION

1.1 SKELETAL MUSCLE

1.1.1 FUNCTION OF SKELETAL MUSCLES

Approximately 40% of the human body is made up of skeletal muscles. Being the largest organ in the body, besides producing motion, generating power, maintaining posture, and stabilizing joints, skeletal muscles are involved in several metabolic processes, nutrient storage, heat production, and hormone secretion [1].

From a metabolic point of view, skeletal muscles are the main site of insulin-stimulated glucose disposal from the bloodstream, responsible for about 80% of the circulating glucose uptake after meals. This process converts glucose into energy or stores it as glycogen, which is essential for sustaining insulin sensitivity and guarding against diseases such as Type 2 Diabetes Mellitus (T2DM) and obesity [2]. T2DM is a chronic metabolic disorder marked by consistently elevated blood sugar levels due to the body's reduced ability to efficiently utilize insulin, as peripheral tissues (e.g., skeletal muscles, liver, adipose tissue) decrease sensitivity and ultimately develop resistance to this hormone. If left unmanaged, this condition can evolve into more serious health issues such as heart disease, kidney problems, or nerve damage [2, 3].

Skeletal muscles are involved in lipid metabolism, especially during endurance exercise when the amount of glucose is limited, and additional energy is required to support muscle contraction [4]. Moreover, skeletal muscles serve as amino acid reservoirs used by other tissues such as the liver, brain, heart, and immune system for the synthesis of tissue-specific proteins during critical catabolic periods, including fasting, long-lasting exercise, or illness. When nutritional and energy needs are met, skeletal muscles predominantly fulfill movement facilitation and posture maintenance [5]. During contraction, skeletal muscles generate heat, which contributes to thermal homeostasis [6]. From an endocrine perspective, in response to contraction skeletal muscles secrete myokines. Myokines are proteins responsible for communication between various body organs including the brain, adipose tissue, liver, and bones. These proteins were

shown to be affecting various processes such as cognitive function, fatty acid metabolism, or bone formation [7 - 9].

Skeletal muscles are exceptionally plastic tissue and muscle mass depends on protein turnover reflecting the rate of protein synthesis and protein degradation within muscle fibers. Muscle growth, characterized by an increase in both muscle mass and fiber size, is influenced by various factors, including a developmental stage (e.g., childhood), mechanical overload (e.g., strength training), or due to hormonal influences (e.g., testosterone). However, various muscles and fiber types can respond differently to the same stimulus. Muscle atrophy, on the other hand, characterized by a decrease in muscle mass and fiber size, can result from factors such as aging, starvation, cancer, extended periods of bed rest, and denervation, among other related conditions [10 - 12].

1.1.2 STRUCTURE OF SKELETAL MUSCLES

Skeletal muscles are one of the three types of muscle tissues found throughout the human body, coming in various shapes and sizes. Other types of tissues such as blood vessels, neurons, and connective tissues are incorporated within the muscle fibers to enable their proper function [13].

Skeletal muscles consist of three layers of connective tissues providing structure and allowing contraction. Muscles are made up of individual muscle cells, also known as muscle fibers due to their length, which contract in response to nerve impulses. Each muscle fiber is surrounded by a thin layer of connective tissue called *endomysium.* These muscle fibers are grouped into bundles called fascicles which add strength and structure. Each fascicle is covered by a slightly thicker connective tissue called *perimysium*. The entire muscle, composed of many fascicles, is wrapped in a tough outer layer of connective tissue called *epimysium*. This outer layer separates the muscles from the nearby tissues and enhances structure and protection.

Skeletal muscles can be attached to bones directly or indirectly through tendons. At one or both ends of the muscle, the tough collagen fibers of the epimysium intertwine with the collagen fibers of tendons. Tendons are connective tissues that are tougher and less elastic than muscles and their role is to firmly attach the muscle to a bone. The tendon meets up with the periosteum, a

fibrous membrane covering the outer surface of the bones, on the opposite side. During muscle fiber contraction, tension is transferred through the connective sheaths, to the tendon which then pulls on the bone causing movement. This continuous connection ensures that when muscles contract, the generated force is efficiently transmitted through these connective tissues leading to performing a wide range of motions with precision and control [13, 14].

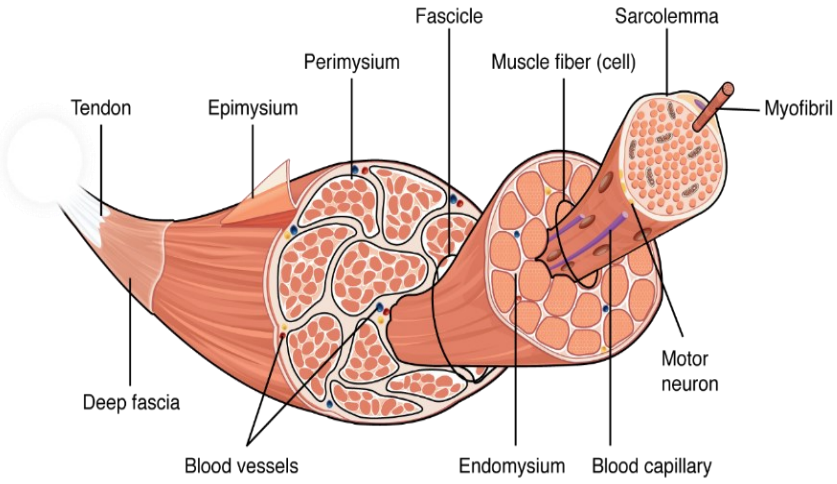


Fig. 1. *Structure of skeletal muscles. (Biga, 2019)*

Muscle cells are elongated and contain multiple nuclei, which allows them to synthesize a multitude of essential proteins to maintain the cells' optimal function. Muscle cells are enclosed by a specific membrane called sarcolemma, inside of which lies the sarcoplasm, corresponding to the muscle cells' cytoplasm. Within a muscle cell, proteins are organized into myofibrils, which extend along the length of the cell and house hundreds to thousands of sarcomeres directly linked to one another. This arrangement gives the skeletal muscles their characteristic striated appearance. The sarcomere, which is the smallest operational unit of a skeletal muscle cell, consists of a well-organized configuration of various proteins and is responsible for generating muscle force and movement. Sarcomeres are composed of overlapping thick (myosin) and thin (actin, tropomyosin, troponin) filaments, among other proteins. When individual sarcomeres shorten, it triggers the contraction of individual skeletal muscle fibers, and as a result, the entire muscle contracts and produces movement [15].

Sarcomere is defined as a myofibril region contained between two Z discs. The dark-striated A band consists of thick myosin filaments stretching across the sarcomere center and extending towards Z discs. These thick filaments are secured at the sarcomere's midpoint (M-line) through a protein called myomesin. The lighter striated I band contains thin actin filaments anchored at Z discs through α-actin. The H zone is in the middle of the A band and contains thick filaments. Generally, skeletal muscles consist of about 20% myosin and about 55% actin by weight. Myosin molecules have a tail and two globular heads that can bind to actin during muscle contraction. Actin filaments contain binding sites for myosin heads. Other important proteins include tropomyosin and troponin, which regulate the interaction between actin and myosin; titin, which maintains the elasticity and structure of muscle cells; and nebulin, which stabilizes actin filaments within sarcomeres [13-16].

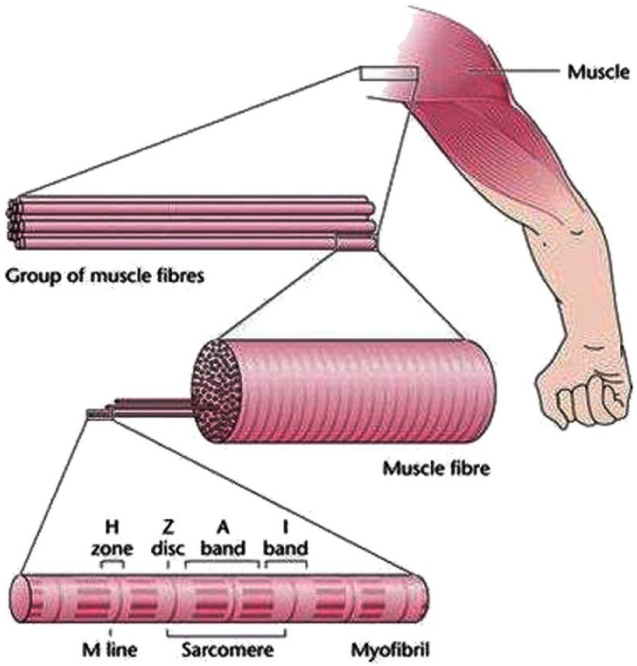


Fig. 2. *Structure of skeletal muscle fibers and sarcomere. (Bloom et al., 1975)*

The Sliding Filament Model of Contraction is a fundamental concept in biology explaining what is happening at the molecular level during muscle contraction. At a relaxed state, tropomyosin wraps around the actin filaments and covers the myosin-binding sites, which prevents the actin-myosin interaction. However, upon receiving nerve impulses, calcium ions are released within the

muscle cells and enter the sarcoplasm. These ions bind to a protein complex called troponin, which is associated with tropomyosin. This binding alters the position of tropomyosin, allowing the myosin heads to interact with actin, forming cross-bridges. Once these cross-bridges are formed, myosin heads pivot and slide along the actin filaments, leading to muscle contraction. Actin filaments are pulled towards the center of the sarcomere. The sliding action causes the sarcomere to shorten. The length of the A band remains unchanged, while the length of the H and I bands decreases, reducing the distance between two Z discs. [13, 17].

Skeletal muscles contain two diverse groups of fiber types, each tailored to perform its specific function. Type I, commonly referred to as Slow-Twitch fibers, are oxidative muscle fibers and well-supplied with mitochondria and capillaries. They predominantly employ aerobic metabolism, using oxygen to generate energy, and they possess mainly glucose and fatty acid oxidative enzymes. Type I fibers favor fatty acids as a fuel source, particularly during low-intensity, endurance tasks. These fibers contract slowly but endure well, making them ideal for prolonged activities. Their red appearance results from a high myoglobin content [18]. Type II, also known as Fast-Twitch muscle fibers, are glycolytic, primarily relying on anaerobic metabolism, which does not require oxygen and is less efficient but produces energy quickly. Glycolytic fibers have a limited capacity for sustained contractions and fatigue more quickly than oxidative fibers. They predominantly use glucose as their primary fuel source, which is converted into ATP through glycolysis [13]. Fast-Twitch fibers are divided into three subcategories – Type IIA (Fast Oxidative-Glycolytic), Type IIB, and Type IIX (Fast Glycolytic), although humans do not appear to have IIB [19]. Type IIA, are capable of both aerobic and anaerobic metabolism, which enables them to perform activities involving bursts of energy for a moderate period. Type IIX, are specifically utilized for intense and rapid movements, relying on anaerobic metabolism. However, they tend to become fatigued quickly. These characteristics make them optimal for short amounts of high-intensity exercise [19, 20].

1.2 PEROXISOMES

1.2.1 STRUCTURE OF PEROXISOMES

Peroxisomes are essential cellular components present in all eukaryotic organisms, including both plant and animal cells. They are typically distributed freely throughout the cytoplasm. These microbodies are generally spherical, with diameters ranging from 0.2 to 1.5 µm. In a mammalian cell, it is common to find several hundred peroxisomes. These structures are enclosed by a semipermeable lipid bilayer membrane containing peroxisomal membrane proteins. These proteins enable the selective passage of metabolites into and out of the organelle and are also involved in peroxisomal biogenesis. The interior of peroxisomes is filled with a granular matrix housing numerous enzymes, cofactors, and substrates involved in various metabolic processes [21, 22].

1.2.2 FUNCTION OF PEROXISOMES IN ANIMAL CELLS

Peroxisomes are highly dynamic organelles that interact with other cellular structures like mitochondria, endoplasmic reticulum (ER), and cytosolic enzymes, to fulfill their critical functions in various catabolic and anabolic processes [23]. A minimum of 50 different enzymes were identified in the mammalian peroxisomal matrix, implying the importance of peroxisomes in a variety of metabolic pathways, including the detoxification of ROS, the α- and ß- oxidation of fatty acids, as well as the synthesis of plasmalogens and bile acids [24, 25].

Peroxisomes facilitate oxidation reactions, wherein oxygen (O_2) is reduced to hydrogen peroxide (H_2O_2) through the activity of FAD-dependent oxidoreductases involved in various cellular processes. These organelles are responsible for approximately 20% of the overall cellular oxygen consumption and can contribute to as much as 35% of the total H_2O_2 production in specific tissues. Given the potentially harmful nature of H_2O_2, peroxisomes contain catalase and other enzymes, which can break down H_2O_2 into water or utilize it for the oxidation of other organic compounds. Imbalanced H_2O_2 levels have been associated with various diseases such as diabetes, cancer, obesity, and kidney damage, which underscores the importance of this process in the overall metabolism [25 - 28].

Oxidative reactions within peroxisomes promote the breakdown of various substrates, such as very long-chain fatty acids (a process referred to as α-oxidation) resulting in the formation of medium-chain fatty acids and other products. However, since peroxisomes lack the electron transport chain, the newly produced shorter-chained fatty acids need to be sent to mitochondria to be further oxidized [26, 29].

In cooperation with ER, peroxisomes provide significant support for lipid biosynthesis through various mechanisms, most notably in plasmalogen synthesis. Plasmalogens are types of ether phospholipids where one of the hydrocarbon chains connects to glycerol through an ether bond instead of an ester bond. These specialized lipids are crucial for cell function and are significant components of membranes in the heart, liver, and brain. The production of plasmalogens begins in peroxisomes and is finished in the ER [26, 30]. When peroxisomes are dysfunctional, as seen in certain medical conditions, plasmalogens are absent in various organs [31].

Peroxisomes collaborate with the ER and lysosomes to enhance the production, transport, and homeostasis of cholesterol, which represents approximately 30-40% of cellular lipids. Moreover, peroxisomes are involved in the initial step of bile acid synthesis, transforming cholesterol into primary bile acids (e.g., cholic acid). Bile acids facilitate the digestion of dietary fats [32, 33].

1.2.3 PEROXISOMAL DYNAMICS

Peroxisomes' number, shape, and size are actively regulated in response to various environmental and developmental signals [34]. Peroxisomes are closely linked to mitochondria, however, in contrast to mitochondria, they lack their own genome [24]. The complex process of peroxisome formation known as biogenesis, relies on the activity of over 30 different peroxisomal (PEX) proteins. Peroxisomal proteins are synthesized from nuclear DNA, translated by ribosomes in the cytosol, and then transported into the peroxisomal matrix using peroxisomal import receptors, Pex5 and Pex7, which recognize specific peroxisome targeting sequences (PTS), PTS1 and PTS2, on the proteins [35, 36].

PTS1 is specifically recognized by Pex5 cytosolic protein receptor, while PTS2 is recognized by Pex7. Following the binding of PTS1-containing proteins to the Pex5 receptor, this complex is

transported to the peroxisomal membrane where it interacts with docking proteins, Pex13 and Pex14, allowing the complex to attach to the peroxisome and be translocated into the matrix. Following translocation, the complex dissociates and PTS1 can execute its metabolic functions. Dissociated Pex5 undergoes recycling, so it can return to the cytosol to initiate the process anew. It was shown that Pex5 has two isomers: Pex5S and Pex5L. In mammalian cells, the interaction between Pex7 and Pex5L is required for the targeting of Pex7 to the peroxisomal membrane. Besides this, the process of PTS2/Pex7 complex translocation is like the one of PTS1/Pex5 [37].

The integral membrane complex comprising Pex2, Pex10, and Pex12, is also associated with the translocation. These proteins share similarities with ubiquitin ligases E3 and are equipped with Really Interesting New Gene (RING) domains, helping the recycling and degradation of PTS receptors through mono- or polyubiquitination processes. There is another complex composed of Pex1, Pex6, and Pex26, supporting the recycling of Pex5 and Pex7 receptors so they can be reused back in the cytosol for executing additional imports [38].

Peroxisomes originate either through *de novo* formation or from already existing peroxisomes. This crucial process relies on Pex3, Pex16, and Pex19, which are essential for accurate localization of Peroxisome Membrane Proteins (PMPs). The *de novo* formation starts with the endoplasmic reticulum (ER) and multiple peroxisomal biogenesis proteins, also known as PEX proteins. As the first step, Pex16 is incorporated into the ER, followed by the inclusion of Pex3 and additional PMPs. This series of events results in the development of pre-peroxisomal vesicles within the ER, which can detach and gradually evolve into fully formed peroxisomes. Once the vesicles detach, Pex19 becomes the key player in the peroxisomal growth, as this protein recognizes and binds newly synthesized PMPs within the cell. Pex19 ensures proper integration of PMPs into existing peroxisomes. The Pex19 complexes then bind to Pex3 and Pex16 located in the peroxisomal membrane and the Pex19-Pex3-Pex16 complex facilitates the import of most PMPs. Peroxisomes can increase in number within the cell via a process referred to as fission, allowing for the adjustment of peroxisome quantity and distribution according to the cell's metabolic requirements. This process requires Pex11ß (an integral membrane protein), fission factors, proteins, and ATP [36, 39, 40].

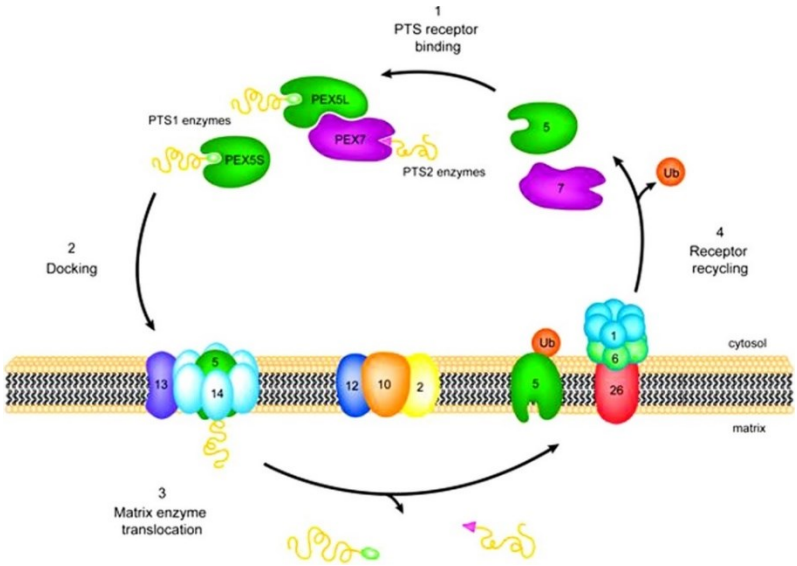


Fig. 3. *Peroxisomal matrix protein import (Braverman et al., 2013)*

Peroxisome homeostasis, manifested by the precise and efficient control of peroxisome number and functionality, must be tightly regulated in response to environmental changes. Peroxisome homeostasis requires not only their effective formation but also the efficient removal of obsolete and dysfunctional peroxisomes, whose half-life is about 1.5 to 2 days [41]. Autophagy is a lysosome-dependent cellular mechanism responsible for degrading and recycling damaged or unnecessary cellular components, to maintain cellular homeostasis [42]. Lysosomes are membrane-bound organelles found in eukaryotic cells, serving as the cell's "recycling centers". During autophagy, lysosomes fuse with autophagosomes to digest the contents. Autophagosomes are double-membrane vesicles, serving as temporary storage units formed during the process of autophagy holding the materials the cell intends to clean up [43].

80% of impaired or aged peroxisomes are removed by the specialized autophagic process termed pexophagy [44, 75]. Generally, the process of selective autophagy requires the ubiquitination of membrane proteins specific to the organelles involved. Confirming this theory, pexophagy is induced by the attachment of a ubiquitin molecule to specific parts of PMPs, such as peroxins, PMP 34, and PMP70, among others [45, 46]. Ubiquitination is initiated in response to stress

conditions and peroxisomal malfunctions [44]. The autophagy process begins with the formation of an autophagosome, which engulfs the targeted peroxisome for its sequestration. Ubiquitinated PMPs are recognized by Ub-binding autophagy adaptors, namely the NBR1 and p62 proteins. Interestingly, when there is an excess of NBR1, p62 is not strictly required. However, their combination significantly improves the efficiency of pexophagy [47]. The autophagosome, which carries the ubiquitinated peroxisome and bound autophagy adaptors, encloses the cargo within its double membrane. Afterward, it fuses with a lysosome containing hydrolytic enzymes, which together create a structure called the autolysosome, and the breakdown of PMPs begins [48]. The breakdown products, such as amino acids, and fatty acids, are then released into the cytoplasm and made available for reuse by the cell. The residual material and waste are eliminated from the cell through exocytosis or other cellular processes [49].

1.2.4 PEROXISOME BIOGENESIS DISORDERS

Peroxisome biogenesis disorders (PBDs), are a group of rare genetic disorders causing defects in biogenesis and function of peroxisomes, resulting in a wide range of clinical symptoms. The development of healthy peroxisomes in mammals relies on the protein products involved in peroxisomal membrane assembly, import of peroxisomal matrix proteins, or peroxisomal division, encoded by PEX genes. Mutations in 14 of these genes in humans have been demonstrated to result in PBDs [40]. PBDs are divided into two categories: Zellweger spectrum disorders (ZSDs) and Rhizomelic chondrodysplasia punctata (RCDP). ZSDs include various subtypes, with Zellweger syndrome being the most severe form involving brain atrophy, facial dysmorphism, renal degeneration, and profound muscular hypotonia [21]. Typically, patients die within a year after birth [50]. Milder forms of ZSDs include neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). The ZSD patients typically show elevated very long-chain fatty acids (VLCFA, $\geq$ 22 carbons) in plasma and decreased plasmalogens in erythrocytes. On the other hand, RCDP is divided into five subgroups (genotypes). Two genotypes, RCDP1 and RCDP5 belong to PBD, while the other three are classified as peroxisomal enzyme deficiencies resulting from mutations in genes responsible for essential peroxisomal enzymes required for plasmalogen synthesis [21].

The diagnosis depends on assessing the peroxisomal functions through biochemical measurements and sequencing of PEX genes. Currently, there are no specific target therapies designed for these conditions. However, recommended management approaches are in place and ongoing research efforts are being pursued [21, 40, 51].

1.3 OBESITY

Obesity is a multifaceted health condition characterized by the excessive accumulation of body fat, which can have detrimental effects on an individual's well-being, quality of life, and life expectancy. Obesity has been on a global rise since 1980, increasing at alarming rates [52]. Traditionally, the primary driver of obesity is perceived to be a prolonged imbalance between calorie intake and calorie expenditure. However, the latest research indicates that obesity is a much more complex issue. Metabolic fitness is shown to be substantially affected by the quality of nutrients, food sources, genetics, epigenetics (e.g., prenatal, and early-life exposure, lifestyle), environmental (e.g., socioeconomic status, food marketing, environmental toxins, cultural norms, social support, screen time) and microenvironmental (e.g., cell-cell interactions, immune cells, pH levels, oxygen levels) factors [53]. With obesity being a widely discussed topic, this field of research is rapidly advancing, and new scientific discoveries continue to emerge.

The most common way to diagnose obesity is to calculate the body mass index (BMI). BMI is determined by dividing a person’s body weight (in kilograms) by the square of their height (in meters squared). An elevated BMI of above 30, stands as a significant risk factor for diabetes, cardiovascular diseases, musculoskeletal disorders, chronic kidney disease, nonalcoholic fatty liver, certain types of cancer, and depression. Besides adding stress on the bodies and minds of patients, managing these conditions can impose an extra burden on the healthcare system. For instance, it is estimated that individuals with obesity have medical expenses that are 30% greater than the medical costs of patients with a normal BMI [52 - 54].

Adipocytes, commonly known as fat cells, play a central role in both storing and releasing fat within the body. Excess calories are transformed into triglycerides and deposited within adipocytes. With continued calorie excess, adipocytes expand in both quantity and size, ultimately causing greater fat retention. In obesity, adipocytes undergo hypertrophy

(enlargement) and hyperplasia (increase in numbers), contributing to an overall body mass increase. Adipose tissues of lean adults represent about 10% of the body weight, while in obese subjects it can be up to 50% [55, 56]. There are two main types of adipose tissue in the human body, including white adipose tissue (WAT) and brown adipose tissue (BAT), with distinct shapes, sizes, functions, and metabolic roles. WAT primarily serves as an energy storage depot, storing excess energy in the form of triglycerides and releasing it when the body needs energy. WAT consists of large, spherical cells called adipocytes, which contain a single, large lipid droplet, which pushes all other organelles to the cell's periphery [57]. This type of adipose tissue also produces hormones and signaling molecules called adipokines that regulate inflammation and metabolism. Excessive accumulation of WAT is the hallmark of obesity [58]. On the other hand, BAT is specialized for thermogenesis, maintaining body temperature and energy expenditure. BAT burns calories to generate heat and is especially active in newborns and during cold exposure. BAT is rich in mitochondria containing a protein called uncoupling protein 1 (UCP1), which uncouples the electron transport chain from ATP production, dissipating energy as heat. This process gives BAT its characteristic brown color and is termed non-shivering thermogenesis. BAT contributes to energy expenditure by burning fat and glucose to generate heat [57].

Adipocytes are considered endocrine cells as they secrete hormones and cytokines involved in the control of metabolism and appetite. Some noteworthy adipokines include leptin, which acts as a hormone responsible for regulating appetite, body weight, and energy expenditure by signaling the brain that sufficient fat stores are present in the body. Another significant adipokine is adiponectin, known for its anti-inflammatory, insulin-sensitizing, and cardioprotective properties, also involved in glucose level regulation and fatty acids breakdown. Adiponectin enhances adipocyte lipid storage, which prevents lipid accumulation. The expression of adiponectin and its levels in the bloodstream is reduced in individuals with obesity, leading to increased fat accumulation, especially in visceral adipose tissue (fat stored around internal organs) [59, 60]. Additionally, there is resistin, where elevated levels are linked to inflammation and insulin resistance. Obese individuals may experience inflammation due to the release of pro-inflammatory agents from both adipocytes and immune cells within the fat tissue [61, 62].

1.3.1 SKELETAL MUSCLES AND OBESITY

It is becoming apparent that obesity and the infiltration of fat into skeletal muscle also significantly influence the development of sarcopenia. Sarcopenia is the age-related involuntary decline in both skeletal muscle mass and strength, which contributes to several negative health outcomes, including frailty, impaired mobility, fatigue, rheumatoid arthritis, and metabolic disorders such as insulin resistance. Sarcopenia is characterized by about 1% muscle loss per year [63]. This phenomenon has been termed sarcopenic obesity and it combines the effects of aging, obesity, and muscle atrophy [64]. Increased body fat was linked to decreased muscle quality and was found to be a predictor of a more rapid decline in lean mass [65]. Excessive body fat inhibits the anabolic effects of insulin, which is essential for protein synthesis stimulation. Moreover, unhealthy adipose tissue releases various adipocytokines (e.g., interleukin-6, tumor necrosis factor-α), which may have detrimental effects on muscle tissue [66, 67].

Additionally, skeletal muscles can become targets of inflammation triggered by obesity. This inflammatory response in both skeletal muscles and adipose tissue can lead to the release of specific cytokine hormones, often referred to as adipo-myokines. Notably, skeletal muscles have the capacity to release interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-15 (IL-15), irisin, myonectin, and myostatin. However, unlike adipokines, these muscle-derived myokines are regulated during exercise and muscle contraction. The release of these circulating factors can either exacerbate or mitigate obesity, inflammation, and insulin resistance, thus influencing overall metabolic health [2].

Having more lean mass is linked to a lowered risk of insulin resistance, metabolic syndrome, cardiovascular issues, and T2DM. Interestingly, genetically altered mice with increased muscle mass showed reduced body fat, resistance to diet-induced obesity, and insulin resistance, suggesting that pathways involved in muscle growth could be potential targets for preventing and treating obesity and its metabolic complications. Moreover, it was shown that myokines produced by muscles, such as irisin, stimulate BAT differentiation and WAT thermogenesis [68, 69].

1.3.2 PEROXISOMES AND OBESITY

Recent studies have uncovered the potential role of peroxisomes in obesity and its associated complications [23, 25, 70]. Obesity and insulin resistance are frequently linked to increased oxidative stress, which arises when the body's antioxidant defenses are surpassed by pro-oxidative processes. Within skeletal muscles, elevated oxidative stress can lead to insulin resistance and hinder the uptake of glucose, a common feature of metabolic issues in obesity. Peroxisomes actively participate in ROS regulation, and any dysfunction can exacerbate oxidative damage [25, 71]. In fact, it was shown that impaired peroxisomal functions in adipose tissue enhanced oxidative stress and inflammation, exacerbating obesity [70]. Moreover, they showed that peroxisomal biogenesis is downregulated in white adipose tissue (WAT) of both diet- and genetically-induced obese mice, further confirming a link between peroxisomes and obesity.

As mentioned above, peroxisomes play a pivotal role in the breakdown of VLCFAs. In obesity, peroxisome-related imbalances in fatty acid metabolism can lead to VLCFA accumulation and increased lipid storage. Recent findings suggest an interaction between peroxisomes and lipid droplets. Lipid droplets are cellular organelles, particularly abundant in adipocytes, crucial for lipid metabolism, energy storage, and signaling. Ether lipids from peroxisomes constitute about 10-20% of the neutral lipids within lipid droplets. Furthermore, both peroxisomes and lipid droplets undergo biogenesis in the same ER subdomain. This connection takes on added significance as reduced circulating ether lipids, which are synthesized partly in peroxisomes, have been linked to obesity [25]. Interestingly, specific types of plasmalogens are found in lower levels in the serum of obese individuals [72]. In specific tissues like BAT, peroxisomes play a crucial role in enhancing thermogenesis and energy expenditure by promoting mitochondrial fission through the production of ether lipids [73]. In fact, peroxisomes and mitochondria are closely related organelles, and their cooperation plays a vital role in various metabolic processes, including energy production [24]. Dysregulated peroxisomal function can negatively impact mitochondrial activity, resulting in decreased energy expenditure and contributing to obesity development [74]. Impaired mitochondrial function has been observed in patients with PBDs [75]. Peroxisome-derived lipids appear to protect against obesity by influencing mitochondrial respiration and

energy expenditure [25]. During the process of adipogenesis (formation of fat cells), peroxisomes increase in number and interact closely with lipid droplets, suggesting an intimate connection between peroxisomes and lipid metabolism. Moreover, peroxisomes regulate adipogenesis by influencing the synthesis of adipogenic ligands, ether lipids, and triacylglycerol. Understanding these roles may have implications for obesity and metabolic disorders [25, 76].

Nevertheless, the underlying mechanistic relationship between peroxisomes in skeletal muscle tissue and their potential involvement in the pathophysiology of obesity remains an area of ongoing investigation.

2. AIM OF THE STUDY

Skeletal muscle, the most abundant tissue of the human body, plays an essential role in locomotion, energy expenditure, physical activity, and breathing and eating. Serving as a primary metabolic site, skeletal muscles regulate the homeostasis of glucose and lipids. Thus, the maintenance of healthy skeletal muscles contributes to whole-body metabolism, reducing the risk of mortality [77, 78].

Peroxisomes are dynamic, widespread organelles, hosting over 50 anabolic and catabolic enzymes involved in several fundamental metabolic pathways, such as ROS detoxification, VLCA degradation, ether lipids (e.g., plasmalogens), and bile acids biosynthesis. Furthermore, peroxisomes engage in crosstalk with other organelles, including mitochondria, endoplasmic reticulum, and lipid droplets. Although it is evident that peroxisomes play a role in multiple pathways relevant to energy metabolism and lipid handling, these organelles have not been extensively explored and many mechanisms remain unclear.

The aim of this study is to investigate the role of peroxisomes in skeletal muscle metabolism in basal conditions and in the presence of a stress-inducing condition – obesity. A better understanding of how peroxisomes control muscle metabolism and function could help in the identification of new therapeutic targets to preserve muscle mass and function in patients affected by both peroxisomal disorders and age-related metabolic disorders, such as obesity and its related complications (insulin resistance, disruption in glucose metabolism, and other metabolic imbalances).

3. MATERIALS AND METHODS

3.1 ANIMAL HANDLING

Animals were handled by specialized personnel under the control of inspectors of the Veterinary Service of the Local Sanitary Service (ASL 16 – Padova), and the local officers of the Ministry of Health. The use of the animals and the experimental protocol was approved by the ethical committee and by the animal welfare coordinator of the OPBA, University of Padova. All procedures are specified in the projects approved by the Italian Ministero Salute, Ufficio VI (authorization numbers Metabolism 328/2022), and were conducted in accordance with the relevant codes of practice for the care and use of animals for scientific purposes. Mice were in a temperature and humidity-controlled animal facility, with a 12-hour light/dark cycle and free access to water and food.

3.2 ANIMAL MODEL

Conditional muscle-specific Pex5-/- mice were obtained by crossing mice bearing Pex5 floxed alleles with transgenic mice expressing Cre recombinase under the control of Myosin Light Chain 1 fast promoter (MLC1f-Cre), which is expressed only in skeletal muscle during the embryonic development. 3 months- old mice were fed both with a standard diet (SD) (MUCEDOLA, 4RF21 certificate) and high-fat diet (HFD) (D12492, Charles River Laboratories International, Inc.) for 16 weeks (water *ad libitum*). Cre-negative mice were used as controls.

3.3 GENOTYPING

Genotyping of muscle-specific Pex5-null mice was determined by analyzing the presence of Cre-recombinase on genomic DNA using a Polymerase Chain Reaction (PCR). An earpiece of each mouse was taken and lysed in 30µL of lysis buffer containing Tris-HCL 1M pH 7.5 and Proteinase K 10mg/mL (Life Technologies) to dissolve cell proteins and obtain free DNA. Samples were incubated at 55°C for 1 hour and then at 95°C for 5 minutes to inactivate proteinase K.

20µL of total volume was prepared for each sample composed of:

- GoTaq Green master mix 2x (Promega): 10µL
- Distilled H_2O: 7µL
- Primer F1, CRE_geno1_f: 0.5µL (previously diluted 1:10 with H_2O)
- Primer R1, CRE_geno1_r: 0.5µL (previously diluted 1:10 with H_2O)
- Sample (previously diluted 1:10 with H_2O): 2µL

Primer sequences are:

- F1: 5'-AATGCTTCTGTCCGTTTGCC
- R1: 5'-ACATTCTCCCACCGTCAGTA

PCR program (40 cycles):

- Step 1 (initial denaturation): 94°C for 3 minutes
- Step 2 (denaturation): 94°C for 45 seconds
- Step 3 (annealing): 61°C for 30 seconds
- Step 4 (extension): 72°C for 1 minute
- Step 5: go to Step 2 for 40 times

The PCR amplicons were analyzed with gel electrophoresis using 1% agarose gel.

3.4 GENE EXPRESSION ANALYSIS

3.4.1 RNA EXTRACTION

For gene expression analysis, total RNA was extracted from gastrocnemius using Trizol, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Trizol, an acid solution that contains phenol, chloroform, and guanidine isothiocyanate, desaturates proteins while preserving RNA integrity. It was added to samples which were then mechanically homogenized with the Tissue Lyser (Quiagen) and metal beads. An extra dose of chloroform was added to separate RNA from cell debris. The overlying aqueous RNA-containing solution was collected, and

RNA was precipitated with 100% isopropanol. RNA pellet was washed with 75% ethanol and finally resuspended in RNAse-free water (GIBCO).

3.4.2 NUCLEIC ACID QUANTIFICATION

RNA quantification was done by using QubiTM (Invitrogen), a fluorometer that allows RNA, DNA, and protein quantification. The QubitTM BR RNA assay kit was used.

RNA purity was assessed by using NanodropTM (Thermo Fisher Scientific), a UV-visible spectrophotometer that returns sample concentration (ng/ul) and two other parameters: A260/280 and A260/230. Values around 1.8-2.2 of both ratios are an index of good RNA purity.

3.4.3 SYNTHESIS OF THE FIRST cDNA STRAND

RNA was retrotranscribed into complementary DNA (cDNA) which was subsequently used as a template for the real-time PCR. 400ng of total RNA was transcribed using the following reaction mix:

- Random primer hexamers (50ng/µl): 1µl – starting point for cDNA synthesis
- Nucleotides (dNTPs) 10mM: 1µl – building blocks for cDNA synthesis
- 13µl of laboratory-grade RNA-free water to adjust the volume of the mixture
- 400 ng of total RNA

The RNA samples were centrifuged and heated at 65°C for 5 minutes to denature any secondary RNA structures. Afterwards, the samples were rapidly cooled on ice, and master mix containing the following components for each cDNA synthesis reaction was added:

- Reaction buffer 5x (Life Technologies): 4µl – for RT enzyme's ideal conditions
- DTT 100mM: 1µl – to maintain the integrity and activity of the enzyme
- RNase Out (Life Technologies): 1µl – prevents RNA degradation by RNases
- SuperScript^{TM} IV reverse transcriptase enzyme (Life Technologies): 1µl
- RNA-free water: 0.5µl

The utilized reaction program was:

- step1 at 25°C for 10min
- step2 at 42°C for 10min
- step3 at 70°C for 10min.

After the completion, each sample's volume was modified to 30µl using RNA-free water.

3.4.4 QUANTITATIVE REAL-TIME PCR

PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) was employed for the Pex5 gene qPCR. 1µl of diluted cDNA was amplified in a total volume of 10µl PCR reactions in a thermocycler (ABI Prism 7000), coupled with a sequence detection system, in 384-well plates (Micro Amp Optical, Applied Biosystems). In each of the wells, 5µl of sample mix (1µl of template cDNA and 4µl of RNA-free water) was combined with 5µl of reaction mix (4.8µl of the SYBR Green Master Mix and 0.2µl of the Primer forward / reverse Mix (50µM)).

The qPCR reaction setting was:

- step1 at 50°C for 2min
- step2 at 95°C for 2min
- step3 of 40 cycles at 95°C for 15s
- step 4 at 60°C for 1 min

Lastly, the differences in Pex5 gene expression between well-type and knockout mice were determined using a relative quantification method. After the qPCR run, the cycle threshold (Ct) values are calculated for both the target gene, as well as the housekeeping gene of choice. In this case, Pan-actin was used as the reference gene to account for variations in sample preparation and qPCR efficiency (E). Pan-actin is an ideal housekeeping gene as its abundance does not change under experimental conditions. To normalize the expression of Pex5 gene to the expression of Pan-actin, the following formula was used:

$$Ratio = \frac{(E_{target})^{\Delta Ct}}{(E_{reference})^{\Delta Ct}}$$

3.5 EchoMRI

Body composition analysis including measurement of lean mass and fat mass content was performed by quantitative nuclear magnetic resonance using EchoMRI^{TM}-100 system (EchoMRI, LLC, TX, USA). The animal was weighed prior to the analysis and the corresponding data were entered into the system. Afterward, the mouse was placed in a specialized plastic container without the use of anesthesia and then inserted inside the EchoMRI system.

3.6 GLUCOSE AND INSULIN TOLERANCE TEST

The status of tolerance for glucose was assessed by the Glucose (GTT) and Insulin (ITT) Tolerance Test (GTT). A 33% glucose solution (2g/Kg) or a 1% insulin solution (0.75 g/ Kg) was administered via intraperitoneal injection following 5-hour fasting (water available *ad libitum*), with venous blood assayed before and at 30-, 60-, and 120 min after the glucose administration using the Freestyle Insulin glucometer. To obtain the most accurate results, animals' stress and discomfort were minimized.

3.7 ELISA - MEASUREMENT OF INSULIN LEVELS

To quantify the concentration of insulin in a blood sample, Enzyme-Linked Immunosorbent Assay (ELISA) was utilized. Blood samples were collected from the retro-orbital sinus and EDTA was used as an anticoagulant to ensure the best preservation of cellular components and morphology of blood cells. After blood collection, samples underwent centrifugation to separate plasma from the blood's cellular components. The isolated plasma was collected and used for the ELISA analysis. ELISA kit (Sigma-Aldrich) comes with specific instructions on the entire procedure. A microplate pre-coated with insulin-specific capture antibodies was used to bind insulin molecules present in the sample. 100µl of each plasma sample was added to the wells on the microplate in duplicate, alongside a series of standards with various known insulin concentrations to create a calibration curve for accurate quantification of the insulin concentration in the specimens. The plate was incubated at room temperature for 2.5 hours, allowing the insulin to properly bind to the immobilized antibodies. Solutions were discarded and washed with a wash solution to wash away the unbound parts of the sample. Afterward, 100µl of biotin-labeled detection antibody

was introduced, and a second incubation took place. Following another washing step, horseradish peroxidase (HRP)-Streptavidin solution was added to each well. The samples were incubated and washed afterward. HRP enzyme recognized the biotinylated antibody, and the amount of HRP enzyme will be directly proportional to the amount of insulin. Wells were incubated and washed again. 100µl of 3,3', 5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated. The chemical reaction caused a pigment to change from transparent to blue. The blue color intensity is directly related to the insulin concentration present in the samples. To stop the reaction, 100µl of stop solution (diluted sulfuric acid) was introduced and acidification of the formed products turned the solution yellow. The absorbance of each solution, which is directly proportional to the amount of the captured insulin, was immediately measured using a microplate reader at 450 nm. The amount of insulin in each well was derived from the interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

3.8 METABOLIC CAGES

Metabolic parameters of laboratory animals, such as oxygen consumption, carbon dioxide production, energy expenditure, and water and food intake were monitored by using the TSE Phenomaster system. Each mouse was individually placed in a freshly clean isolated cage containing sensors to measure oxygen consumption, carbon dioxide production, food and water compartments. Prior to the beginning of the experiment, gas calibration was performed to ensure accurate data collection. Mice remained in the cages for 3 consecutive overnight periods. For this study, the first 24-hour acclimatization period was excluded from the analysis.

3.9 TREADMILL PROTOCOL

The exercise performance was assessed by maximal treadmill running time. For acute exercise studies, mice were acclimated and trained on a LE8710M treadmill (Harvard Apparatus). On days 1 and 2, all animals were acclimated for 10 min at the speed of 9 m/min, and a foot shock of 0.2 mA intensity was applied. On day 3, mice were subjected to a 10-degree uphill single bout of running starting at the speed of 10 m/min and a foot shock of 0.2 mA intensity. 10 min later, the

treadmill speed was increased at a rate of 1 m/min every 5 min until exhaustion. The exhaustion was defined as the point at which mice spend more than 5 s on the electric shocker without attempting to resume running, even after the increase of the food shock intensity to 1 mA.

3.10 ORGAN AND TISSUE COLLECTION

8-month-old animals were sacrificed by cervical dislocation. Organs and tissues were collected and weighed immediately after euthanasia and directly frozen in liquid nitrogen.

3. 11 STATISTICAL ANALYSES

The data are displayed as the mean ± SEM. The predetermined level of statistical significance was set as $P < 0.05$. Statistical evaluations of two experimental groups of the same genotype were performed through a paired t-distribution test. For comparisons involving more than two groups, a 2-way analysis of variance (ANOVA) was employed. However, to ensure the reliability of ANOVA, conducting a normality test beforehand is a necessary step, as ANOVA assumes that the data within each group or combination of factors are normally distributed.

4. RESULTS

4.1 MUSCLE-SPECIFIC PEX5-KO MICE AS A MODEL TO UNRAVEL THE IMPACT OF PEROXISOMAL DYSFUNCTION IN SKELETAL MUSCLES

To investigate the role of peroxisomes in skeletal muscle function both in basal and metabolic stress conditions (such as high-fat-induced obesity), a muscle-specific Pex5 knockout mouse model (hereafter referred to as Pex5 KO) was generated. Pex5 is essential for peroxisomal functions as this protein is involved in the import of most peroxisomal enzymes into the peroxisomal lumen. To generate this model, Pex5 floxed mice (hereafter referred to as Control or Ctrl) were crossed with a transgenic line expressing Cre recombinase (CRE) under the control of Myosin Light Chain 1 fast promoter (MLC1f).

To validate the successful Pex5 deletion, we monitored Pex5 gene expression in the gastrocnemius muscle using Real-Time PCR. Our results confirmed that in both dietary conditions Pex5 mRNA levels in KO mice were significantly reduced compared to Ctrl mice (Figure 4). Moreover, a significant difference was observed between Ctrl mice on different diets, pointing out a close relation between peroxisome remodeling and adaptations to muscle metabolic stress, such as obesity. Given that Pex5 deletion has been linked to impaired peroxisomal functionality [70, 79], and that previous results in the lab showed that Pex5-null peroxisomes are peroxisomal protein import deficient (unpublished data), it is reasonable to infer that peroxisomal dysfunction might occur also in our experimental model. **Thus, Pex5-null mice could be considered as a suitable model to unravel the impact of peroxisomal alterations in skeletal muscle both in normal and in metabolic stressed conditions.**

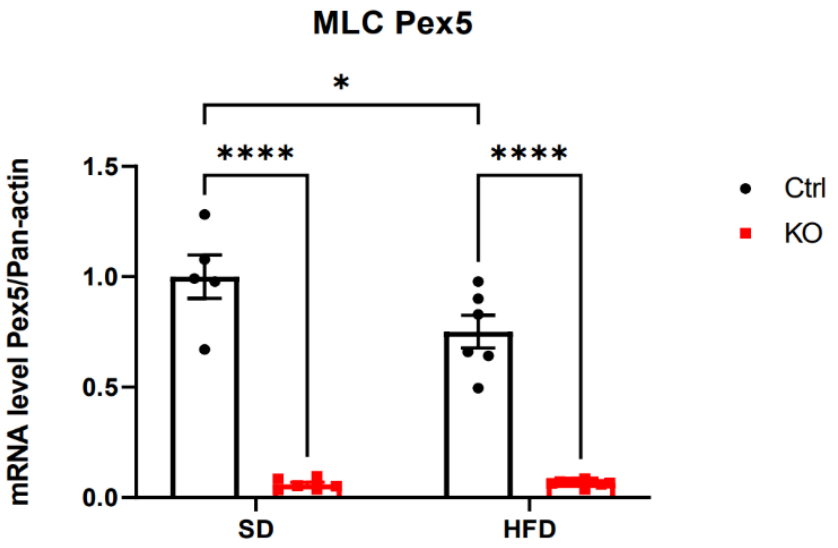


Figure 4. *Pex5 gene expression in gastrocnemius in Ctrl and KO mice treated with a standard diet (SD) and high-fat diet (HFD). Data were normalized to the Pan-actin housekeeping gene. Results are expressed as means ± SEM. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.2 BODY WEIGHT GAIN IS HIGHER IN PEX5 KO MICE SUBJECTED TO THE HIGH-FAT DIET

To study the role of peroxisomes in skeletal muscle function during basal and metabolic stress conditions, such as diet-induced obesity, 3-month-old Ctrl and KO mice were fed both with a standard (SD) and a high-fat diet (HFD) for a 4-month period. Mice's body weight was monitored weekly to verify the gradual weight gain and the development of obesity in mice exposed to HFD compared to those on SD. Figure 5a shows a noticeable increase in body weight gain among all mice subjected to HFD compared to those on SD, which confirms the HFD effectiveness. Concerning mice fed with SD, both Ctrl and KO mice displayed similar body weight gain patterns over time. Conversely, from the first month of treatment onward (Figure 5b, c), Pex5-null mice on HFD gained substantially more body weight compared to Ctrl mice in the same diet group. **These results suggested that the absence of functional peroxisomes affects the net gain of body weight under metabolic stressed conditions, but not under basal conditions.**

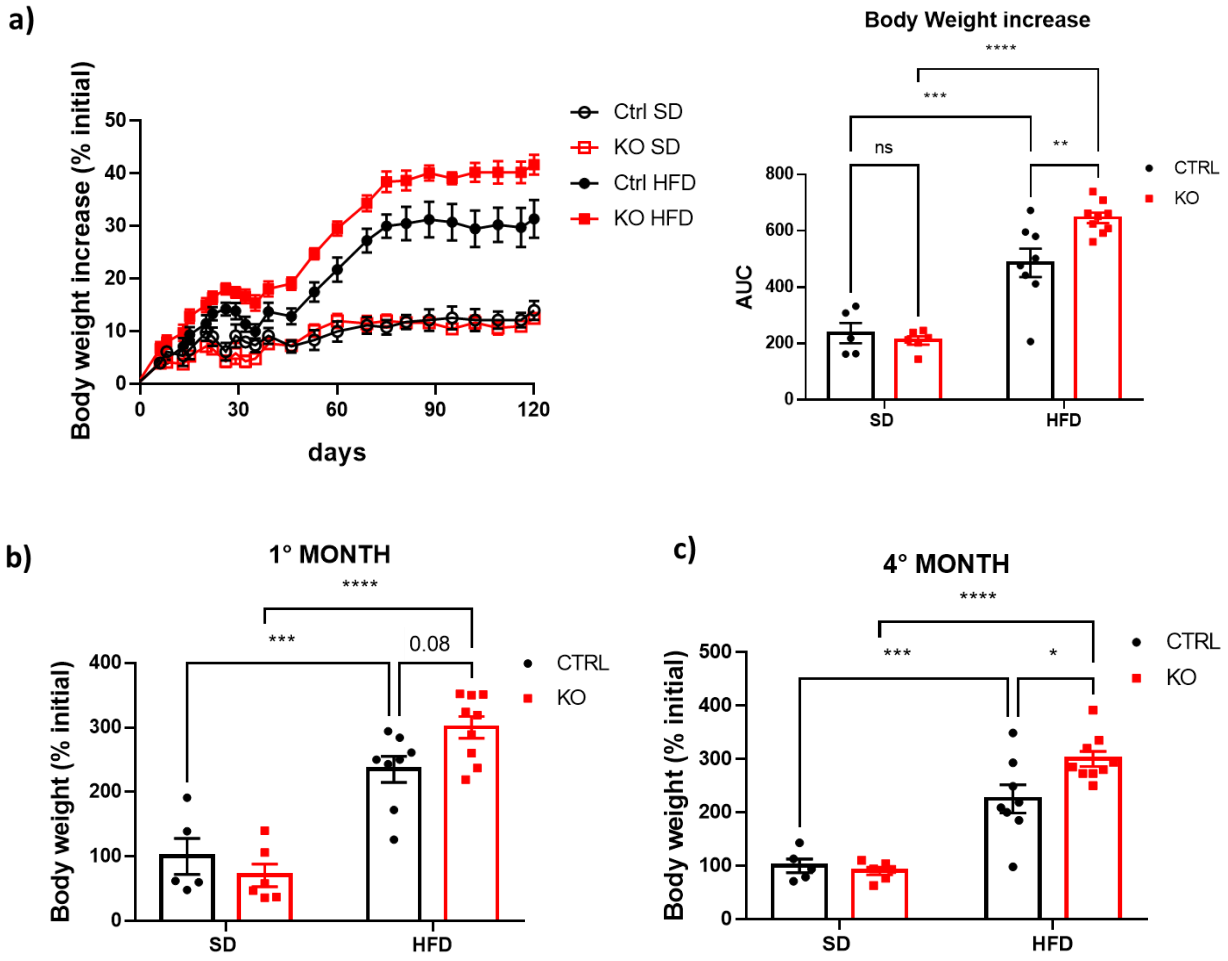


Figure 5. a) *Body weight gain and area under the curve (AUC) of Ctrl and Pex5 KO mice fed with SD and HFD.* ***b-c)*** *Weight gain during months 1 and 4 of the experiment. Data were normalized to Ctrl mice on SD for each time point. Results are expressed as means ± SEM. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.3 PEROXISOMAL DYSFUNCTION IN SKELETAL MUSCLES INDUCES FAT ACCUMULATION IN OBESITY

To uncover reasons for the observed body weight increases between Ctrl and KO mice on HFD, lean, and fat mass were monthly monitored by EchoMRI in all groups. Lean mass accounts for muscles, bones, organs, tissues, and fluids, while fat mass comprises solely the content of fat. Figures 6a and b show that the lean mass remained constant across all groups throughout the entire four-month period, irrespective of Pex5's presence and diet treatment. On the other hand, the fat mass of all mice subjected to HFD experienced a significant increase over time (Figures 6c and d). Moreover, nearly significant differences both during months 1 and 4 were observed in the

fat mass content between Ctrl and muscle-specific Pex5-null mice on HFD (Figures 6e and f). **Taken together, these data suggested that alterations of peroxisomal function in skeletal muscles induce fat accumulation under obesity-induced stressed conditions.**

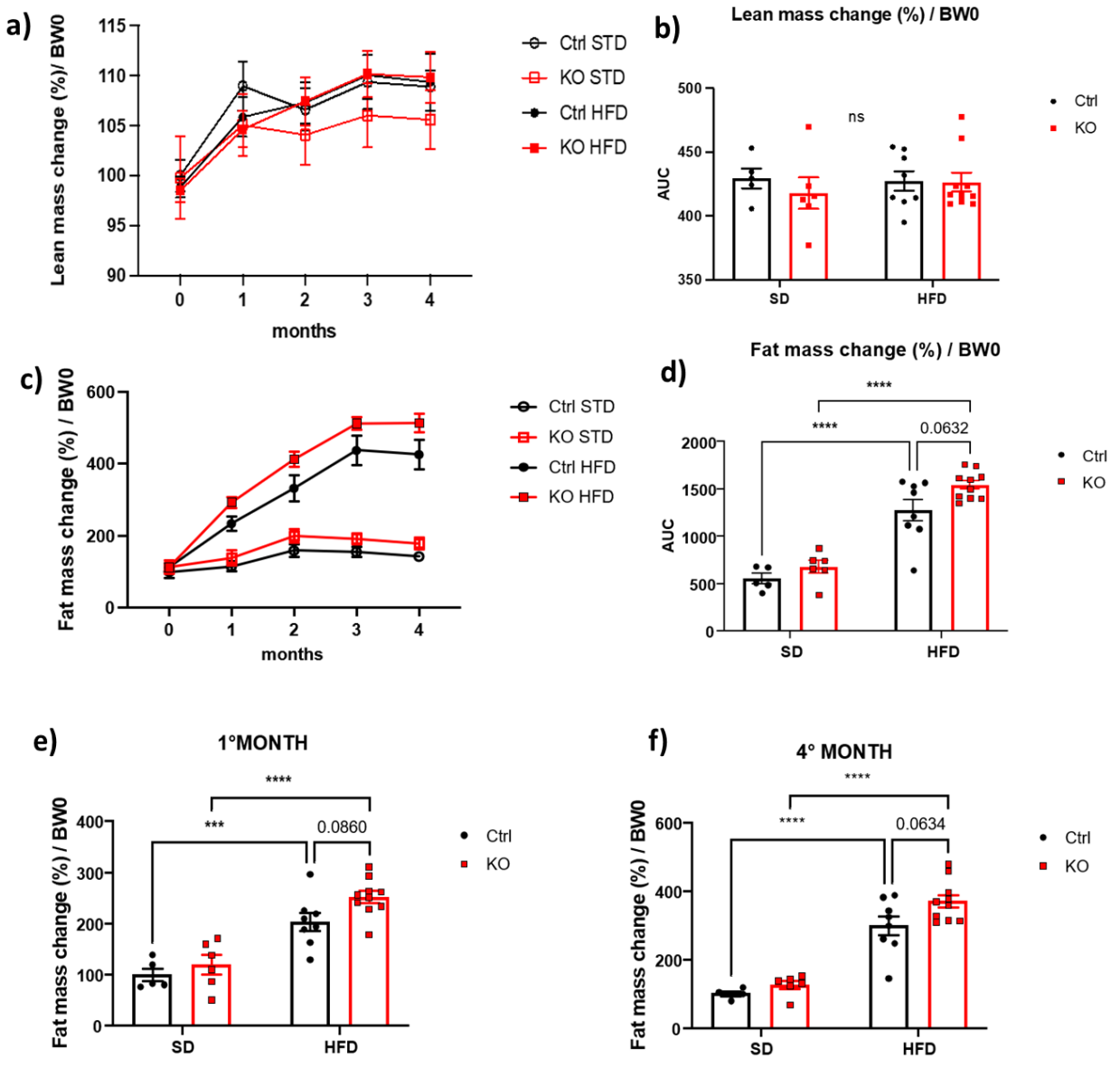


Figure 6. *EchoMRI analysis of Ctrl and KO mice in SD and HFD at different time points.* ***a)*** *lean mass change,* ***b)*** *AUC of lean mass change,* ***c)*** *fat mass change,* ***d)*** *AUC of fat mass change,* ***e)*** *1st month of fat mass change,* ***f)*** *4th month of fat mass change. Data were normalized to initial body weight. Results are expressed as means ± SEM. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.4 PEROXYSOMAL DYSFUNCTION DOES NOT AFFECT ORGAN AND TISSUE SIZE

As both lean and fat mass were not altered among Ctrl and KO mice on HFD, we collected and weighed tissues and organs to understand whether the increased body weight gain in KO mice on HFD was attributed to changes in organ or tissue size.

Acting as a stable and easily measurable indicator of overall body size, the tibia length was used for normalizing organs' weight. As depicted in Figures 7a and b, the size of the heart and liver in all subjects remained constant, showing no notable variations. However, irrespective of the presence of Pex5, all mice subjected to HFD displayed notably elevated levels of white adipose tissue (WAT) (Figure 7c) and brown adipose tissue (BAT) (Figure 7d) compared to their counterparts on SD.

As the primary focus of this study is to investigate the role of peroxisomes in skeletal muscles, different hindlimb muscles were weighted and data normalized to tibia length. In Figures 7e-g., it is evident that no notable variations were observed in the gastrocnemius and Tibialis anterior (TA) muscles' weight among the subjects, irrespective of the Pex5 presence or the diet. Soleus and the Extensor Digitorum Longus (EDL) were also examined. Regarding the soleus muscle, it was observed that the weight of KO mice on HFD was significantly higher compared to KO mice on SD. Conversely, concerning the EDL muscle, a significant difference was noted between Ctrl mice on different diets. **The findings suggested that overall peroxisomal dysfunction in skeletal muscles does not impact the size of organs and tissues**. **However, it appears that obesity can lead to an increased weight of the soleus muscle when properly working peroxisomes are absent.**

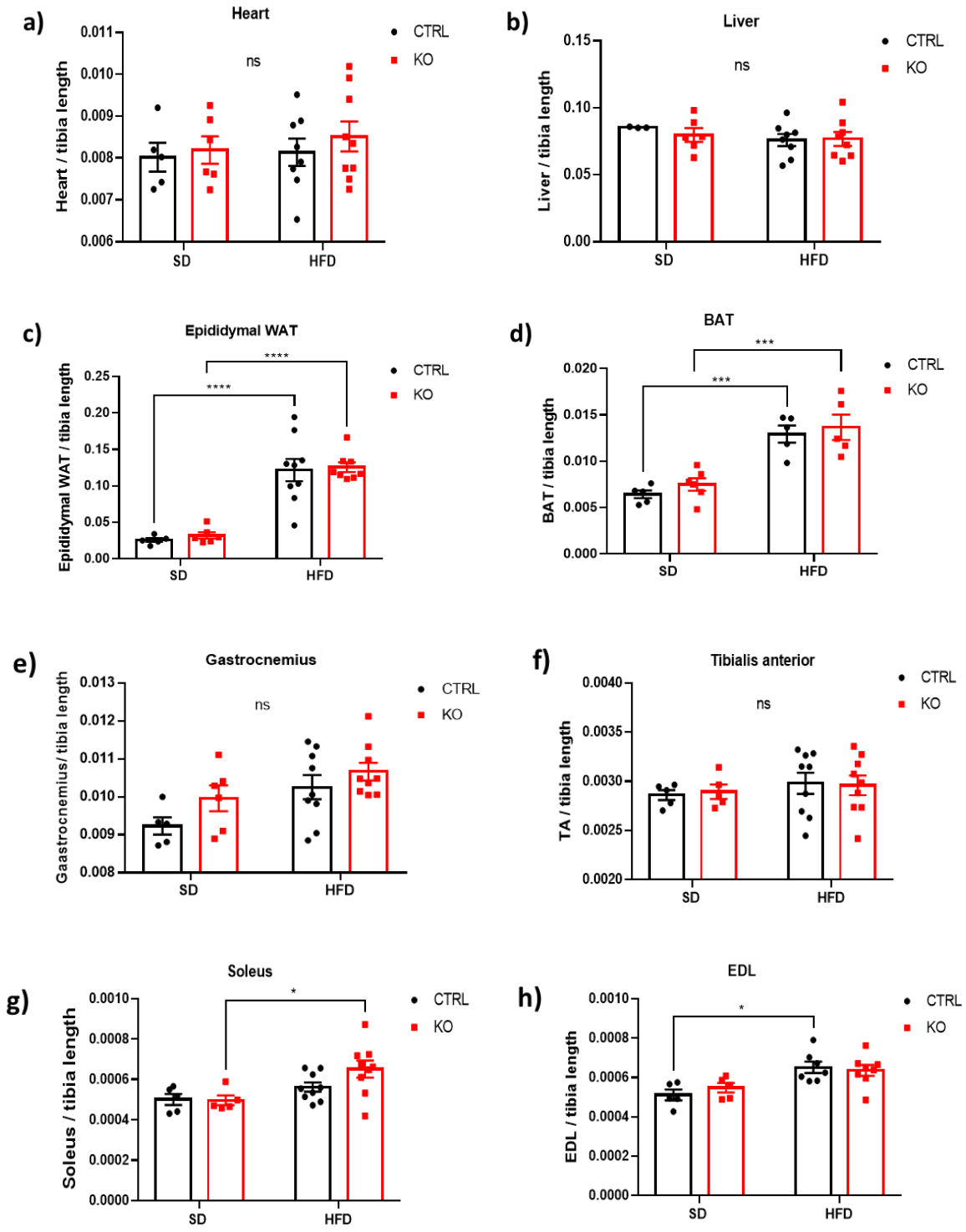
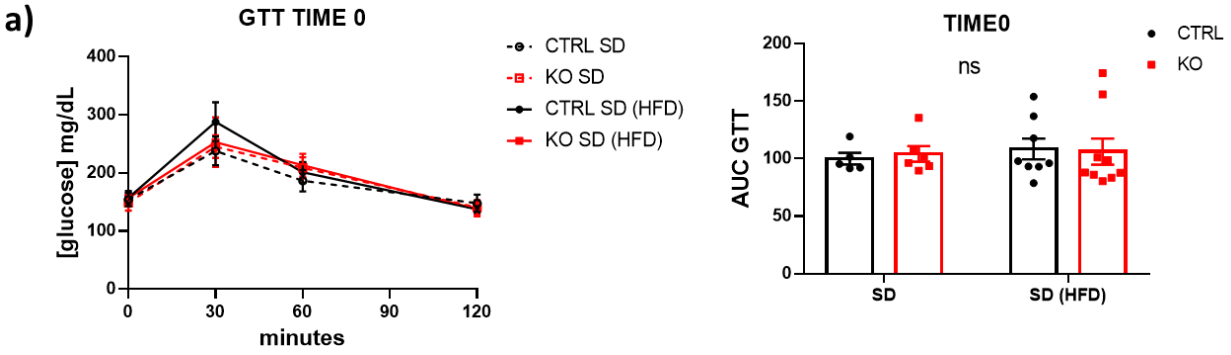


Figure 7. *Organs and muscle size of all subjects normalized to tibia length: heart **(a)**, liver **(b)**, WAT **(c)**, BAT **(d)**, gastrocnemius **(e)**, tibialis anterior **(f)**, soleus **(g)**, EDL **(h)**. Results are expressed as means ± SEM. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.5 PEROXISOMAL DYSFUNCTION IN SKELETAL MUSCLES DOES NOT ALTER GLUCOSE SENSITIVITY

As skeletal muscle is crucial in glucose uptake, we aimed to see whether skeletal muscles' peroxisomes are involved in glucose homeostasis. To assess this point, both Ctrl and KO mice fed with SD and HFD were subjected to Glucose Tolerance test (GTT), a standard experimental technique to evaluate glucose metabolism and the body's ability to maintain balanced blood sugar levels.

As shown in Figure 8a, initial comparison at time 0 (age: 3 months old), prior to the initiation of HFD treatment, revealed no significant distinctions among all subjects involved in the experiment, confirming a consistent baseline for all mice prior to HFD initiation. Our results showed that both after the first and the fourth month of HFD treatment when mice were respectively 3 and 7 months old, both groups of mice on HFD showed higher AUC compared to those on SD (Figure 8b and c). However, no differences among Ctrl and KO subjects on any diet were observed at any given time point. **Collectively, these results suggested that metabolic stress, such as obesity, and the presence of dysfunctional peroxisomes in skeletal muscle do not affect glucose response in either of the experimental conditions.**



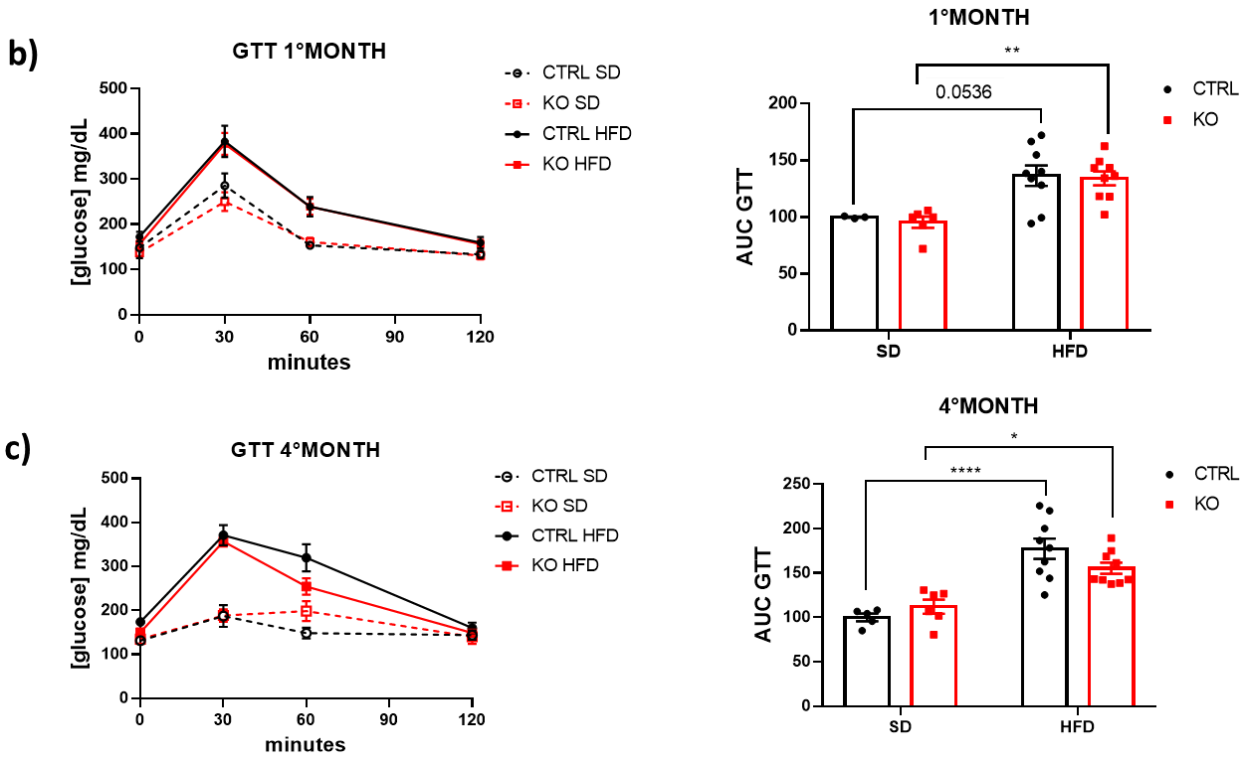


Figure 8. GTT test with *Area under the curve (AUC) before the diet **(a)**, after 1 month **(b)**, and 4 months **(c)** of HFD treatment. For each month, data were normalized to Ctrl mice on SD. Significance *p<0,05; **p<0,01; **p<0,001.*

4.6 INSULIN SENSITIVITY IS NOT ALTERED IN SKELETAL MUSCLES WITH PEX5-NULL PEROXISOMES

Besides GTT, we performed the Insulin Tolerance Test (ITT) to uncover any possible changes in glucose metabolism due to peroxisomal dysfunction. During this test, insulin is administered to lower blood sugar levels and monitor how quickly and effectively blood sugar decreases. All Ctrl and KO mice on both diets (SD, HFD) were subjected to ITT to evaluate how their bodies respond to insulin.

As depicted in Figure 9a, at the start of the experiment, when mice were 3 months old, the ITT of all subjects showed no noteworthy differences among all the participating mice, confirming a consistent baseline before starting the HFD treatment. Notably, one month into HFD, both Ctrl and KO mice on HFD exhibited a significantly higher AUC compared to their counterparts on SD,

pointing to insulin resistance in both HFD groups (Figure 9b). Interestingly, after 4 months of the HFD treatment, i.e., when mice were 7 months old, the increasing trend on HFD was no longer observed, and all groups maintained similar AUC values (Figure 9c). There were no significant distinctions noted among Ctrl and KO groups at any given time point, regardless of the diet. **These findings confirmed that HFD treatment results in impaired insulin sensitivity and that this latter is not affected by dysfunctional peroxisomes in any of the investigated conditions.**

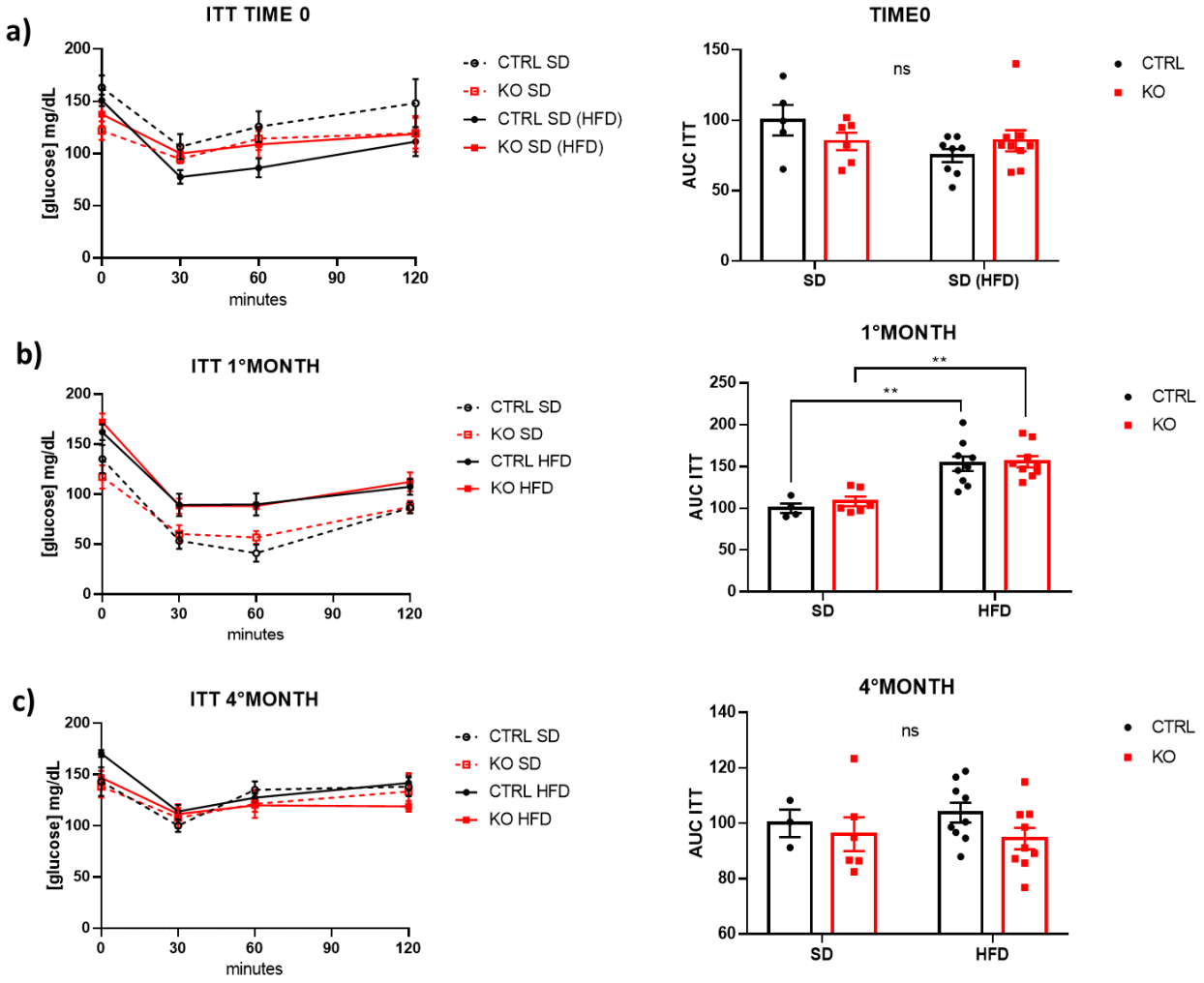


Figure 9. ITT test with *Area under the curve (AUC) before the diet **(a)**, after 1 month **(b)**, and 4 months **(c)** of HFD treatment. For each month, data were normalized to Ctrl mice on SD. Significance *p<0,05; **p<0,01; **p<0,001.*

4.7 HIGH-FAT DIET EXACERBATES INSULIN LEVELS WHEN SKELETAL MUSCLE PEROXISOMES ARE DYSFUNCTIONAL

To further investigate the link between peroxisomes and glucose, which is delivered in skeletal muscles by insulin, we monitored insulin levels in Ctrl and KO mice in both diet conditions by carrying out ELISA assays.

Figure 10. illustrates that the blood insulin concentration levels exhibited a trend of increase in all mice exposed to HFD, underscoring the influence of HFD. During the 2nd month, HFD had a substantial impact on insulin levels in the KO mice, while there were no notable differences among Ctrl groups subjected to different diets (Figure 10a). Moreover, a significant difference was observed between Ctrl and KO mice on HFD. Similarly, in the 4th month, there was a nearly significant difference only between KOs fed with different diets (Figure 10b). Furthermore, although the difference between KO and Ctrl mice on HFD was not substantially significant, an increasing trend is observed in the HFD-fed KO mice compared to the HFD-fed Ctrls. **These results suggested that the absence of functional peroxisomes increases the sensitivity to the adverse effects of HFD in insulin production, implying the importance of peroxisomal function in blood insulin level regulation.**

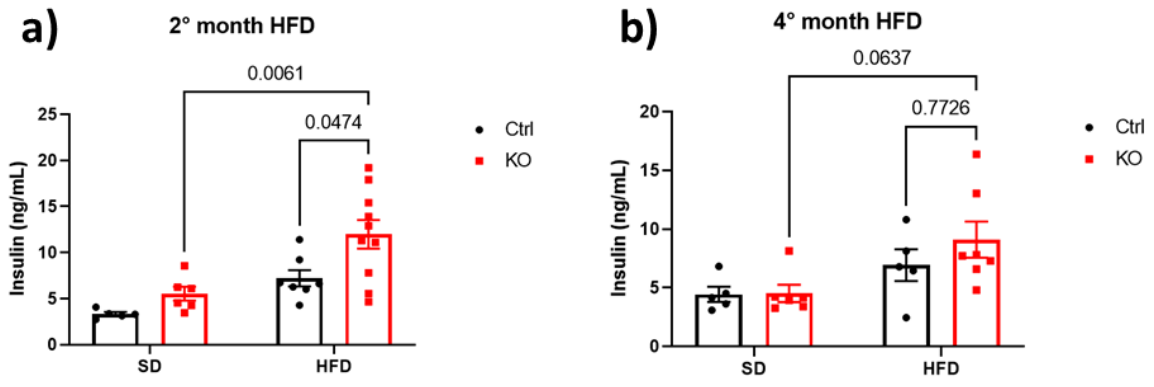
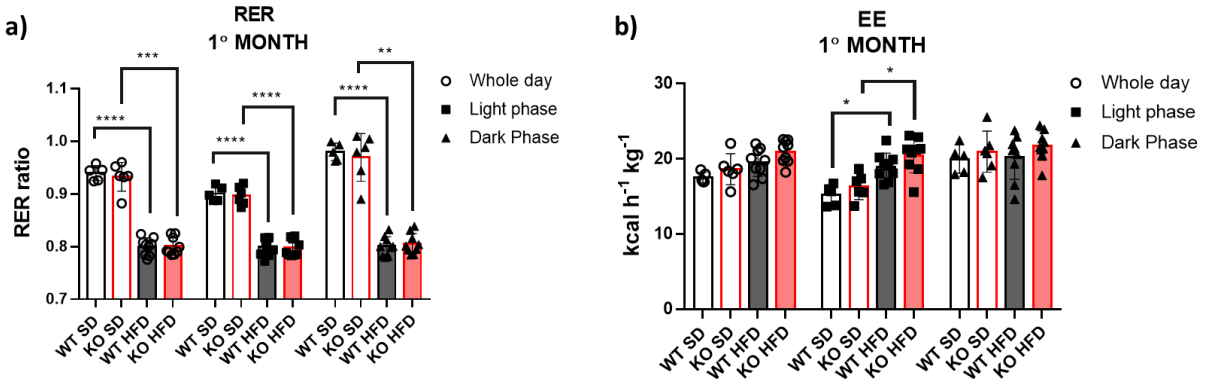


Figure 10. *ELISA-obtained blood insulin concentration levels after 1 month **(a)** and 4 months **(b)** of HFD treatment. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.8 DYSFUNCTIONAL PEROXISOMES IN SKELETAL MUSCLES DO NOT AFFECT ENERGETIC METABOLISM

Next, we investigated whether the presence of dysfunctional peroxisomes in skeletal muscles could affect the whole energy metabolism, both in basal and in obesity-stressed conditions. To reach this aim, the indirect calorimetry method was used: all mice were individually placed in single cages and the volume of O_2 produced and CO_2 released were monitored to calculate the Respiratory Exchange Ratio (RER) and the Energy Expenditure (EE).

Energy metabolism analysis was done after one month (Figures 11a, b) and four months (Figures 11 c, d) of the HFD exposure. As anticipated, at both time points, all mice on HFD had RER values close to 0.8 (preference for fat oxygenation), whereas all SD-treated mice had RER above 0.9 (preference for carbohydrate oxygenation). However, there were no differences between Ctrl and KO on the same diet. Concerning the EE values, a significant difference was observed during the light phase (less active phase of mice) of the first month between Ctrl and KO mice on different diets. However, this difference was no longer observed during the last month of treatment. Moreover, there were no discernible differences between Ctrl and KO mice within the same diet groups. **Taken together, these data suggested that alterations in skeletal muscle peroxisomes do not alter energy metabolism either in normal or in obesity-stressed conditions.**



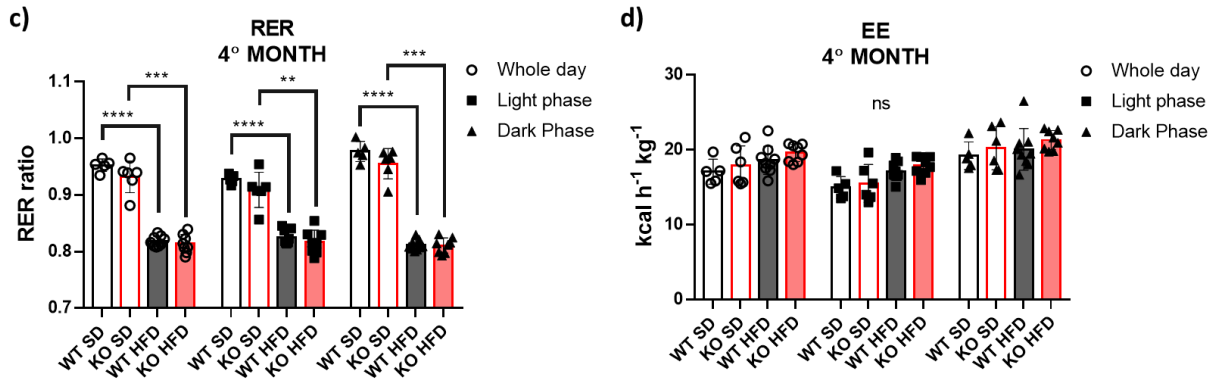


Figure 11. *RER and EE metabolic cage analysis of Ctrl and KO mice after 1- **(a, b)** and 4- months **(c, d)** of SD or HFD treatment. Data are presented as the mean ± SEM. Significance *p<0,05; **p<0,01; ***p<0,001.*

4.9 DYSFUNCTIONAL PEROXISOMES AFFECT MUSCLE PERFORMANCE UNDER METABOLIC STRESS CONDITION

Next, we aimed to investigate whether the lack of functional peroxisomes might influence muscle performance. Running experiments were conducted during the 4th month of HFD, comparing Ctrl and KO on HFD. Both distance and running time were measured, providing insights into mice's muscle health and overall fitness. On average, Ctrl mice on HFD ran about 750m, while KO mice on HFD ran about 550m (Figure 12a). In terms of time, Ctrl mice were able to run for about 55 minutes and KO mice for about 40 minutes (Figure 12b) Both measurements significantly differed between Ctrl and KO mice on HFD. **These results conveyed the impression that in stressed conditions, the lack of functional peroxisomes in skeletal muscles might affect muscle performance, suggesting a link between muscle health and peroxisomal fitness.**

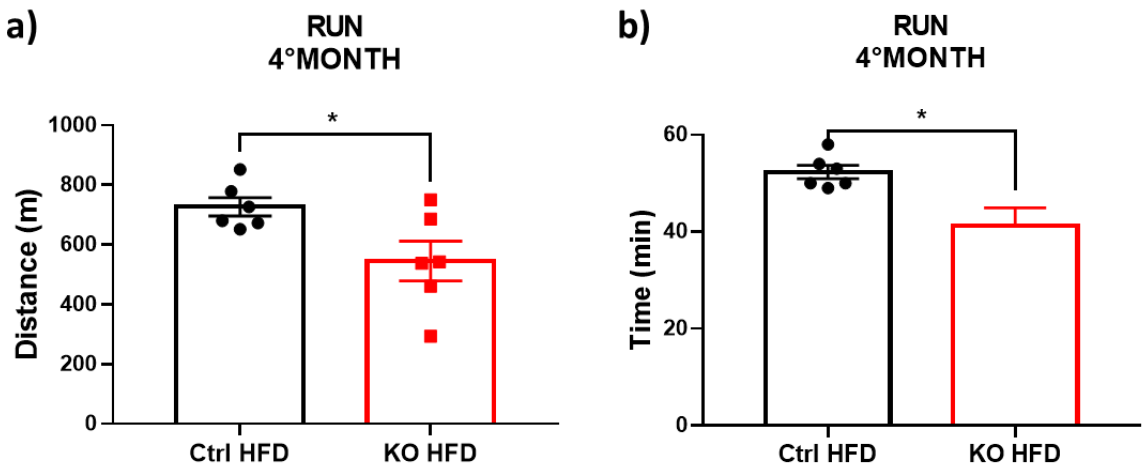


Figure 12. *Treadmill run of Ctrl and KO mice on HFD after 4 months of treatment, measurement of distance* ***(a)*** *and time* ***(b).*** *Data are presented as the mean ± SEM.* *******, $P < 0.05$; **, $P < 0.01$; **, $P<0.001$.

5. DISCUSSION

Skeletal muscle is one the most abundant and dynamic tissues in the human body, accounting for approximately 40% of the total body mass. Skeletal muscles play a major role in various bodily functions, including movement enabling, posture maintenance, force, and power generation. Metabolically, this type of muscle tissue contributes to basal energy metabolism, serving as a storage site for essential substances like amino acids and carbohydrates, producing heat, and consuming a significant portion of oxygen and fuel during physical activity. Notably, skeletal muscles serve as a reservoir of amino acids required for the synthesis of organ-specific proteins. The amino acid release also aids in maintaining blood glucose levels during periods of starvation [1]. Additionally, skeletal muscles produce and release signaling molecules called myokines, playing a role in inflammation, and communication between different tissues in the body [7]. Importantly, reduced muscle mass impairs the body's response to stress and chronic illness, highlighting its significance for disease prevention and overall health maintenance [80]. Peroxisomes are small, membrane-bound structures found within eukaryotic cells. One of their primary functions is their involvement in lipid metabolism, playing a crucial role in the breakdown of fatty acids. This makes peroxisomes particularly vital in tissues with high metabolic demands, such as the liver and kidneys [81]. Considering that skeletal muscles are major contributors to energy metabolism, it is reasonable to explore the potential distinctive functions of peroxisomes within these tissues. Remarkably, peroxisomes also participate in the synthesis of specific lipids, including plasmalogens, which are essential for maintaining the integrity and fluidity of cell membranes. These organelles are involved in cellular defense mechanisms, by safeguarding against reactive oxygen species through the containment of enzymes such as catalase and peroxidase [23, 39]. Additionally, peroxisomes have been linked to obesity [26]. For instance, the maintenance of peroxisomal homeostasis in adipose tissue was shown to attenuate obesity features [70]. Our investigation revealed a potential correlation between peroxisomes within skeletal muscles and obesity, as we observed a reduction in Pex5 gene expression in control mice on a high-fat diet (HFD) compared to their counterparts maintained on a standard diet (SD) [see Figure 4]. Therefore, our aim was to explore the role of peroxisomes in the metabolic pathways

of skeletal muscles under both basal and stress conditions induced by a high-fat diet leading to obesity.

To do that, a muscle-specific Pex5 knockout mouse model was established. The muscle-specific Pex5 gene deletion was initially assessed through Cre-recombinase PCR genotyping and then confirmed by Real-time PCR. This assessment revealed that all Cre-positive mice had a significant reduction in Pex5 gene expression compared to the Cre-negative control mice (see Figure 4). The deletion of Pex5 has previously been associated with dysfunctional peroxisomes in various tissues, including adipose tissue [70] and the peripheral nervous system [82]. Prior experiments in our laboratory have demonstrated that Pex5-null peroxisomes in skeletal muscles exhibit defects. Immunostaining analysis against PMP70 (a peroxisomal marker) in single FDB fibers revealed that KO mice display PMP70-positive puncta. However, when transfected with the SKL-GFP plasmid to assess peroxisomal functionality, Pex5-null mice showed a cytosolic distribution rather than punctate localization. This implies an impairment in peroxisomal protein import within peroxisomes. In addition, in Ctrl mice, PMP70 was colocalized with ACAA1 (Acetyl-CoA Acyltransferase 1), a protein that is synthesized in the cytoplasm and then transported into the peroxisomal matrix for VLCFA degradation. This colocalization was not observed in KO mice, further indicating defects in peroxisomal protein matrix import. In essence, Pex5-null mice exhibit PMP70-positive structures resembling peroxisomal ghosts (like those seen in Zellweger disease). Pex5 KO mice have been previously utilized to study the role of dysfunctional peroxisomes in the Zellweger syndrome, a condition that affects the entire body [83]. These structures are import-deficient residual peroxisomal membranes with minimal or no matrix content. Thus, all the prior (unpublished and not shown) findings supported the suitability of our muscle-specific Pex5 KO model for investigating the impact of dysfunctional peroxisomes in skeletal muscles.

To reach our aim, 3-month-old mice were fed with SD and HFD for four months. The successful establishment of diet-induced obesity was validated by a significant increase in body weight gain observed across all HFD-treated subjects compared to those on the standard diet (Figure 5a). Notably, both Ctrl and KO mice on SD displayed similar body weight gain patterns over time, suggesting that under basal conditions, the absence of functional peroxisomes does not

substantially influence the overall body weight gain (Figures 5b and c). However, Pex-5 null mice on HFD exhibited a substantial increase in body weight gain relative to Ctrl mice within the same dietary group. This notable disparity in body weight gain under metabolic stress conditions highlights the potentially critical role of functional skeletal muscular peroxisomes in regulating body weight during diet-induced obesity (Figures 5c and d).

To understand the cause of the observed body weight increase, body weight composition was monitored monthly through EchoMRI analyzer which measures body fat, lean, and free water masses. The analysis revealed the efficiency of the HFD as all mice subjected to this treatment gained more fat mass over time relative to mice on SD (Figures 6c and d). Unexpectedly, the lean mass, which encompasses muscles, bones, organs, tissues, and fluids remained relatively constant across all groups over the entire duration of the study (Figures 6a and b), irrespective of the presence of Pex5 and the dietary regimen. Previous research has linked obesity to a reduction in muscle quality and a faster decline in lean mass [66]. Therefore, in an HFD-induced obesity context, we would have expected to see a reduction in the lean mass over time in obese mice, more emphasized in the presence of dysfunctional peroxisomes which are necessary for organs' health and functionality. Several factors could account for this lack of change in lean mass between the two dietary groups, including possible compensatory mechanisms, the contribution of other tissues, and possibly an insufficient duration of the experiment. It would therefore be ideal to repeat the experimental procedure with a new group of mice and extend the treatment to possibly see any changes. Moreover, to further validate the data, the analysis could also be repeated by inducing muscle-specific peroxisomal dysfunction in ob/ob mice to investigate the crosstalk between skeletal muscles and WAT mediated by peroxisomes. Ob/ob mice are a strain of genetically modified leptin-deficient laboratory mice commonly used in obesity research. As a result, these mice are unable to regulate their body weight and become markedly obese, with excessive fat accumulation and various metabolic abnormalities [84].

On the other hand, our data reported that KO mice in HFD gained more fat mass compared to Ctrl mice. This observation offers a possible explanation for the higher overall body weight gain observed in KO mice. One could think that under obesity conditions, defective peroxisomes can

induce an impairment in lipid uptake in the skeletal muscles thus inducing fat accumulation in other organs. In addition, skeletal muscles harboring dysfunctional peroxisomes could also be unable to sustain the degradation of a large amount of fatty acid, especially VLCFA, introduced with the diet. This could result in the establishment of a crosstalk between muscles and other metabolic organs, such as liver and adipose tissue, that would therefore accumulate the fatty acids not degraded from the muscles. To understand where this fat accumulation occurred and to see whether peroxisomal dysfunction could induce change in organs' size (atrophy or hypertrophy), various tissues and organs were collected and weighed. In contrast to our initial assumptions, the size of two pivotal organs, the heart, and the liver, remained constant across all experimental groups (Figures 7a and b), which suggests that peroxisomal dysfunction within skeletal muscles did not influence their size. We anticipated observing variations, particularly in the weight and morphological characteristics of the liver, due to the possible fat accumulation resulting from fatty acid breakdown disruption in peroxisome-deficient muscles. To further explore this phenomenon, Oil Red O (ORO) staining, a histological technique used to visualize lipid droplet accumulation within tissues, including the liver, could be performed.

Concerning the adipose tissues, all HFD-treated mice displayed notably elevated levels of epididymal WAT and BAT compared to their counterparts on SD (Figures 7c and d). The increase in adipose tissue is a well-established response to high-fat diets and reflects the body's adaptive response to excess energy intake [85, 86]. However, we would have expected to see a difference between Ctrl and KO on HFD which would have explained fat accumulation in KO subjects. Likely there are other types of adipose tissue that were not collected, such as visceral and abdominal adipose tissue, that could be responsible for fat increase.

Turning attention to skeletal muscles themselves, various hindlimb muscles were weighed (Figures 7e - h). The gastrocnemius and tibialis anterior (TA) are key components of the mouse hindlimb and have distinct structures and functions. Gastrocnemius is a white, relatively large muscle, and is composed primarily of fast-twitch IIA fibers, making the muscle well-suited for activities that require short bursts of intense power [87]. TA muscle is located at the front of the lower leg and consists of predominantly fast-twitch IIB, and IIX fibers, with a smaller contribution

of IIA fibers [88]. Both the gastrocnemius and TA muscles showed no notable variations in weight among all subjects regardless of the presence of Pex5 or dietary conditions, suggesting that peroxisomal dysfunction did not affect the size of these muscles. However, differences emerged when examining the soleus and Extensor Digitorum Longus (EDL) muscles. Soleus is located deep in the calf region of the hindlimb, with a red appearance due to its high myoglobin content, which is characteristic of oxidative, slow-twitch fibers [88]. ELD muscle is a relatively small muscle, primarily composed of fast-twitch IIA and IIB muscle fibers with a lower mitochondrial content. It has a pale appearance due to its lower myoglobin content compared to slow-twitch muscles [89]. The soleus muscle in KO mice on HFD showed a significantly greater weight compared to KO mice on SD. There was a slight increase in the weight of soleus muscle in Ctrl mice on HFD, compared to Ctrl mice on SD. Therefore, these results suggest that the impact of dysfunctional peroxisomes on soleus weight was aggravated when combined with an obesity-inducing high-fat diet. An increase in soleus muscle weight might be attributed to lipid accumulation. To substantiate this hypothesis, one could examine muscle fibers under electron microscopy to identify the presence of lipid droplets. Alternatively, BODIPY staining, a fluorescent dye for lipid visualization within tissues, could be employed for verification.

On the contrary, a significant difference in EDL muscle weight emerged only between Ctrl mice on different diets. A slight increase in EDL size was also observed among KO mice on different diets. These findings indicate that, unlike in the case of soleus muscle weight, diet alone can affect the size of the EDL muscle, even with properly functioning peroxisomes. The complex regulation of organ and tissue size involves various signaling pathways and interactions between various physiological processes [90], which may not be primarily governed by peroxisomal function within skeletal muscles. The study's focus on a specific set of organs and muscles may not account for potential variations in other tissues (e.g., the brain, as dysfunctional peroxisomes can disrupt the supply of essential fatty acids and metabolites to the brain; kidneys, as altered lipid metabolism can influence kidney function; or pancreas, as this tissue is responsible for insulin secretion and glucose metabolism) and metabolic processes that could respond differently to peroxisomal dysfunction in skeletal muscles. Additionally, it is important to acknowledge that the collecting

and weighing of tissue and muscle samples may be susceptible to procedural human errors that could have influenced the outcomes.

Glucose serves as the primary energy source for cells [91]. To facilitate the uptake of glucose by cells and lower blood sugar levels, the pancreas secretes a hormone called insulin [92]. The Glucose Tolerance Test is a valuable tool for assessing metabolic well-being, insulin sensitivity, and potential issues related to glucose metabolism. A standard response to the GTT involves a temporary rise in blood sugar levels following the introduction of glucose. This spike triggers the pancreas to release insulin, allowing cells to absorb glucose. As a result, blood sugar levels gradually return to their initial baseline as cells utilize glucose. Abnormal glucose metabolism is associated with metabolic disorders such as diabetes, obesity, and cardiovascular diseases [93]. Throughout this study, both Ctrl and KO mice on the HFD exhibited higher AUC values compared to those on the SD, indicating the development of hyperglycemia (elevated blood sugar levels) in response to HFD. High-fat diets are known to contribute to the development of impaired glucose tolerance [94]. However, there were no significant differences observed among Ctrl and KO mice on any diet, which may indicate that diet composition and calorie content have a more dominant influence on blood sugar levels compared to the genotype. It appears that muscle-specific peroxisomes may not have a direct or major role in regulating blood sugar levels.

Since insulin is involved in glucose metabolism, together with GTT, we performed the Insulin Tolerance Test. Insulin plays an important role in regulating blood sugar levels, enabling glucose uptake, driving energy metabolism, facilitating energy storage in the form of glycogen, promoting amino acid entry into cells, and many more [95]. During an ITT, insulin is administered to induce a rapid decrease in blood glucose levels. As blood sugar levels decrease, the body's counterregulatory mechanisms should kick in to prevent severe hypoglycemia (abnormally low blood sugar levels). These mechanisms include the release of hormones like glucagon, epinephrine, and cortisol to raise blood sugar levels back to a safe range [96]. Unhealthy trends during an ITT include a blunted or delayed drop in blood glucose levels after insulin administration, which can indicate insulin insensitivity. Insulin insensitivity is a condition where cells do not respond efficiently to insulin, resulting in elevated blood sugar levels and this

condition is often triggered by obesity [2, 97]. As anticipated, after one month of HFD treatment, the insulin sensitivity of all HFD groups significantly decreased compared to their SD counterparts. However, an interesting observation is that after 4 months of HFD, this trend was no longer evident. Surprisingly, mice on an SD attained similar levels as mice on an HFD. This could be attributed to potential insulin insensitivity, which may arise because of growth and aging in the mice. Nonetheless, our expectations were for a higher AUC indicating greater insulin insensitivity in the HFD group compared to the SD group, as the HFD group presumably faced a more challenging metabolic environment. Notably, as no significant differences emerged between Ctrl and KO groups at any given time point, regardless of the diet, it appears that muscle-specific dysfunctional peroxisomes do not have a discernible impact on insulin sensitivity within the experimental conditions.

Measuring the concentration of insulin in blood samples is another way to provide valuable information on various physiological and pathological processes. In the context of our study, normal insulin levels typically reflect healthy glucose metabolism and insulin sensitivity. Elevated insulin levels often signal reduced cell responsiveness, including skeletal muscle cells, to the effect of insulin. To keep up with blood sugar management the body increases insulin production which is the hallmark of type 2 diabetes mellitus [3]. Conversely, diminished insulin levels may indicate insufficient pancreatic insulin production, which is characteristic of type 1 diabetes mellitus. Insulin levels can be impacted by a wide range of factors, including diet composition, physical activity, stress, sleep patterns, genetic predisposition, the presence of metabolic disorders, inflammation, and other contributors. Therefore, insulin levels are the product of the interplay among these factors [95, 98]. The results demonstrated that all mice exposed to HFD exhibited an increase in blood insulin concentration levels compared to those on the SD (Figure 10), suggesting the influence of the diet on insulin production, which is a typical response to a high-fat diet and obesity [94, 97, 98]. Notably, during the 2^{nd} and 4^{th} months of HFD treatment, the KO mice in HFD showed a substantial increase in insulin levels compared to the SD-fed KO mice, while there were no significant differences among the Ctrl groups on different diets. These results suggest that the absence of functional peroxisomes increases susceptibility to the adverse effects of HFD on insulin sensitivity. Prior research showed that excess accumulation of lipids impairs

insulin sensitivity in skeletal muscle [99]. Therefore, a possible explanation is that as peroxisomes are involved in fatty acid metabolism, their dysfunction can lead to lipid accumulation. Increased lipid accumulation, particularly in skeletal muscle, can disrupt insulin signaling pathways, leading to diminished insulin sensitivity. Moreover, significant differences emerged between KO and Ctrl mice on HFD during the 2nd month. During month 4, the differences were no longer significant, however, an increasing trend was observed in the HDF-fed KO mice compared to the HFD Ctrls. This could be because KO mice on HFD are gaining more fat, and as the amount of circulating free fatty acids increases, insulin receptors are impaired, and insulin is no longer efficiently sensed by the cells. Eventually, pancreas produces more insulin.

Indirect calorimetry is a method used to estimate energy expenditure (EE) and determine the respiratory exchange ratio (RER) by measuring the exchange of oxygen (O2) and carbon dioxide (CO2) gases during metabolic processes [100]. Additionally, our metabolic results on the consumption of oxygen and production of carbon dioxide provide insight into the individual phases of the day, light phase versus dark phase, to account for differences in metabolic processes and behavior that occur during each phase. Mice are nocturnal animals, typically being more active during the night, which is referred to as their dark phase. On the other hand, mice tend to rest and sleep more during the daylight hours, referred to as the light phase. RER is the ratio between VCO_2 production and VO_2 consumption, shedding light on the type of fuel (fat or carbohydrates) metabolized for energy production. RER values close to 1.0 indicate that the dominant energy source is carbohydrates. Conversely, values close to 0.7 or below indicate that fat is the main energy source. In case RER values fall between 0.7 and 1.0, it means that both fuel sources, fats, and carbohydrates, are contributing to energy production and the dominance of one over the other depends on the proximity of the value to 0.7 or 1.0 [100]. Energy expenditure (EE) refers to the total amount of energy utilized or consumed by an organism within a defined timeframe, and it is calculated based on the amount of oxygen consumed and carbon dioxide produced. EE encompasses three elements: (1) resting metabolic rate, (2) physical activity, and (3) thermic effect of food [101]. The resting metabolic rate is the amount of energy that the body requires to maintain its essential functions and some of the factors it depends on are age, gender, body composition, hormonal levels, and genetics. Physical activity represents the energy used for

bodily movements, when muscles are engaged, for instance walking, running, and playing. The thermic effect of food is diet-induced thermogenesis stemming from digestion and nutrient processing. Different types of nutrients require varying amounts of energy to be metabolized. Protein has the highest thermic effect, requiring about 20-30% of the energy it provides. Carbohydrates require approximately 5-10% of their energy content and fats having the lowest thermic effect require about 2-3% of the energy they contain [102]. Our analysis revealed that after both one month and four months of HFD exposure, all mice on HFD exhibited RER values close to 0.8, indicating a preference for fat as a source of energy (Figures 11a and c). In contrast, SD-treated mice had RER values above 0.9, indicating a preference for carbohydrate metabolism. This is in line with the expected metabolic response to the dietary fat and carbohydrate content. Interestingly, there were no discernible differences in RER values between Ctrl and KO mice on the same diet. Nonetheless, given the observed increase in fat accumulation in KO mice on an HFD and their reduced capacity to efficiently degrade fatty acids, it was anticipated that RER values near 1 would be evident in HFD KO mice. This expectation stems from their heightened reliance on glucose for ATP production. Concerning the EE results (Figure 11b and d), HFD-fed mice had significantly increased EE values during the light phase of the first month, which may represent short-term adaptations to dietary changes, and these differences may have resolved over time as mice acclimated to their diets. The lack of significant differences in EE during the 4th month suggests that initial disparities leveled out or that other factors could be influencing EE more prominently at this point in the experiment. In general, a higher EE can be caused by positive factors, for instance, increased physical activity or increased metabolism, as well as negative ones, such as increased muscle imbalances (muscles need to work harder to perform tasks) and the potential need for muscle repair mechanisms [102]. Nonetheless, consistent with the RER data, there were no notable differences in EE between Ctrl and KO mice within the same diet groups. Altogether, these results suggest that alteration in skeletal muscle peroxisomes do not have a substantial impact on overall energy metabolism, both in normal and obesity-stressed conditions. Some of the possible explanations could be compensatory mechanisms that maintain metabolic homeostasis in response to peroxisomal dysfunction.

The treadmill experiment revealed that KO mice on an HFD covered both a shorter distance and ran for a shorter duration, compared to Ctrl mice on an HFD (Figures 12a and b). These results suggest a connection between peroxisomal fitness and muscle health, especially under conditions of metabolic stress. The reduced endurance observed in Pex5-null mice may be attributed to the involvement of peroxisomes in the conversion of fatty acids into energy, a critical process for sustaining endurance activities such as running. As a result, muscle performance, particularly in prolonged physical activity, may be compromised. The use of an HFD as a dietary condition adds another layer of complexity, as HFDs are known to induce metabolic changes, including lipid accumulation and insulin insensitivity, which can negatively affect muscle function [94, 99]. Other factors, such as muscle inflammation, oxidative stress, and changes in substrate utilization, may also contribute to the observed differences and should be explored in future studies.

To sum up, our study unveiled the consequences of impaired peroxisomal function in muscle tissue on various aspects, including weight gain, body composition, tissue size, muscle metabolic health, and endurance activity. Notably, dysfunctional peroxisomes had a significant impact on body weight gain and fat accumulation under HFD-induced obesity conditions. While HFD treatment led to impaired glucose and insulin sensitivity, this impairment was not influenced by peroxisomal dysfunction in muscle tissue. However, peroxisomal dysfunction in skeletal muscles seemed to increase the sensitivity to the adverse effects of HFD in insulin production. Nonetheless, in stress conditions, the lack of functional peroxisomes in skeletal muscles appeared to affect muscle performance. In conclusion, these findings emphasize the complex interplay between peroxisomal function, metabolic stress, and various physiological parameters. Dysfunctional peroxisomes exhibit distinct effects on different aspects of metabolic physiology. These results shed light on the importance of peroxisomes in overall metabolic health and offer valuable insights for future research in this field.

Although we see some differences in the muscle-specific KO mice compared to wild-type mice, before drawing definitive conclusions, additional experiments are required to strengthen our findings. These experiments could include Hematoxylin and Eosin (H&E) staining to study morphology and cellular components of skeletal muscles, cross-sectional area measurements to

detect potential signs of muscle atrophy, fiber type analysis to see a potential change in the proportion of glycolytic and oxidative fibers within skeletal muscles, force measurement in vivo to additionally assess muscle health and performance. Future research may explore the physical and metabolic crosstalk between peroxisomes and other organelles (like mitochondria and the endoplasmic reticulum) in the context of obesity. If subsequent experiments substantiate the adverse impact of peroxisomal dysfunction on obesity, muscle force, and muscle performance, as a next step, one could investigate the modulation of peroxisomal biogenesis and activity to determine if the restoration of peroxisomal function leads to improvements in the overall phenotype. This approach may offer valuable insights into potential therapeutic interventions.

This project put the basis in the understanding of the role of peroxisomes in muscle metabolism, which would help the maintenance of muscle mass and force in patients affected by peroxisomal disorders and obesity, thus ameliorating their whole life conditions.

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