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Implications for lipid induced sarcopenia; *Ishige okamurae* and its active components prevent palmitate impaired skeletal myogenesis

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Summary

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Sarcopenia, defined as the loss of muscle mass and strength with increasing age, leads to disability, frailty, morbidity, and mortality. Anorexia and cachexia lead to the loss of muscle mass. Besides decreased strength, the loss of muscle mass also negatively affects aerobic capacity and metabolic rate, ultimately reducing function and quality of life. Obesity, another health concern, is defined as abnormal and excessive body fat. In recent years, the prevalence of obese individuals in both middle and old age has increased. Similar to sarcopenia, the risk of obesity increases with age. The emerging and challenging epidemiological trends in developing countries include sarcopenia and obesity. The prevalence of obesity combined with sarcopenia is referred to as sarcopenic obesity/ obese-sarcopenia, which exhibits synergistic complications of both syndromes.

Obesity causes substantial changes in fat metabolism, leading to fat deposition in non-adipose tissues such as skeletal tissue. The development of obesity is associated with high-lipid diets, predominantly saturated fatty acids such as palmitic acid (16:0, PA). PA is the most common saturated fatty acid in the human body. Excess fatty acid infiltration into skeletal tissues alters metabolic signaling. Moreover, growth molecular signaling and catabolic pathways that support the proper functioning of skeletal cells are also affected. Individuals with sarcopenic obesity have greater risks of metabolic disorders compared to those for sarcopenia or obesity alone. Strong and healthy muscles are vital for the handling of overload created by obesity. Skeletal muscle myogenesis is of the utmost importance in maintaining muscle mass and regenerative capacity. However, lipid overload disturbs skeletal myogenesis.

The study evaluates association of PA in the myotubes formed via *in vitro* serum deprivation and the negative effects of fatty acids on skeletal muscle. The myocellular mechanisms in skeletal myogenesis are researched to understand its implications.

Plant species that grow in the marine environment; namely, seaweeds or sea vegetables, have become an emerging sustainable nutritional source and an essential part of healthy human diets, mostly in the East Asian region. They are a good source of vitamins and minerals, fiber, and polysaccharides that support low caloric intake. Furthermore, seaweeds contain pigments, proteins, polyunsaturated fatty acids (PUFAs), and phenolic compounds. Polyphenols are an important component of marine algae (brown algae) that provide functional food and pharmaceutical significance. However, studies reporting their use as interventions in muscle-related research are limited.

The study examined a non-cytotoxic concentration of PA to provide mechanistic insights into PA-impaired skeletal myogenesis and potential medicinal and dietary interventions through edible brown seaweed, *Ishige okamurae* (IO). C2C12 cells were examined for myogenic markers, adipogenic factors, and regenerative capacity through growth regulators against PA interference to assess IO and purified diphloroethohydoxycarmalol (DPHC) as potential treatments. Both IO and DPHC improved myogenic marker (myogenin, MyoD, and MyHC) levels. While PA down-regulated myogenic markers while improving adipogenic factors (PPARγ, c/EBPα, A-FABP), DPHC significantly arbitrated the negative effects. DPHC treatment also improved phosphorylation of the growth regulatory PI3K/Akt/mTOR axis over the adverse effects of PA. The results of this study suggested regulatory mechanisms through which the bioactive components IO and DPHC based on the sustainable utilization of *I. okamurae* inhibited the PA-induced impairment of skeletal myogenesis.

Generation of reactive oxygen species (ROS) is closely related with the ectopic lipid accumulation due to improved flux of energy substrate such as excessive dietary fat, thus influence cellular dysfunction. Pathogenesis of diabetes and linked sarcopenia is associated with the activation of pathways related to oxidative stress and muscular mitochondrial dysfunction. Further, mitochondria function as a signaling platform for multiple biological responses including immunity and metabolism. Literature provides evidence on the function of mitochondrial ROS (mtROS) in the activation of nucleoside oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) inflammasomes and the regulation of innate signaling pathways. Controlled levels of mtROS are vital for the innate host defense against pathogens, though unrestrained amounts may lead to pathologies including chronic inflammation. The Nlrp3 inflammasome consisting of apoptotic speck-containing protein with a CARD (ASC) and inactive pro-caspase-1 conveys inflammatory signals downstream by cleaving pro-IL-1β to IL-1β. The secreted IL-1β and IL-18 delivers and continues inflammatory signaling. Palmitate influences the Nlrp3 inflammasome, mtROS as well as pattern recognition receptors (PRRs). Ultimately, this could lead to apoptosis which in myocellular environment is a tissue weakening.

Experimental results demonstrate PA improved cell injury, where myotube injury was significantly attenuated by IO and DPHC. Moreover, PA promoted mtROS generation leading to Nlrp3 activation as well as transduction of NF-κB signaling. Notably, PA increased the Nlrp3 complex formation as evident by the immunofluorescence analysis while DPHC substantially abolished it. The PA-induced Nlrp3 activation was successfully reversed by MCC950, a Nlrp3 inhibitor to confirm the significance of Nlrp3 inflammasome formation in IL-1β and IL-18 release. This confirms the vital role of Nlrp3 inflammasome in the release of IL-1β and IL-18. Mito-Tempo, a mtROS scavenger

remarkably reduced PA-induced Nlrp3 inflammasome activation. Additionally, PA-stimulated production of IL-1β and IL-18 were as well inhibited with the Mito-Tempo treatment. Taken together, the results implicate mtROS production induced by PA alters cellular dynamics and plays a central role in the Nlrp3 inflammasome mediated IL-1β and IL-18 expression. Collectively, DPHC is a potent alleviator of mtROS where it reverses mitochondrial dynamics thus providing mitigation of inflammasome activation. Further into the research, ishphloroglucin A (IPA), another active component from IO exhibited skeletal muscle regenerative potential. Palmitate induced skeletal myogenesis effects were hampered by the compound where it also substantially inhibited the Nlrp3 driven skeletal dysfunction.

A better understanding and of the molecular pathways of lipid induced sarcopenia can have profound implications in the development of therapeutic interventions. Thus, the results of this study suggest the potential for treatments based on the bioactive components IO, DPHC, and IPA with the sustainable utilization of the *I. okamurae* marine algae.

Keywords: palmitic acid; skeletal myogenesis; sarcopenia; *Ishige okamurae*; diphloroethohydoxycarmalol; ishphloroglucin A

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Part I: Background and significance

1.1 Introduction

The term “Sarcopenia” which is derived from Greek implies the loss of flesh, conveys the weakness of muscle. Sarcopenia is defined as the loss of muscle mass and strength that occurs with aging which leads to disability, frailty, morbidity, and mortality rates. Further, anorexia, dehydration, and cachexia as well serve as causes of loss of muscle mass. [1, 2]. The loss of muscle mass accompanies the loss in strength thus the aerobic capacity and metabolic rate, ultimately contributing to reduced function and quality of life. The daily living activity performing capacity is reduced where greater physical disability is observed in individuals suffering from sarcopenia. Other than the dramatic impact on the quality of life of each individual, this burdens up the public health sector up to significant levels. As per reports, sarcopenia attributed $18.5 billion in the US in 2000, which is a 1.5% approximation of total healthcare expenditure [3].

Though the prevalence of sarcopenia is difficult to be estimated due to the impracticability of assessing muscle mass, vivid methodologies were implemented over the past two decades while new techniques have come into introduction up to date. As per the statistics, 5- 13% of the adults in the 60- 70 age range, and 11- 50% aged 80 or above, suffer from sarcopenia [4]. This broadness of the statistics is influenced via the heterogeneity of the study populations and the different techniques used. Due reason, the “International Working Group of Sarcopenia” developed a unanimity definition to diagnose and screen such conditions with the intention of identifying the patients with sarcopenia [5]. Distinctive recommendations were made including a two-part evaluation based on patient history and health status with a physic functional examination. The positive individuals then undergo a whole body, dual-energy X-ray absorptiometry (DXA) scan for confirmation [6].

Occurrence of sarcopenia diverges among populations depending on the definitions implemented to assess the situation. A report by Bischoff-Ferrari states an incidence of 3% to 24% above 65 years [7], where enforcing European Working Group on Sarcopenia in Older People (EWGSOP) criteria demonstrated deviated values [8]. The agreement between multiple working groups for sarcopenia is to define it through a combined approach of muscle mass and quality. An elaborative review by Chen and colleagues discuss sarcopenia as a challenge among the wider community of Asians. Further, the Asian Group for Sarcponeia (AWGS) intends to encourage research related to sarcopenia [9]. Though the cross-sectional studies verify the association of low muscle mass to functional deterioration, the results were inconsistent in the time-course study. Concerns over methods to determine the affected populations are brought out as reasons for this [10]. A comprehensive review on the epidemiology of sarcopenia by Kan (2009) illustrates the prevalence of sarcopenia depending on different cohorts and definitions used in each study [10]. Accordingly, in an American cohort (New Mexico Elder Health Survey; NMEHS) 15-25% under the age of 70 years were sarcopenic when the disorder was defined as an appendicular muscle mass index < 2 SDs compared with the mean of reference. Application of muscle mass index < 2 SDs compared with the mean value was later referred to as the Baumgartner criteria. Exceeding the age of 80 witnessed 50% of men and 40% of women enlisted as sarcopenic [11]. A study by Lauretani et al. (2003) (InCHIANTI study), implemented knee extension isometric torque, handgrip, lower extremity muscle power, and calf area as indicators of sarcopenia. Indicators measure greater than 2 SDs below the mean was considered as sarcopenia [12]. The results revealed a difference in sarcopenic percentage population depending on the age group while it was measured in reference to calf muscle cross-sectional area. A systematic study among Asians was undertaken with elderly community-dwelling Chinese volunteers in

Hong Kong, where it assessed the prevalence and risk factors associated with sarcopenia [13]. Muscle mass was measured via dual-energy X-ray absorptiometry and muscle mass 2 SDs below the mean was defined as sarcopenic. In a population of 70 years and older, the incidence of sarcopenia was reported as 12.3% (men) and 7.6% (women). A sarcopenic prevalence of 0.8% women and 1.3% men (over 60 years of age) was reported in a survey conducted in the Korean population [14]. Smith and the team (2021), address the issue of sarcopenia in low- and middle-income countries (LMICs). They have found that food insecurity plays a vital role in the prevalence of sarcopenia [15]. Thus, the study suggests concerns about food insecurity as an effective approach. Multiple macro and micronutrient deficiencies are associated with food insecurity. This may lead to energy deficiency, consequential body composition alterations due declined basal metabolic rates, ultimately leading to inclined risk of sarcopenia [16]. Conversely, a small population (n=168, adults aged ≥60) study conducted in Mexico reported no significant association between food insecurity and sarcopenia [17]. An elaborative discussion by Daskalopoulou et al. (2020), mentions the incidence of both sarcopenia and sarcopenic obesity to be associated with sociodemographic, bio-clinical, and lifestyle factors in LMICs [18]. The study involved individuals aged ≥65 in selected countries in North, South America, and Asia (Cuba, Dominican Republic, Mexico, Puerto Rico, Peru, and China). Multiple factors including, gender, marital status, academic background, personal wealth are suggested to be considerably influencing body fat and alterations in skeletal muscle mass, thus expressing sarcopenia. Another fact brought into attention is the limited healthcare facilities among the LMICs. However, focused health promotion programs and cost-effective primary prevention programs may shed light under these circumstances in LMICs.

Techniques used to evaluate sarcopenia revealed that aging not only influenced changes in muscle mass but also in composition and material properties [19]. From puberty until the fifth decade of life, the fiber size and the number remain stable in a muscle. Beyond this point, the decrement of the fiber size and number initiate and become obvious [20]. The decline of the motor units reduces the force production. A direct impact is made upon the fast-twitch type II muscle fibers rather than that on slow-twitch type I muscle fibers [21]. Type II fibers use glycogen glycolysis as the primary source of ATP and possess the largest cross-sectional area (CSA). The maximal power generation is observed through type II fibers where it plays an important role in such activities as weight lifting and sprinting [19]. Power et al. (2010) reports on muscle wasting; marathon runners use type I fibers are 40% slower than in their youth while they reach the age of 80s, where weightlifters are weaker by 60% [21].

Muscle mass maintenance is a balance between protein synthesis and degradation systems. Muscle mass increment is possible with either protein synthesis incline or protein degradation decline, whereas muscle mass decrement occurs with the opposing involvement of the above two factors. Aging influences the protein balance via decreased expression of hormonal factors and increased inflammatory factors [6, 19]. Poor nutrition contributes to sarcopenia. Protein and essential supplemental intake via food play a vital role in the fulfillment of energy balance, eventually eradicating sarcopenia. The seafood diet; a broad food category with thousands of species produced from capture and aquaculture methods, consists of key macro and micronutrients such as high-quality proteins, minerals, vitamins, and healthy fats. These determine its functional food and pharmaceutical significance. Thus, seafood is receiving much attention as a source of a healthy and sustainable diet [22].

Given the above-abstracted elements, prevention and treatment of sarcopenia will improve the quality of life and reduce the economic burden. This review article summarizes current knowledge on sarcopenia focusing on (i) the importance of skeletal muscle mass and function, (ii) aging-related changes, (iii) molecular pathways involved, and (iv) the role of seafood as a treatment.

1.2 Muscle atrophy

1.2.1 Importance of skeletal muscle, mass, and function

Skeletal muscle tissue is dynamic and possesses plasticity. This accounts for the muscle contraction power and movement that are required for a stability maintenance. Almost half the body weight is comprised of skeletal muscle. Further, it serves as a safe storage facility for protein (50–75% of whole-body proteins). The muscle tissue contains water (75%), protein (25%), carbohydrates, fat, and minerals (5%) [23]. Myofiber or the muscle cell is the construction unit of skeletal muscle. Individual muscle fiber size and number, determine the size and strength of the whole muscle though, fat infiltration as well the connective tissues could alter the affiliation [24]. Morphologically, the cells contain multiple nuclei where protein synthesis of each region is regulated independently but coordinated, where each territory is referred to as nuclear domains [25]. A whole muscle is attached to the skeletal system via the tendons and is consisted of muscle fibers. The outer layer of the whole muscle is the epimysium which the inner muscle fibers are surrounded by another connective tissue layer, the perimysium. Muscle fiber further could be dismembered into the level of myofibrils and is approximately 1 cm in length and 100 μm in diameter [23, 26]. Satellite cells located in between the sarcolemma and the basal lamina are important in muscle growth, repair, and regeneration.

Myofibrils consist of sarcomeres, the sections in between z discs and is the residing area for actin and myosin which are the main proteins (70- 80%). Actin and myosin are the driving force of the muscle cells. The sarcomere is the basic structural and functional component of the striated muscle [27]. The subunit of the sarcomere is the myofilament where several myofilaments come together to form a single sarcomere. The proper functioning is assured via three major components in the sarcomere; the thick filament,

thin filament, and titin. Myosin is the dominant component in the thick filament and actin assembles the thin filament. The sarcomere and the sarcoplasm consist of further proteins such as tropomyosin, titin, and nebulin [28]. The total number of sarcomeres within a myofibril depends on the muscle fiber length and diameter. The power is generated via the interaction between actin and myosin filaments. Myosin component attaches to the actin framework, which a chemical transformation sequence induces the breakdown of adenosine triphosphate (ATP), thus causes force and movement. The mechanism that explains the force generation and movement was first proposed in 1954 and referred to as the "Sliding filament theory" [29]. The detailed assessment of the above is out of scope in this review article.

Muscle fiber cellular elements include the transverse tubular system (T tubule), sarcoplasmic reticulum (SR), and the mitochondrial system. The T-system is an invagination of the surface membrane thus physically contiguous with sarcolemma. The transverse invaginations extend across the longitudinal axis of the muscle fiber. This serves as the message conveyer from motoneuron to myofibrils. A uniform distribution of excitation signal is ensured throughout the fiber. These also involve the exchange of Ca^{2+} with the cytoplasm through sodium-calcium exchangers. This system is dynamic and could change its volume depending on osmotic stress and other factors [30, 31].

Sarcoplasmic reticulum is complex compared to the T tubules and stores calcium which is required in muscle activation [26]. The muscle calcium homeostasis is maintained by SR (Calsequestrin and SERCA proteins) holding the responsibilities in the storage, release, and reuptake [23]. At each sarcomere, the T system retains a close spatial association with the SR. Simply, SR determines muscle function and performance. Calsequestrin (CSQ), is a high capacity, low affinity Ca^{2+} binding protein in the SR.

Ryanodine receptor-Ca^{2+} release channels (RyRs) which are located at the SR terminal; cisternae, releases the Ca^{2+} in the SR [32-34]. The sarco (endo) plasmic reticulum Ca^{2+}-ATPase (SERCA), is responsible for the back sequestration (reuptake) of the Ca^{2+} into the SR [35].

The other element, mitochondria are spread throughout the cell. These are reported as non-discrete organelles and are interconnected via a reticular network by which each other communicate [36]. The extremely dynamic nature of the mitochondrial density is non-surprising given its responsibility as the main generator of cellular energy. This contains all the enzymes related to the oxidation of the high-energy substrates. The resulting ATP is used for muscle contraction, ion transport, and other cellular mechanisms [26]. Moreover, mitochondrial density is important in the determination of muscle endurance properties.

Motor neurons innervate muscle fibers. The whole muscle is innervated by a motor neuron where the individual fibers are then innervated separately forming a branching structure at each hierarchical level. The signal for the activation of the skeletal muscle commences in the motor cortex of the brain and is successfully traveled through the spinal cord to be transmitted via the motor neurons to every single fiber (neuromuscular junction) in the unit, ensuring the simultaneous contraction [37]. The electrical impulse received by the motor neuron (pre-synaptic) is transferred to the muscle fiber (post-synaptic) through chemical means using acetylcholine released from the axon end of the neuron. This causes the discharge of Ca^{2+} from the SR, ultimately activating the contractile mechanism [19, 38].

The noteworthy discrepancy in the mechanical and metabolic machinery of individual fibers lay the base for heterogeneity of human skeletal muscle. The abundant prevalence

of fiber type varies among different muscles. Multiple fiber types among a single muscle reflect the adaptation of the muscle to different activities imposed by the motor neurons. Thus, muscle protein isoform identification has become a valuable and reasonable approach in the investigation and identification of muscle fiber types [39]. Depending on the specific type of myosin present in the fibers, motor units are segregated into three distinct categories. Though, the physiological properties measured via the means of oxidative and glycolytic potentials do not proportionally associate with the myosin heavy chain isoform, merely. Lang et al. (2010), review a slow motor unit and two fast motor units, depending on the specific type of myosin expressed in each [19]. The motor units which are consisted of a minute number of fibers and type I myosin are recognized as slow motor units. In contrast, fast fatigable motor units consist of the highest number of fibers where its fibers possess the largest cross-sectional area (CSA). The energy transduction of slow motor units are relatively slower in rate and the contractile velocity is less, hence, appropriate for precise and finite activities. The morphological appearance is reddish due to the rich contents of mitochondria and myoglobin. This allows the oxidative metabolism of triglycerides and carbohydrates for sustainable delivery of ATP. Therefore, these are well suited for sustained aerobic exercise. The fast fatigable motor units are composed of type IIx myosin and are fast in energy transduction compared to slow motor units. Glycogen breakdown is the main source of ATP and the fibers are poor in mitochondria, thus a relatively higher amount of energy could be supplied in a short time. Quick movements such as weightlifting and sprinting recruit fast fatigable motor units employing its maximal power production. The third type of motor unit exists named fast fatigue-resistant, which possesses intermediate properties of the above two types. This consists of type IIa myosin. The fourth type of muscle fiber is discussed in a comprehensive review by Schiaffino S. and Reggiani (2011), and worth referring to them

for detailed study [40]. Though this review summarizes points of three fiber types, the units could be more elaborately dissected into several other categories depending on the physiological, metabolic, and morphological extents.

Depending on the duration and the intensity of the activity involved in the metabolic energy production pathway is selected to sustain the muscle actions. Muscle fibers can readily use the energy stored as ATP and creatine phosphate (CP) as well as undergo anaerobic glycolysis and oxidative phosphorylation. The energy demand increases in proportion to the intensity of the activity. Anaerobic glycolysis provides immediate ATP, but muscle cellular homeostasis is affected due to the end products (H^+ and inorganic phosphates). Exhaustive exercise leads to the depletion of phosphocreatine (PCr) stored in the muscles. The utilization of PCr and anaerobic glycolysis to produce the energy required for the muscle depends on the intensity, duration, and the type of activity involved [41]. The oxidative phosphorylation which utilizes the mitochondrial network yields the energy to be harnessed at a long hour and high-intensity events. The metabolic oxygen demand is supported via a network of capillaries. The relationship between the demand and the supply of oxygen was first described by August Krogh which came into fame as the “Krogh cylinder model” [42]. Accordingly, oxygen tension in muscles is low at rest, where oxygen consumption increase by 10-fold in muscle work.

Carbohydrates and lipids are the dominating sources of substrates for ATP production. Glycogen is stored in the muscle tissue and is readily available when required. The glucose uptake to the muscle cells occurs through the insulin-independent pathway. The carbohydrate store is not large enough to be oxidized for an extended duration and could be exhausted in few hours of intense work. Further, the muscle stores fatty acids derived from triglycerides which serve as the substrate for lipid metabolism. Amino acids derived

from muscle protein degradation as well contribute towards total energy metabolism but are only used in the complete absence of carbohydrates [43, 44]. Alterations in protein balance do not typically change the number of myofibers but instead cause an increase or decrease in the size (i.e. cross-sectional area) of individual myofibers, which is ultimately reflected as an increase (hypertrophy) or decrease (atrophy) in whole muscle volume and mass. It is the primary reservoir of amino acids that can be mobilized into the blood to be used for synthesis of proteins by other tissues or as a hepatic gluconeogenic substrate for maintenance of blood glucose during the fasting state.

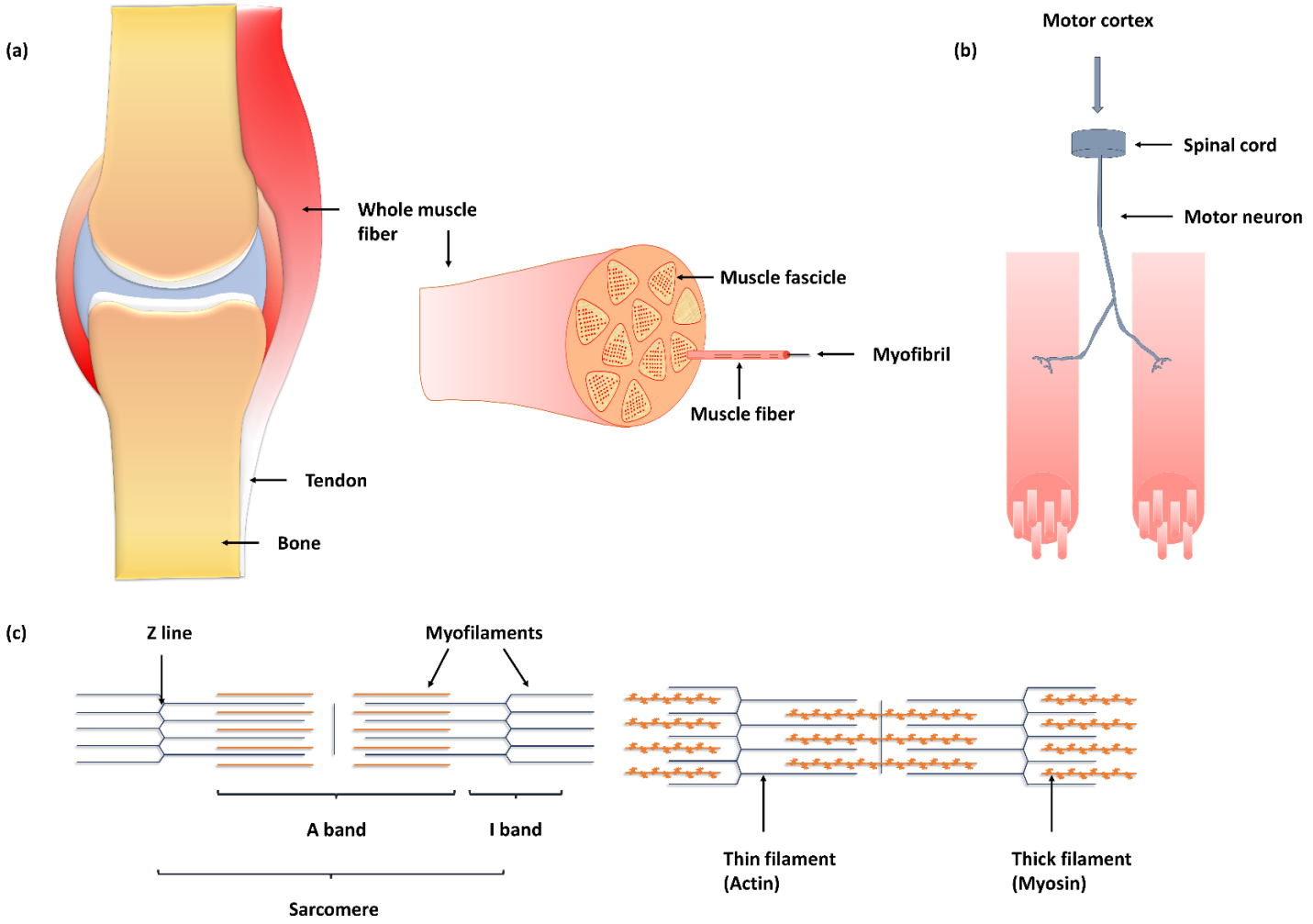

Figure 1- 1. *Structure of skeletal muscle hierarchical illustration. (**a**) Whole muscle and muscle fiber in each level, (**b**) muscle fibers connected to the motor unit, (**c**) contractile protein arrangement.*

1.2.2 Obese-sarcopenia

There are several publications rationalizing seafood as a nutritional intervention to either sarcopenia (this review provides evidence from literature) or obesity [45-48]. However, studies reporting seafood used as interventions in obesity-muscle-related research are limited. To satisfy the health benefits through nutritional approaches, two contradicting attitudes have to be addressed simultaneously. It's the gaining of the muscle mass and the losing of the fat mass. Thus, body composition has to be considered instead of bodyweight alone. A ketogenic diet is suitable in this regard. It contains low carbohydrate and primarily contain proteins and fat. Seafood is one such type of diet. Suckkov et al. (2021) suggest a seafood ketogenic diet for aging-related sarcopenic obesity [49].

Furthermore, literature provides evidence on research conducted rationalizing nutrition and muscle health. Yokohama et al. (2021) provide a detailed report on how nutrient-derived dietary patterns are associated with sarcopenia [50]. The cross-sectional study involved a sample of community-dwelling older Japanese. The suggested reduced risk regression dietary pattern was characterized by a high intake of fish and seaweeds as marine food sources. These food intakes were inversely associated with sarcopenia in community-dwelling older Japanese. Research conducted focusing on middle-aged and elderly Korean populations, consumption of white rice, fish, and seaweeds were related with the lower incidence of declined muscle mass in both men and women [51]. This implies above stated dietary pattern evidenced to help protect against loss of skeletal muscle mass. Excessive calorie intake leads to high body fat mass dealing with the dietary pattern. Lower muscle mass is associated with obesity leads to sarcopenic obesity. As obesity and sarcopenia share similar pathological pathways, obesity could increase the risk of sarcopenia development.

Literature research provides evidence regarding how obesity-related factors could influence sarcopenia and potential marine natural product interventions. Though these have not directly addressed sarcopenic obesity. Among them, diphloroethohydoxycarmalol (DPHC) isolated from *Ishige okamurae* reportedly showed the potential to inhibit inflammatory myopathy [52]. Moreover, DPHC promoted cytosolic Ca^{2+}, resulting in increased glucose transport to myotubes to modulate blood glucose levels [53]. Yang et al. proposed the potential of brown algae to improve glucose metabolism in skeletal muscle in a murine high-fat diet (HFD) model [54]. Diphloroethohydoxycarmalol (DPHC) associated with inflammatory myopathy was assessed in detail as an inhibitory agent of tumor necrosis factor (TNF)-α, demonstrating its action through the NF-κB and MAPK pathways [52]. These studies attracted attention to *I. okamurae* as a material in the cosmeceutical, nutraceutical, and functional food sectors.

1.2.3 Ageing related changes in muscle structure and function

Sarcopenia is a term suggested by Rosenberg in the late 1980s, referring to the loss of muscle mass related to the ageing process [55]. The term has been reinvented in different times extending its idea and scope with an in-depth and advancing understanding of the incidence. Low muscle strength including muscle quantity and quality was implemented as the 2018 operational definition, where it was revised in 2019 considering disease of the muscle/muscle failure intending lower muscle strength as the key feature. Hence, muscle strength is a better predictor of clinical outcome compared to muscle mass. Eventually, sarcopenia is defined as the loss of muscle mass and strength that is accompanied by a decline in physical activity, functionality, and performance.

Vivid indexes are used in the evaluation of sarcopenia. Among them; appendicular skeletal muscle mass index (ASMI) (ASM/ height^2) and skeletal muscle index (SMI) are most commonly implemented. Though the indexes are important and practical in serving clinical purposes, they are not accurate due to the non-uniform behavior of sarcopenia [56]. The disease condition is categorized into three severity stages according to the European Working Group of Sarcopenia; Pre-sarcopenia, Sarcopenia, and Severe sarcopenia. The initial stage indicates muscle mass reduction alone in which the others exhibit muscle mass plus strength and performance decrement [8]. Epidemiological studies show that total body mass considerably increases within the second to fourth decade of life and lean body mass (LBM) to be declined to start from the second decade for both men and women [56].

Table1- 1. *Sarcopenia severity categorized accordingly with the European Working Group on Sarcopenia in older people*

Pre-sarcopenia	**Sarcopenia**	**Severe sarcopenia**
Reduced muscle mass	Reduced muscle mass	Reduced muscle mass
Muscle strength not reduced	Reduced muscle strength or performance	Reduced muscle strength
		Reduced performance

(Cruz-Jentoft et al., 2010)

The decrease in muscle mass is influenced by both individual fiber size and number. The decline in the muscle fiber size is referred to as muscle atrophy where the fiber number reduction condition is hypoplasia. It is evidenced that type II fibers undergo atrophy compared to type I. Further, loss of CSA of type II fibers is obvious [57]. While reaching old age, fiber type interchange as well reinnervates from type I to type II lead to co-expression of both type MHC-I and MHC-II [58]. During the earlier stages, sarcopenia was documented with the use of ultrasound imaging which led to the overestimation of

muscle CSA in older subjects. This was due to the presence of fat and connective tissue which are non-separable in ultrasound scanning. Though, the problem was overcome with the CT and MRI scan enabling investigators to reach different levels as well differentiate between contractile and non-contractile components [59].

The muscle architecture is affected in sarcopenia leading to changes in the spatial arrangement of the muscle fibers. Muscle power is generated via the force into the velocity of shortening. Muscle force is the number of sarcomeres present in parallel formation on a CSA while shortening velocity depends on the number of sarcomeres placed in a series of a given fiber length. Reaching old age leads to decrease fascicle length implying a loss of sarcomeres in series reducing muscle shortening velocity. Ageing leads to the accumulation of advanced glycation end products (AGEs) due to protein glycation. The contractile properties of myosin proteins are altered via AGEs, causing decrements in shortening speed. Muscle stiffness is increased due to intramuscular cross-linkages. The decline in the number of actomyosin cross-bridges and reduced excitation-contraction (EC) coupling contribute towards force drop. The EC uncoupling is influenced by the degeneration in the function of dihydropyridine receptors where it controls Ca^{2+} release from SR [56, 60, 61]. Moreover, mechanical properties of the tendons considerably deteriorate in old age contributing to tendon stiffness. Tendons are responsible for the force transmission from muscle to the skeletal system. Several in-vivo studies have reported on the reduction in force in older individuals due to alterations in muscle optimal length and reduced tendon stiffness affecting time vital to decelerate body mass to prevent free falls [62, 63].

Morphological changes in the muscle tissue are attributed to the infiltration of muscle tissue components by lipids and such conditions are referred to as myosteatosis [64]. The

bone marrow precursor cells possess the potential of exhibiting both myocytic and adipocytic phenotypes in which ageing influences the latter. Lipid deposition in the muscle tissue provides another source of adiposity and is acknowledged as myocellular lipid which results from the reduced oxidative capacity of triglycerides due to ageing [65-67]. The particular condition in which sarcopenia is associated with obesity due to lipid accumulation is known as sarcopenia obesity. The given condition adds further burden to the locomotion and helps sustain sarcopenia through the collaboration of macrophage-mediated pro-inflammatory cytokine release (IL-1, IL-6, and TNF-α). The crosstalk between adipocyte-myocyte is believed to occur through adipokines (leptin, adiponectin, and resistin from adipocytes). Skeletal muscles are affected by leptin and stimulate lipolysis and insulin sensitivity. These processes eventually reduce the muscle quality in older obese individuals [65, 68].

Muscle regenerative capacity is reduced in the ageing process due to the loss of functional ability and the number of satellite cells. These are myogenic precursor cells vital in muscle repair and growth. The cells reside a quiescent period until they become required in action. Thus proliferate and differentiate into myoblasts [69]. Some reports find the loss of capacity of satellite cells is to be involved with the age-related changes in molecular mediators given that myogenic regulatory factors (MRF) [70]. Another approach to the matter describes the decline in the Notch activation. Satellite cell behavior is mediated by a transmembrane receptor known as Notch. Activation of the particular receptor is influenced by mitogen-activated protein kinase (MAPK) activity. Though MAPK activity is reduced with ageing [71, 72].

The survival and functioning of the skeletal muscles are necessarily sustained by proper motor neuron activity. With advancing age, the motor disturbances increase and are

reported to be the responsible cause for the loss of one-third of the skeletal muscle mass due to the interplay between two cell types [73]. Senescent motor neurons are characterized by several phenotypes including alterations in the dendritic tree size, and decline in synaptic input. Neurodegeneration is observed with ageing expressing neuroaxonal dystrophy (NAD) with axon terminal enlargements ultimately paving the way to impaired neurotransmission via disconnection [74]. With the death of motor neuron cells, muscle fibers undergo denervation and adjacent motor neurons reinnervate the particular muscle fibers via axonal sprouting. Thus, the reinnervation ratio increases. Though, the reinnervation process does not operate in its full potential with ageing, and leads to incomplete recovery of denervated muscle fibers [75, 76]. A resulting factor of this is the alteration of slow and fast axonal stimuli, with the declined amount of myelinated neurons. Further comments are published concerning the age-related changes of the neuromuscular junction (NMJ). NMJ is the site of the transfer of the electrical transduction produced by the nervous system to the muscle fiber. The process occur via the means of chemical synapse. Ageing process results in morphological changes in the NMJ leading to functional alterations of signal transduction [77]. Further, pre-terminal ends start exhibiting thinning, swelling, and sprouting. Simultaneously, postsynaptic terminals exert decrement in size and number along with the density of postsynaptic folds [78]. Though, NMJ is one of the widely discussed topics concerning sarcopenia, a detailed analysis of it is beyond the scope of this review.

Skeletal muscle tissue constantly involves in the turnover of protein to amino acids. Muscle mass is maintained through the dynamic balance between protein synthesis via free amino acids and degradation of proteins resulting in free amino acids. A constant muscle mass is ensured given the rates of synthesis and degradation to be balanced [19]. Although, over time, deficits in the system can result in muscle loss. The muscle mass of

young adults cares for approximately 30% of the whole-body protein turnover. The value alters to 20% or less when reaching the elderly stage [79]. The ageing population requires greater protein intake (1.14 g kg^{-1} day^{-1}) compared to younger (0.8 g kg^{-1} day^{-1}) as per the nitrogen balance studies published. Though in sarcopenia, protein intake exhibits inverse proportionality to age.[80]. Further, the elderly sarcopenic population deviates its protein turnover rate towards the catabolic stage expressing anabolic resistance. The decreased sensitivity of leucine is currently understood to be the cause of anabolic resistance among others such as changes in the efficiency of mammalian target of rapamycin (mTOR), and blunt situations in the phosphorylation of kinases (p70S6K). Inversely, the IGF-1 levels are depressed in the elderly.[81-84]. The skeletal muscle protein degradation is controlled via the ubiquitin-proteasome-proteolytic pathway (Ub pathway) and with achieving old age the expression of this expands. The process involves a series of enzymatic steps (E1; Ub activation, E2; engagement of target protein, E3; transfer by Ub-protein ligase from E2 to target proteasome complex) in protein degradation. Inflammatory cytokines (TNF-α, IL-6) and hormones (cortisol, angiotensin) including reactive oxygen species promote the pathway [19, 81]. Further, insulin resistance is observed to be increased with advancing age and is substantially impaired in type 2 diabetes. Insulin is important in glucose homeostasis as well as in the protein turnover in the skeletal muscle. Insulin is appeared to be correlated with the mechanisms of IGF-1, where IGF-1 binds to the skeletal muscle cell surface activating signaling pathways responsible for anabolic, anticatabolic, and antiapoptotic processes. Though, IGF-1 is produced via two sources; in the liver via GH mediation and in the muscle cell itself. With the advancing age, GH production declines leading to reduced IGF-1 levels, hence protein synthesis and muscle cell function impairments are obvious [19]. Moreover, testosterone has a growth effect on skeletal muscle and is mediated via androgen receptors. It's reported on the ratio of

testosterone and serum cortisol to be correlated with the muscle CSA and strength [85], as cortisol is a potent stimulus to protein catabolism. With age, the circulation levels of hormones alter, possibly contributing to sarcopenia.

A fact of utmost importance related to muscle atrophy is its relation to oxidative stress, chronic inflammation, and mitochondrial dysfunction. The molecular pathways of each component may converge influencing the protein balance ultimately affecting the mass of the muscle fiber [86]. The imbalance between the oxidants and antioxidants is referred to as oxidative stress. The factors that influence sarcopenia do not act in isolation, and many of the pathways do overlap with oxidative stress. The metabolic products accumulate over time damaging the cell components, particularly mitochondria and DNA sequences. Advancing age leads to alterations in the mtDNA [87-89], and would cause skeletal muscle cell apoptosis and structural abnormalities. Hence, the metabolic functions are affected. The effects on the electron transport chain alter the respiration function [90]. The skeletal muscle apoptosis and production of ROS are induced by mitochondrial dysfunction and SR stress. The events are prominently triggered by superoxide anion and hydrogen peroxide. The effect of mitochondrial damage on muscle tissue disorders such that osteoporosis, hypertrophy, and sarcopenia were discovered and published by Trifunovic et al (2004) [91]. Further, a correlation between neuromuscular degeneration alongside mitochondrial dysfunction was reported recently combining its relation to the lack of antioxidant enzyme superoxide dismutase leading to sarcopenia in transgenic mice [92].

The correlation between muscle protein metabolism and inflammation has gained much interest. The chronic state of slight increments of the plasma pro-inflammatory mediator concentrations (TNF-α, IL-6) is referred to as low-grade inflammation and demonstrates

cells leaving cell cycle consequently entering cellular senescence state. The state further induces the production of pro-inflammatory cytokines and over-activation of NF-κB [93]. Sarcopenic elderly were reported to exhibit elevated circulating concentrations of IL-6 and TNF-α. Further, it is published to be a better predictor in morbidity and mortality of older subjects [94]. These findings address the age-related changes in muscle and inflammatory mediators.

Table1- 2. *Age related changes in muscle structure and function*

Potential changes	Association with sarcopenia
Muscle mass decrement	***Protein turnover;*** Protein turnover rate shifted towards catabolic stage expressing anabolic resistance. Blunted expression of anabolic pathway proteins (mTOR, p70S6k, IGF-1). Protein degradation pathway complications (Ub pathway). Insulin resistance. Declined growth hormone production. Altered expression of testosterone and serum cortisol.
	Muscle fiber; Muscle mass is influenced by both the muscle fiber size and number. Muscle atrophy and hyperplasia are associated with muscle fiber. Type II fiber atrophy/ loss of CSA is obvious during aging.
	Lipid infiltration; Lipid infiltration referred to as myosteatosis. Improved adipocytic phenotype in aging process. Macrophage mediated pro-inflammatory cytokine release. Thus, the muscle quality in older obese individuals are reduced.
Muscle power decrement	***Muscle fascicle length, AGE, EC uncoupling, Stiffness;*** Decreased fascicle length implies loss of sarcomeres leading to reduced muscle shortening velocity. Accumulation of AGEs affect the myosin proteins. Intramuscular cross-linkages cause muscle stiffness. Reduced EC coupling contributes towards force drop. Affected dihydropyridine receptors. Reduced tendon stiffness.
Muscle regeneration capacity decline	***MRF;*** Muscle regeneration capacity reduced due to the loss of the satellite cells and the altered performance of myogenic regulatory factors (MRFs) with aging. This affects proliferation and differentiation. Notch activation reduction.
Motor neuron impairment	Increased motor neuron disturbances cause loss of one-third of skeletal muscle mass. Neurodegeneration observed through NAD and axon terminal enlargements. Incomplete recovery of denervated muscle fibers. Functional alterations in NMJ.
Oxidative stress	Muscle atrophy is related to oxidative stress. Accumulation of metabolic products trigger the situation. Metabolic functions are affected. Possible muscular dysfunction and neuromuscular degeneration. Elevated concentrations of inflammatory cytokines.

Ub pathway, ubiquitin-proteasome pathway; mTOR, mammalian target of rapamycin; p70S6k, ribosomal protein S6 kinase beta-1; IGF-1, insulin-like growth factor 1; CSA, cross-sectional area; AGE, advanced glycation end-product, EC, excitation-contraction; MRFs, myogenic regulatory factors; NAD, neuroaxonal dystrophy; NMJ, neuromuscular junction

1.2.4 Molecular pathways involved in sarcopenia

Regulation of the protein turnover via molecular pathway activation is a method of maintaining muscle mass and performance of adults against different pathophysiological conditions. The muscle mass depends on the protein and cell turnover [95]. Muscle mass could enhance either via incline in the protein synthesis or decline in protein degradation, while the muscle mass could shrink due to the opposite activity of the protein turnover. A major barrier to the active management of sarcopenia is the scarce understanding of the molecular mechanisms. This section will summarize the molecular pathways regulating muscle growth and the behavior of the particular pathways reaching sarcopenia.

The distinctive positive regulatory mechanistic pathway of muscle growth is the serine/threonine kinase Akt driven, mTOR involved signaling. Akt is activated through phosphatidylinositol-3,4,5-triphosphates produced by PI3K. The activation of the Akt is further induced by IGF-1 and insulin. Muscle growth impairment due to reduced muscle fiber and size with the muscle-specific inactivation of the IGF-1 receptor was reported indicating its vital role in muscle hypertrophy [96]. Protein synthesis is stimulated by Akt thorough mTOR and its downstream effectors. Published data evidence, declined postnatal growth following muscle-specific mTOR knockout [97]. mTOR is a two-part multiprotein complex, comprised of mTROC1 and mTORC2. mTORC1 which contains RAPTOR is responsible for the regulation of protein synthesis, mitochondrial, and ribosomal biogenesis. Further, catabolic processes such as autophagy are also influenced by mTORC1. Mechanical loading, growth factors, as well as nutrient feeding, can induce mTORC1 activation [98]. Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase (p70S6K) is the main effector molecules of mTORC1 which involve in protein synthesis. The cap-binding protein eIF4E is repressed by the 4E-BP1. The intriguing behavior of S6K proteins in the Akt

pathway is evidenced by the experiments involving S6K1 null mice, where they exhibited blunted hypertrophic response against activated Akt response against IGF-1 [99]. mTORC2 is involved in the Akt-FoxO signaling and contains RICTOR. Rapamycin is a specific mTOR inhibitor and directly impacts mTORC1 though reported to affect mTORC2 as well. Rapamycin could act indirectly via inhibiting Akt, hence de-repressing FoxO. Given the above conditions, FoxO behavior should always be monitored while rapamycin treatment [100]. Even though the facts from the literature prove about molecular pathways involving PI3-K/Akt/mTOR levels decline with advancing age in skeletal muscles, some researchers report conflicting results. Non-significant protein levels (Akt, mTOR, RAPTOR, S6K1, and 4E-BP1) in aged muscles were reported by Sandri et al (2013) [101]. Similarly, Markofski et al (2015), discuss the effect of hyper-phosphorylation of mTORC1 in elderly skeletal muscle contributing towards insulin resistance [102].

Myostatin, earlier referred to as growth and differentiation factor-8 (GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily. It is reported that myostatin continuous to express in the skeletal muscle tissue since embryogenesis. Further myostatin null mice exhibited muscle mass growth throughout the life span due to muscle fiber hyperplasia and hypertrophy [103]. The double muscling phenotype in cattle was observed due to the mutations in the myostatin gene [103, 104]. These findings led to the understanding of the function of myostatin as a negative regulator [103]. Myostatin first binds to a type II receptor (ActRIIB) and the complex forms (ligand/receptor), where its kinase then activates the type I receptor. This in turn leads to the phosphorylation of the Smad proteins (Smad2/3). These proteins are transcription factors in the cytoplasm. The assembly between Samd2/3 with Smad4 forms a heterodimer that translocates to the nucleus thus activate the transcription of target genes [105]. The muscle fiber size

regulation via myostatin is suggested relating to satellite cells. The satellite cell activation, proliferation, and differentiation causes increased fiber size. Studies have substantiated the act of myostatin in upregulating p21 (a Cdk inhibitor), thus negatively regulating cell cycle progression and support maintenance of the quiescent status of satellite cells [106]. Moreover, myostatin impacts the reduction of myogenic regulatory factors (MRFs – MyoD, Myf5, Myogenin, MRF4) affecting myoblast proliferation and differentiation [107]. MyoD plays a crucial role in the repair of the damaged skeletal muscle and is involved in the regulation of muscle cell differentiation [108]. Siriett et al (2007), discuss the enhanced muscle regeneration via the antagonizing myostatin, subsequent myoblast, and macrophage migration through inclined levels of Pax7 and MyoD [109]. Research concerning myostatin treatment has revealed it to act on the ubiquitin-associated gene (atrogin-1, MuRF-1) expression upregulation. Further, Akt phosphorylation was reported to be inhibited, hence rising FoxO1 levels supporting atrophy-related expression incline. Nevertheless, the NF-κB (p65 subunit) expression was not changed during the experiments, concluding the activity of myostatin to be NF-κB independent mechanism [110]. Another fact is that, the rapamycin mTOR partially dependent Smad 2/3 pathway inhibition could promote muscle hypertrophy independent of satellite cells. These particulars endorse the cross-talk of myostatin and Akt pathways at different levels. Therefore myostatin inhibitors combined with IGF-1/Akt promoters would deliver promising drugs in muscle growth [111]. Even though contrasting results have also been published. Carlson et al. (2008) reported sarcopenic muscle with elevated expressions of TGF-β and Smad3 excluding myostatin [112]. A study conducted with vastus lateralis biopsies of healthy young and old subjects extracted RNA stated no difference in the circulating myostatin levels related to age [113]. Similarly, non-significant alterations of

myostatin levels between young and elderly sarcopenic men were recorded by Ratkevicius et al (2011) [114].

1.3 Proteolytic systems in skeletal muscle

For example, the turnover rates of myofibrillar proteins (i.e. myosin, actin) are several times slower than that of non-myofibrillar proteins because of the way they are organized into complex multi-protein structures. Protein degradation and synthesis pathways occur independently. The degradation of proteins in skeletal muscle, including the ubiquitin-proteasome, autophagy-lysosome, caspase, and calpain pathways. Notably, the ubiquitin-proteasome, autophagy-lysosome, and caspase-mediated proteolytic systems are regulated by common upstream molecular signaling pathways. As a consequence, the activities of all three systems are frequently increased by the same initiating stimuli.

1.3.1 Ubiquitin-proteasome system

Cell proteolytic systems regulate the overall muscle homeostasis, in consequence, control protein turnover. The two most important cell proteolytic mechanisms are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system. The UPS is an energy-consuming mechanism (ATP) that acts via three enzymes and identified proteins are degraded by the addition of ubiquitin (Ub). The system consists of two distinctively identifiable processes, ubiquitin activation, and conjugation. Ub is initially complexing with Ub-activating enzyme (E1) in an ATP-dependent manner. Subsequently, Ub is transferred to Ub-conjugating enzyme (E2) and is catalyzed by Ub-ligase enzyme (E3) to be conjugated with the target protein. The process is continued up to a minimum of four Ub monomers and moved through the 26S proteasome where they will be degraded into short peptides [115]. In a situation of increased association of ubiquitin to muscle proteins and upregulation of ubiquitin-proteasome subunit, transcription lead to declined muscle mass. Additionally, efficient blockade of inclined protein degradation by

proteasome inhibitors and raise of proteasome ATP dependent activity influences muscle atrophy via UPS [116]. MAFbx/Atrogin-1 and MuRF1 are collectively termed atrogens (E3 ubiquitin ligases). These were observed to be increased during muscle atrophy induced by denervation and considered as sensitive markers of protein degradation [117]. Intriguingly, atrogin-1 knockdown resulted in muscle loss prevention during fasting [118], where dexamethasone-induced muscle atrophy resistance was reported in the absence of MuRF1 [119]. Growth-related and survival pathway proteins appear to be the substrates of atrogin-1. MyoD degradation is enhanced by atrogin-1, inhibiting myotube formation [120]. An important activator of protein synthesis, eukaryotic translation factor 3 subunit 5 (eIF3-f) ubiquitination is promoted by atrogin-1 [121]. Atrogin-1 interacts with sarcomere proteins, enzymes related to glycolysis, and mitochondrial proteins linking it to both structural and functional proteins [122]. In contrast, target substrates of MuRF-1 are structural protein titin, troponin1 including myosin heavy chain, further including proteins associated with glycolysis and glycogen metabolism [123, 124]. The atrogenes are related to the FoxO transcriptional factors [125]. Phosphorylated Akt suppresses protein degradation via promoting FoxO phosphorylation other than its involvement in the mTORC1 mechanisms. The dephosphorylated form of FoxO has the potential to translocate to the nucleus, hence initiate the transcription of target genes supporting catabolic roles (MuRF1 and atrogin-1) [125, 126]. With regard, the activity of Akt on FoxO could alleviate muscle atrophy. Atrogene transcription is further stimulated via oxidative stress, pro-inflammatory cytokines, and glucocorticoids. The expression of the atrogenes was reported to be regulated in inflammation and oxidative stress via the p38 mitogen-activated protein kinase (MAPK), and the nuclear factor-kappaB (NF-κB) pathways [127]. Contradictory findings of the behavior of atrogenes were reported. Old and young subjects demonstrated inclined ubiquitin expression [128].

Further, a study conducted by Raue et al (2007), concluded that rest and resistance exercise both contribute towards muscle loss with age-inducing atrogin-1 levels [129]. However, considering the function of UPS, it may suggest regulation of atrogenes would be a possible mechanism to preserve muscle mass with advancing age, though further investigations are required with enhanced sample size. The ubiquitin-proteasome (UbP) system degrades ~80-90% of intracellular protein. During chronic illness-induced atrophy, the UbP is the primary system responsible for the accelerated degradation of myofibrillar proteins.

1.3.2 Caspase-mediated proteolysis (need to construct from the beginning)

They cleave a wide spectrum of cytosolic targets. Activation of caspase-3 is an important step in the progressive loss of muscle protein due to catabolic stressors. Caspase-3 is activated by upstream caspases that are involved in both the extrinsic (death receptor-initiated) and intrinsic (cytosolic) caspase cascades. The release of cytochrome c and other factors from the mitochondria by enhancing the activity of permeability pores in the outer membrane. Release of cytochrome c from mitochondria induces the formation of a signaling complex that activates caspase-9 and subsequently caspase-3. However, skeletal muscle cells are different than most other cell types because they are post-mitotic and multinucleated, and activation of apoptotic pathways in the traditional sense does not cause loss or death of the entire muscle cell or fiber. It is clear that the activation of caspase-3 is a key factor that triggers the progressive loss of muscle protein in catabolic illness.

1.3.3 Autophagy-lysosome system

Autophagy is a process that clears the dysfunctional cytoplasmic components in living cells via interacting lysosomes. This is an essential housekeeping process in skeletal muscle as a major metabolic organ. As the muscle tissue acts as a source of amino acids during times of stress and starvation, it requires and is efficient to recycle damaged components as well as to degrade proteins to meet the energy demands. Macroautophagy is the process by which the skeletal muscle formulate cytoplasmic components for degradation by lysosomes [130]. Further, this process is vital in the remodeling of cellular architecture. The autophagy mechanism is controlled by the Atg proteins where it involves three steps; initiation, nucleation/ autophagosome formation, and lysosome fusion/ degradation. The initiation is induced by the lack of IGF-1 (nutrient-dependent), thereby inhibiting of Akt/mTOR pathway. During this period, Akt is inactive while AMPK is active resulting in inactivation of mTOR and FoxO activation, where it would support the transcription of atrogenes, hence linking this to the 26S proteasome degradation as well. Inactive status of mTOR would inhibit phosphorylation of unc51-like kinase-1 (ULK1) leaving it to initiate nucleation [131, 132]. Further, nucleation requires beclin-1 complex, Atg7, Atg10, and E1/E2 ubiquitin ligases, thus forming autophagosome. Lysosome fusion completes the process [130]. The trimeric protein complex in the autophagy ULK-1/FIP200/Atg13 is regulated by mTOR [133]. The action of mTOR was reported to be contradictory about autophagy in some research, where inhibition of mTOR only was insufficient to alter the muscle autophagic flux. Research results delivered mTORC1 knockout to be effective towards inhibition of atrophy where mTORC2 the rapamycin independent component knock out increased autophagy. The data were correlated with the FoxO3 in the promotion of the autophagy mechanism [134]. Intriguingly, the upstream molecules of mTOR such as Akt are considered to play a vital

role in skeletal muscle autophagy. This implies, the regulation of autophagy is a two-way mechanism; Akt/FoxO involved transcription-dependent pathway and mTORC1/ULK1 mediated transcription-independent pathway [130]. Depending on these facts, a matter of interest is whether autophagy a protective or a detrimental procedure. It is a process of necessity and fine equilibrium would ensure the protection of muscle mass, preventing muscle wasting. Masiero and Sandri (2010), experimented on Atg7 knockout mice to block autophagy in skeletal muscles. Results expressed unexpected muscle atrophy and myofiber degeneration concluding the critical role of autophagy in the myofiber maintenance to avoid toxic compounds and dysfunctional organelle accumulation [135]. Muscle wasting with activated autophagy was reported by Risson et al (2009) concerning the mTOR. The inactivated mTOR complex defects in dystrophin leading to severe myopathy [97]. These studies signify the importance of the necessity and regulation of autophagy for skeletal muscle function.

Lysosomes are membrane-bound vesicles with an acidic internal environment that contain an assortment of hydrolases that degrade a variety of molecules. At least a dozen lysosomal proteases, known as cathepsins, work together to fully degrade proteins to amino acids. Lysosomes are the end-point of pinocytosis, endocytosis, phagocytosis, and autophagy pathways. The cell uses four main mechanisms to regulate the entry of substrates into the lysosome; (1) plasma membrane proteins are endocytosed and fuse with endosomes which are trafficked to the lysosome; (2) selected proteins are directly transported through the lysosomal membrane in a chaperone mediated process; (3) micro autophagy; (4) macro autophagy. Lysosomes are particularly poised to degrade stroma proteins, such as those bound in the membrane or located in the extracellular matrix, which have entered the cell through pinocytosis or receptor-mediated endocytosis. However, it does contribute to overall proteolysis during atrophy. Several small, Ub-like

protein factors (LC3, GABARAP, GATE16, and Atg12) are required for nucleation and elongation of membranes into augophagosomes. Two other essential proteins involved in autophagosome elongation include Atg (autophagy-related) 5 and Atg7. While a basal level of autophagy is required to maintain cell survival and organelle turnover it appears that excessive autophagy is detrimental to muscle mass. Conditions that cause muscle atrophy may induce excessive autophagy by damaging the mitochondria.

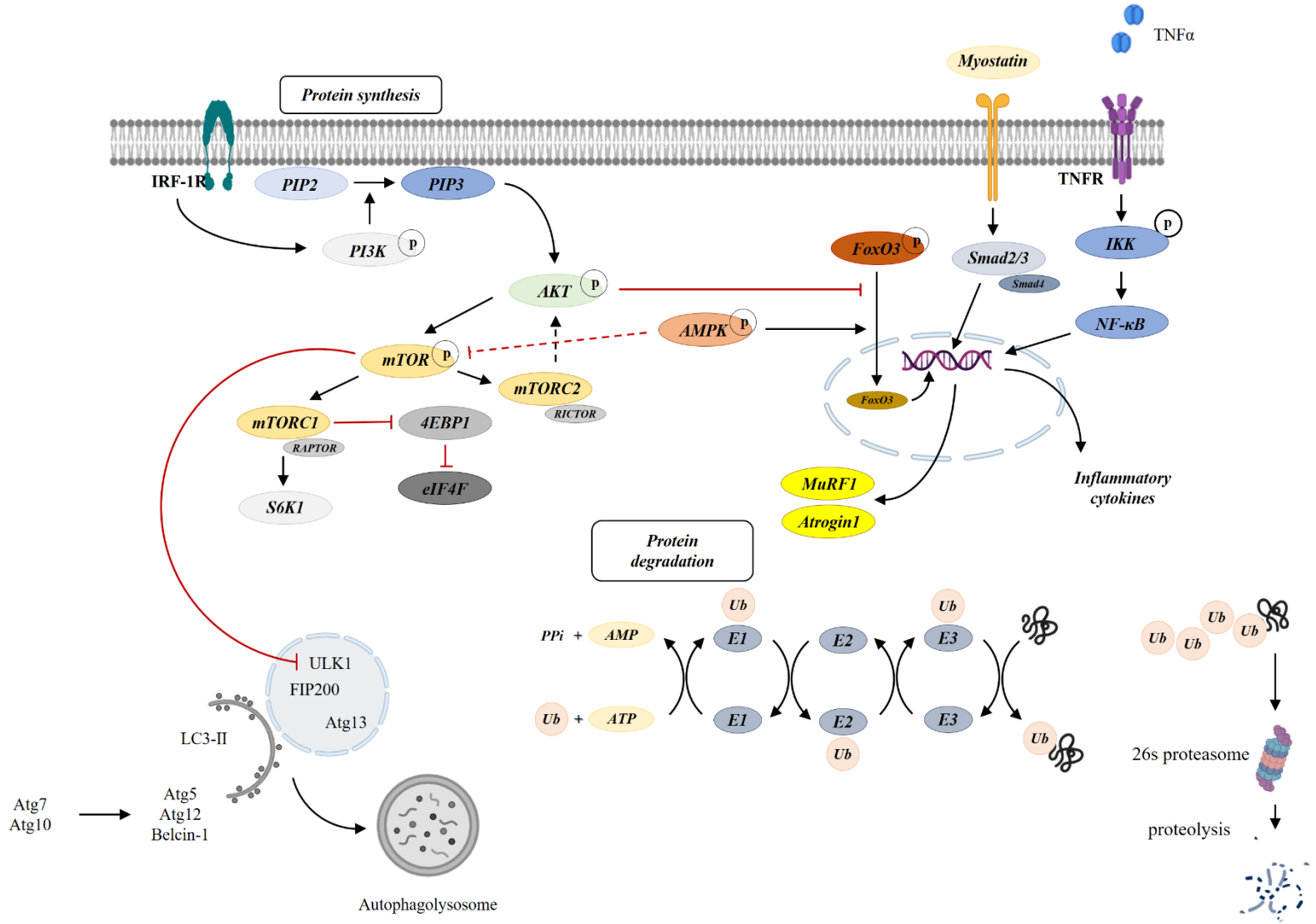

Figure 1- 2. *Overview of selected signaling pathways.*

1.4 The role of seafood as a preventive measurement for sarcopenia

Sarcopenia is associated with numerous factors such as genetic influences, deficiencies in the anabolic hormonal levels, exceeded amounts of inflammatory cytokines, and insulin resistance. Malnutrition is a key concern among the elderly population. Insufficient dietary intake leads to reduced muscle protein synthesis which creates a negative energy balance, eventually supporting sarcopenia. It is required to meet the challenges of increasing life span. Bed rest due to injury and aging results in muscle loss with reduced appetite and exercise. Nutritional supplementation would highly benefit the elderly population in this regard [136]. Hence, vicious cycle continuous, muscle strength and physical performance decline with aging influence the risk of poor nutrition, where poor nutrition contributes to sarcopenia. Nutritional supplements associated with physical exercise exhibited promising results as an efficacious method of preventing and treating sarcopenia. Even though direct hormonal supplements could increase muscle power and function, waves of side effects have limited the usage of them as potential treatments in long term. Thus, this section will be discussing seafood nutritional supplementation as a potent treatment for the prevention of sarcopenia.

Marine organisms have many bioactive components, which are not reported in terrestrial organisms [137]. The abundance of high-quality proteins, n-3 polyunsaturated fatty acids (PUFA.s), and other nutrients, such as minerals, trace elements, and vitamins, make seafood a popular option as the supplementary [138]. Furthermore, much evidence indicated those functional ingredients could effectively reduce the risk of type 2 diabetes, possibly some cancers, and aging-induced sarcopenia [139-141]. Based on those viewpoints, we have known that seafood can be considered as an essential option of supplementation. It has been mentioned that starting in late middle age, skeletal muscles atrophy, and muscle tissue exhibit morphological changes (e.g., infiltration with

noncontractile material, such as fat and connective tissue), decreasing muscles' ability to create and retain force and negatively impacting daily activities (walking and lifting items) [142]. Functional nutrients found in seafood have emerged as an effective therapeutic strategy by changing the gene expression of growth regulatory factors in the muscle that are consistent with anabolism and increased the gene expression of master regulators of mitochondrial function for maintaining appropriate muscle mass and function in clinical care for older persons [143-145].

To date, we have known that eating fish rather than meat or poultry was associated with a lower risk of a range of adverse cardiovascular outcomes [146]. Thus, the diet patterns have been changed, the "white meat" such as fish has become popular. In Asia and the Mediterranean Sea, seafood is used as the common food in daily life. Due to the price is similar to other meat, people tend to choose fish as a better protein and n-3 PUFA resource. Further, seafood cost depends on the country or region associated with its landscape. Numerous epidemiological studies have looked into the link between marine dietary items and age-related disorders, such as cardiovascular disease and sarcopenia [141, 147, 148]. For example, based on epidemiological research, older people are more likely than younger people to consume insufficient protein [149]. As a result, the recommended protein consumption is 1.2 g/kg/day [150]. Additionally, compared to pork and beef, the protein from fish is much easier to digest. And also, fish contain large amounts of omega-3 fatty acids, an unsaturated fat that benefits the heart rather than increasing the risk of heart disease [151]. Thus, based on that vital evidence, we could encourage seafood consumption as a good option for older people to enhance their body composition.

Accordingly, to the definition (FAO 2009), seafood living resources include marine, freshwater fish, other aquatic animals, and plant species (seaweed) that grow in aquatic environments. It has become an essential part of the human diet. It's been reported, 15% of average per capita animal protein intake to be compensated through fish (FAO 2009) [152]. Considering the above values, seafood is receiving much attention as part of a healthy diet. Essential vitamins (A, B_3, B_6, B_{12}, and D) including mineral ions such as calcium, potassium, zinc, iron, iodine, selenium, magnesium increase the value of seafood. Even though it's a positive source of fatty acids (FA), the saturated FA content is reported to be low and serves as a major source of essential long-chain FAs (n-3) [153]. Fish contains omega-3, vitamin D, and E including magnesium which suggests its value as a functional food to prevent sarcopenia [154].

Seafood polyunsaturated fatty acids (PUFAs) express significant biological activity compared to plant-based PUFA. Neurological development, growth, and immune responses are affected by the n-3 PUFAs in seafood [152]. These include, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). The EPA and DHA ratio in marine phytoplanktons is high, thus help accumulation in the food chain. The habitat and the type of fish determine the amount of EPA and DHA. Though, fatty fish (salmon, mackerel) exhibit a higher proportion of n-3 PUFAs compared to lean fish (cod, halibut) while shellfish are reported to have low levels [155]. Chronic low-grade inflammation associated with aging is referred to as inflammaging and was discussed as a distinct contributor to the development of sarcopenia in earlier sections. Hence, attenuating inflammaging would be an alternative approach to overcome sarcopenia. Even though anti-inflammatory drugs are in use to decline inflammatory responses to improve physical performance in the aging community, it holds risk on adverse events. In such situations, omega-3 PUFAs

could potentially serve as an alternative. The plasma level increment of pro-inflammatory cytokines is suggested to develop and progress sarcopenia affecting muscle catabolic and anabolic signaling [156, 157]. The cell structural, functional properties are assisted by the EPA and DHA concentrations in the cell membrane. Though EPA and DPA can be produced through ALA to compensate for insufficient dietary intakes, DHA is essential to be supplemented through proper diet [158]. Custodero et al. (2018), reviews the potentially averting functional decline due to aging via reducing systemic inflammation. The intervention of pre-selected biomarkers of inflammation (IL-6 and C-reactive protein) was examined. The meta-data analysis reported on the potential of omega-3 PUFAs in reducing the mentioned biomarkers [159]. A study involving middle and late adulthood with the supplementation of EPA and DHA significantly reduced the circulating pro-inflammatory cytokine levels (IL-6, IL-1β, and TNF-α) supporting the effective dietary intervention against inflammaging [160]. In the elderly age, the potential to perform the physical exercise as well the protein intake is blunted, which is referred to as anabolic resistance. Omega-3 PUFA would provide a source of overcoming the issue via muscle protein synthesis increment with the stimulation of the mTOR signaling pathway. The importance and the significant role of this intermediate protein were discussed in the previous section. Yoshino et al. (2016), reports on n-3 PUFA included dietary supplements as a potent muscle therapy. This research associates decrement of the inhibition of mTOR signaling via n-3 PUFA about calpain and ubiquitin-mediated proteolysis. At the same time, respiratory electron transport as well as oxidative phosphorylation including extracellular matrix were increased with n-3 PUFA therapy. Conversely, the G-protein coupled receptor (GPCR) associated pathways were augmented by the treatment [145]. Fish oil-derived omega-3 PUFA therapy was subjected to an experiment by Gordon et al. (2015), as a treatment for age-associated

reduction of muscle mass and function. The clinical study reports on the increased thigh muscle volume and handgrip strength over six months of PUFA therapy [161]. Aging influences insulin resistance leading to anabolic resistance. Literature indicates increased insulin sensitivity with omega-3 PUFA treatment in mid-age men [162]. Suggested benefits over muscle health due to omega-3 supplementation include amplified nerve conduction related to muscle activation, mitochondrial function improvement resulting in oxidative energy production, and muscle hypertrophy [142].

Amino acids required for the synthesis of muscle proteins are supplied by the dietary proteins. The muscle mass, strength, and nitrogen balance are suggested to be maintained through proper protein supplements. The optimal protein intake of the elderly is elaborately reviewed by Wolfe et al. (2008) [163]. The recommended daily average (RDA) of protein intake is 0.8 g kg^{-1} day^{-1}, though receiving the adequate amount is challenging for the adults [164]. Despite the fact, aged muscle express blunted response against the anabolic signal of protein synthesis, but following a protein-rich meal, the induction potential is mounted [154]. Skeletal muscle is the largest protein reservoir and can be immediately utilized to supply amino acid in a fasting or stress situation. Hence, adequate protein intake is necessary to overcome the negative protein balance. Under particular conditions of reduced energy intake, optimal protein content can be achieved via diet composition modulation [165]. Overfeeding would only increase fat deposition. Aquatic animal muscle tissue proteins are considered comparatively valuable than other animal-derived proteins. The dietary essential amino acid concentration determines the protein's nutritional value. Concerning human requirements, aquatic proteins contain dietary essential amino acid composition compared to vegetable proteins. Lack of strong collagenous fibers and tendons make seafood proteins easily digestible and absorbable [166]. Seaweed protein content varies depending on the species, where red seaweeds

exhibit the highest dry weight. *Porphyra yezoensis* was reported to express 47% of proteins, while *Palmaria palmata* followed as 35% according to dry mass. Both species are used as sea vegetables [167]. Fish protein consumption is reported to reduce epididymal adipose tissue and plasma triglyceride levels, and enhance skeletal muscle weight (fast-twitch) [168]. Among other nutrients, amino acids are allocated with much interest due to their ability to not only contribute towards the structure of proteins but also their involvement as signaling molecules [169]. Lysine and methionine are two essential amino acids abundantly found in seafood proteins. Generally, fish and shellfish are favorable sources of essential amino acids [166]. Postprandial muscle protein synthesis is of utmost importance to maintain muscle mass. Leucine, an essential amino acid, in particular, stimulates key anabolic processes contributing to muscle protein synthesis and compensates for blunted signals in the elderly. Amino acid mixtures with increased leucine contents were reported to be associated with postprandial muscle protein response in the elderly [170]. Marine seaweed amino acid composition has been a subject of study frequently. Though a high proportion of it is composed of aspartic and glutamic acids other amino acids also contribute up to a significant extent. *Laminaria digitata* a brown seaweed, *Ulva pertusa* a green seaweed, and *Porphyra tenera* (red seaweed) were reported to exhibit noteworthy amounts of leucine [167]. Leucine as well exerts anti-catabolic properties further assisting anabolism through inhibition of protein breakdown. A metabolite of leucine, β-hydroxy-β-methylbutyrate (HMB) naturally occurs in human muscle affecting protein balance. Supplementation of HMB was reported to be preserving muscle mass [171]. Taurine is one such sulfur containing amino acid (2-aminoethane-sulfonic acid) ubiquitously present in animal tissue. It’s a non-essential amino acid, thus not incorporated in macromolecule formation. Though, this is important in several physiological processes, particular mechanisms are not totally

elucidated [172]. Taurine exhibits skeletal muscle strengthening through its role in the excitation-contraction coupling. The dihydropyridine receptor (DHPR), is a voltage gated Ca^{2+} channel in the T-tubule system, where ryanodine receptor (RYR1) is expressed in the SR. Mechanical interaction of the both through Ca^{2+} release triggers the muscle contraction. With advancing age, skeletal muscles undergo functional impairments due to structural changes. The uncoupling of DHPR-RYR1 alters the SR Ca^{2+} release, declining myoplasmic Ca^{2+} elevation, resulting lower Ca^{2+} supply to contractile proteins. Thus, Ca^{2+} uncoupling is an alteration that leads to skeletal muscle weakness [173]. Dutka et al. (2014), reports the potential of taurine to increase the rate of SR Ca^{2+} uptake with a possible action through SR lumen in both type I and type II muscle fibers [174]. Physiological aging is influenced by ER stress being one of the most important Ca^{2+} stores. Impairments of Ca^{2+} regulation improve ER stress due to accumulation of protein misfolding ultimately leading to apoptotic pathways via caspase mechanisms. Handling Ca^{2+} homeostasis would pave the way to inhibit the activation of apoptotic pathways [175]. Proposed mechanisms of protection via taurine include Ca^{2+} level modulation and membrane stabilization and osmolality maintenance. The calcium levels are suggested to be mediated by handling Ca^{2+} by sarcolemma and mitochondria. Taurine builds up interaction with membrane proteins and distorts membrane proteins to alter their function. Thus, changes in the intracellular ion concentrations (Ca^{2+}) are acquired. Taurine is also reported to exhibit detoxifying ability against hypochlorous acid to generate taurine chloramine which is comparatively less toxic and involves immune system modulation [176]. The nuclei turnover of adult skeletal muscle is limited under normal physiological conditions, where upon injury rapidly initiate to prevent muscle mass loss. Satellite cell activation is a distinctive process that would induce myogenic regulatory factors [177]. The quality and quantity of satellite cells, as well as the regeneration behavior, are

influenced via the systemic environment. With regard, taurine assist modulate the ROS generation and inflammatory cytokines allowing satellite cells to enhance their activity [178]. Enzyme hydrolysates from *Hippocampus abdominalis* were assessed for their muscle growth capacity in vitro and in vivo systems by Kim et al. (2019). The study explains the potential of alcalse hydrolysate of *H. abdominalis* to enhance muscle growth via inhibiting myostatin-Smad driven negative signaling, where it would improve IGF1-Akt driven positive pathway [179]. Further, purified peptides from the same specie was reported to improve skeletal muscle differentiation through p38MAPK/ Akt axis [180].

The distinct regulatory and signaling molecule of muscle fibers is Ca^{2+}. Thus, the variable expression of proteins related (i.e. calpains) to Ca^{2+} signaling determines the contractile properties of muscle. A study involving 1339 aged Korean adults indicated a correlation between the level of daily calcium intake and appendicular skeletal muscle mass [181]. A detailed review on calcium ion in skeletal muscle was published by Brechtold et al. (2000), explaining its crucial role in muscle function [182]. Magnesium is the second abundant mineral in the body second to potassium. It is involved in vivid physiological functions such as enzyme activation. A study conducted on magnesium supplementation revealed it would enhance the free and total testosterone levels supporting the physical activity [183]. Another study reported a strong correlation between dietary magnesium level and skeletal muscle mass [184]. Selenium is a trace element that exerts its biological activities through selenocysteine. This is involved in the protection of muscle from oxidative damage. Selenium deficiency is related to muscular dystrophy [185].

Creatine is present in the human body and produced in the liver, pancreas, and kidney, where it would be transported to the sites of utilization (skeletal muscle), for a high-energy generation. Arginine, glycine, and methionine are the primary substrates of

creatine production. Muscle meat including fish is an abundant source of creatine, tuna, and salmon in particular. Free creatine is translated to phosphocreatine by creatine kinase ultimately utilized as an instant source of energy during resistance training. Creatine supplementation is reported to be affecting inclined levels of intracellular osmolality and muscle glycogen storage upregulation [186, 187]. Further reports elaborate its potential in strengthening muscle strength while reducing cellular damage and inflammation due to intense physical exercise [188, 189].

Although vitamins do not serve in structural functions or significant energy production, they are essential for multiple physiological purposes. Due reason, the vitamin requirement in the diet is minute. Seaweed is a source of vitamins, antioxidants, and pigments due to its natural habitat of exposure to direct sunlight during its lifespan. Water-soluble vitamins such as vitamin C are abundant in *Ecklonia arborea* and *Gracilaria changii* [190]. Further, algae contribute B-group vitamins, vitamin A and vitamin E. Vitamin A is important in the antioxidant defense mechanism, where it would act to prevent oxidative stress-related pathogenesis of sarcopenia [191]. Marine carotenoids are referred to as astaxanthins, which are naturally distributed in marine organisms such as fish (shrimp, crab) and fish (salmon). Due to its potential of elevating the PGC-1α (peroxisome proliferator-activated receptor-γ coactivator), also referred to as mitochondrial nutrient [192]. Similarly, vitamin E is considered a biological antioxidant inhibiting the lipid peroxidation of long-chain PUFAs [154]. Whereas, vitamin B_6 deficiency leads to neurological disorders affecting motor neurons ultimately weakening distal movement [193]. Another member of the B-vitamin complex is vitamin B_{12}, that its deficiency exhibit neuromuscular symptoms. Vitamin C is a renowned nutritional supplement against oxidative stress and is considered a vital hydrophobic antioxidant due to its role in the regeneration of vitamin E in the cell membrane. Further,

it downregulates the vitamin E radicals generated. [191]. Combined supplementation of vitamin C and E has driven the total lean mass gain [194]. Among all vitamins, D-vitamin is a widely discussed topic with their involvement in sarcopenia. Vitamin D is associated with skeletal functions such as bone metabolism, where its deficiency enhances fracture risk. Declined levels of vitamin D were reported to be influencing immune and neurodegenerative disorders [154]. Due to its association with muscle mass, strength, and neuromuscular functions, deficiencies were published to be correlated with significant decline in physical performance, increasing the risk of fall and fracture [195]. Vitamin D stimulates protein synthesis hence muscle growth via binding with specific nuclear receptors in muscle tissue. The particular receptor expression is reported to be declined with age. Moreover, vitamin D help maintain calcium phosphate homeostasis [196, 197].

An important component of marine algae (brown algae) is polyphenols which help determine the value of functional food and pharmaceutical significance. Though, studies reporting its intervention in muscle-related research are limited. Among them, diphloroethohydoxycarmalol (DPHC) isolated from *Ishige okamurae* was communicated about its potential to inhibit inflammatory myopathy [198]. Moreover, DPHC demonstrated cytosolic Ca^{2+} promotion which in result affected glucose transport to myotubes to modulate blood glucose levels [199].

Supplementation of the above-discussed components beneficially effects as a treatment against sarcopenia. Nutritional proteins and healthy functional agents from seafood can be implemented in the role of preventing sarcopenia. Though it requires further studies regarding dosage, frequency, and sexually dimorphic effect in the usage. Resistance training alongside nutritional supplementation has not been considered as a topic of discussion in this review. As dietary supplement seafood would positively influence protein kinetics in the aging population.

Table1- 3. Nutritional factors as supplements against sarcopenia.

Intervention	Effect on sarcopenia	Research reference
Omega-3 PUFA	Dietary intervention against inflammaging. EPA+DHA treatment significantly reduced the levels of IL-6, IL-1β, and TNF-α.	(Tan et al., 2018)
	GPCR associated pathway augmentation. Mitochondrial function and extracellular organization related pathway regulators were increased, proteolysis involved regulators (clapain and ubiquitin) decreased by n-3 PUFA treatment. Inhibition of key anabolic regulator mTOR was decreased. GPCR signaling related pathways were increased.	(Yoshino et al., 2016)
	The clinical study reports on the increased thigh muscle volume and handgrip strength over six months of PUFA therapy. Therapy exhibited reduced decline of muscle mass in aged adults which supported physical independence.	(Smith et al., 2015)
	Increased insulin sensitivity with omega-3 PUFA treatment in mid aged men. Correlation between n-3 PUFA concentrations in red cell phospholipids with improved insulin sensitivity. Further, free fatty acid levels and CRP concentrations were lowered.	(Albert et al., 2014)
Protein, amino acid	Reduce epididymal adipose tissue and plasma triglyceride levels, and skeletal muscle weight increment. Fast-twitch muscle weight was increased.	(Kawabata et al., 2015)
	Leucine, an essential amino acid, in particular stimulates key anabolic processes contributing muscle protein synthesis and compensate for blunted signals in elderly. Amino acid mixtures with increased leucine contents were reported to be associated with postprandial muscle protein response in elderly.	(Koopman et al., 2009)
	Supplementation of HMB was reported to be preserving muscle mass. The test was conducted during 10 days of bed rest.	(Deutz et al., 2013)
	Ca^{2+} accumulation in the SR was facilitated by taurine. Ca^{2+} affinity towards SERCA was probably involved.	(Dutka et al., 2014)

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fatty acid; mTOR, mammalian target of rapamycin; CRP, C-reactive protein; HMB, β-hydroxy-β-methylbutyrate; SR, sarcoplasmic reticulum; SERCA, sarco (endo) plasmic reticulum Ca^{2+}-ATPase; PCr, phosphocreatine; GLUT-4, Glucose transporter type 4; PGC-1α, PPARγ coactivator 1 alpha; DPHC, diphloroethoxycarmalol

	Enzyme hydrolysates of *Hippocampus abdominalis* as a functional food material from marine animals to grow muscle cells and to enhance muscular exercise capacity.	(Kim et al., 2019)
	Peptides from *Hippocampus abdominalis* improve muscle differentiation and endurance performance via p38MAPK/ Akt signaling.	(Muthuramalingam et al., 201
Minerals	A study involving 1339 aged Korean adults indicated a correlation between the level of daily calcium intake and appendicular skeletal muscle mass. Concluded as, an inverse relationship between daily calcium intake and sarcopenia.	(Seo et al., 2013)
	The strong correlation between dietary magnesium level and skeletal muscle mass indices. Indicated the importance of dietary magnesium for muscle health.	(Hayhoe et al., 2019)
	Selenium deficiency is related to muscular dystrophy.	(Orndahl et al., 1982)
Creatine	Due to creatine supplementation, the PCr availability inclines and lactate accumulation declines in muscle.	(Balsom et al., 1995)
	Oral glucose tolerance of healthy individuals was improved by the combined creatine and protein supplementation. Muscle GLUT-4 and glycogen contents in human skeletal muscle were increased by oral creatine supplementation.	(Derave et al., 2003)
	Exhaustive resistance exercise caused inflammation and cell damage were downregulated by creatine supplementation.	(Santos et al., 2004)
Vitamin	Skeletal muscle PGC-1α elevation was influenced by astaxanthin. Hence, mitochondrial aerobic metabolism activates, accelerating lipid utilization.	(Liu et al., 2014)
	Combined supplementation of vitamin C and E have driven the total lean mass gain.	(Bjornsen et al., 2016)
	The study reveals the potential of vitamin D and calcium supplementation to reduce fall by 49% in vitamin D deficient elderly women.	(Bischoff et al., 2003)
Phlorotannin	Inflammatory myopathy inhibited by *Ishige okamurae* isolated diphloroethoxycarmalol (DPHC).	(Kim et al., 2020)
	Ishige okamurae isolated diphloroethoxycarmalol (DPHC) improves cytosolic Ca^{2+} which in result affects glucose transport to myotubes to modulate blood glucose levels.	(Yang et al., 2021)

EPA, eicosapentaenoic acid; DHA, docosapentaenoic acid; IL-6, interleukin 6; IL-1β, Interleukin 1 beta; TNF-α, Tumor necrosis factor-α; GPCR, G-protein coupled receptor; PUFA, polyunsaturated fatty acid; mTOR, mammalian target of rapamycin; CRP, C-reactive protein; HMB, β-hydroxy-β-methylbutyrate; SR, sarcoplasmic reticulum; SERCA, sarco (endo) plasmic reticulum Ca^{2+}-ATPase; PCr, phosphocreatine; GLUT-4, Glucose transporter type 4; PGC-1α, PPARγ coactivator 1 alpha; DPHC, diphloroethoxycarmalol

1.5 Conclusions

Human function and mobility are supported by the skeletal muscle tissue through its cellular components and structural design. Aging represents complex aetiology allied with immune, hormonal, and neuronal alterations which ultimately affect daily physical activities. Sarcopenia, a condition related to aging and express the risk of losing functional independence. Protein balance attending its inclined anabolism and decelerated catabolism, as well as the muscle regeneration potential alongside satellite cell activation involving molecular pathway mechanisms, are crucial in overcoming sarcopenia. Cellular survival pathways (Akt/ mTOR) to generate protein in demand and proteolytic mechanisms (UPS/ autophagy) to maintain clear cellular structure and function are of utmost importance in the understating and regulation of sarcopenia. Nutritional interventions about overcoming the consequences of sarcopenia have received much attention. With respect, nutritional supplements from seafood sources are being suggested as positive feedback to counteract inflammaging, oxidative stress, and insulin resistance accompanied with sarcopenia. Despite the fact that sarcopenia is attributed simply as a decrement in physical potential, the complex biology behind age-associated muscle loss is the center of gravity which as researchers we should extend understanding.

1.6 Goal of the study

While exercise training favors maintenance of muscle mass, for critically ill patients it is very difficult and often not a feasible option to counteract muscle loss. Additionally, targeted treatments such as proteasome inhibitors have been largely ineffective at maintaining muscle mass suggesting that ideal therapies would affect multiple proteolytic systems. A safe nutritional therapy is an ideal option. Since the overarching objective of this project is to better understand how IO/IPA/DPHC prevents the atrophy-inducing responses to palmitate, we conducted experiments to elucidate how palmitate and IO/IPA/DPHC affect Akt signaling, inflammasomes, and proteolytic signaling.

This information could provide a rationale for the use of IO/IPA/DPHC supplementation as a treatment for chronic illness-related muscle atrophy. Such an approach would be attractive because it is a nutritional therapy that has numerous other positive effects that are also typically dysregulated in conditions associated with muscle wasting and it poses a low chance for eliciting other undesired side effects.

References

1. von Haehling, S., J.E. Morley, and S.D. Anker, *An overview of sarcopenia: facts and numbers on prevalence and clinical impact.* J Cachexia Sarcopenia Muscle, 2010. **1**(2): p. 129-133.
2. Morley, J.E., et al., *Sarcopenia.* J Lab Clin Med, 2001. **137**(4): p. 231-43.
3. Janssen, I., et al., *The healthcare costs of sarcopenia in the United States.* J Am Geriatr Soc, 2004. **52**(1): p. 80-5.
4. Morley, J.E., et al., *Frailty and the aging male.* Aging Male, 2005. **8**(3-4): p. 135-40.
5. Fielding, R.A., et al., *Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia.* J Am Med Dir Assoc, 2011. **12**(4): p. 249-56.
6. Gumucio, J.P. and C.L. Mendias, *Atrogin-1, MuRF-1, and sarcopenia.* Endocrine, 2013. **43**(1): p. 12-21.
7. Bischoff-Ferrari, H.A., et al., *Comparative performance of current definitions of sarcopenia against the prospective incidence of falls among community-dwelling seniors age 65 and older.* Osteoporos Int, 2015. **26**(12): p. 2793-802.
8. Cruz-Jentoft, A.J., et al., *Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People.* Age Ageing, 2010. **39**(4): p. 412-23.
9. Chen, L.K., et al., *Sarcopenia in Asia: consensus report of the Asian Working Group for Sarcopenia.* J Am Med Dir Assoc, 2014. **15**(2): p. 95-101.
10. Abellan van Kan, G., *Epidemiology and consequences of sarcopenia.* J Nutr Health Aging, 2009. **13**(8): p. 708-12.
11. Baumgartner, R.N., et al., *Epidemiology of sarcopenia among the elderly in New Mexico.* American Journal of Epidemiology, 1998. **147**(8): p. 755-763.
12. Lauretani, F., et al., *Age-associated changes in skeletal muscles and their effect on mobility: an operational diagnosis of sarcopenia.* J Appl Physiol (1985), 2003. **95**(5): p. 1851-60.
13. Lau, E.M., et al., *Prevalence of and risk factors for sarcopenia in elderly Chinese men and women.* J Gerontol A Biol Sci Med Sci, 2005. **60**(2): p. 213-6.
14. Kim, Y.S., et al., *Prevalence of sarcopenia and sarcopenic obesity in the Korean population based on the Fourth Korean National Health and Nutritional Examination Surveys.* J Gerontol A Biol Sci Med Sci, 2012. **67**(10): p. 1107-13.
15. Smith, L., et al., *Association between Food Insecurity and Sarcopenia among Adults Aged ≥65 Years in Low- and Middle-Income Countries.* 2021. **13**(6): p. 1879.
16. Kurpad, A.V., S. Muthayya, and M. Vaz, *Consequences of inadequate food energy and negative energy balance in humans.* Public Health Nutr, 2005. **8**(7A): p. 1053-76.
17. Perez-Zepeda, M.U., *Food Insecurity and Its Association with Sarcopenia Elements.* Innovation in Aging, 2017. **1**(suppl_1): p. 50-51.
18. Daskalopoulou, C., et al., *Factors related with sarcopenia and sarcopenic obesity among low- and middle-income settings: the 10/66 DRG study*. Scientific Reports, 2020. **10**(1): p. 20453.
19. Lang, T., et al., *Sarcopenia: etiology, clinical consequences, intervention, and assessment.* Osteoporos Int, 2010. **21**(4): p. 543-59.

20. Faulkner, J.A., et al., *The aging of elite male athletes: age-related changes in performance and skeletal muscle structure and function.* Clin J Sport Med, 2008. **18**(6): p. 501-7.
21. Power, G.A., et al., *Motor unit number estimates in masters runners: use it or lose it?* Med Sci Sports Exerc, 2010. **42**(9): p. 1644-50.
22. Troell, M., M. Jonell, and B. Crona, *The role of seafood in sustainable and healthy diets.* 2019.
23. Frontera, W.R. and J. Ochala, *Skeletal muscle: a brief review of structure and function.* Calcif Tissue Int, 2015. **96**(3): p. 183-95.
24. Fortin, M., et al., *Paraspinal muscle morphology and composition: a 15-yr longitudinal magnetic resonance imaging study.* Med Sci Sports Exerc, 2014. **46**(5): p. 893-901.
25. Allen, D.L., R.R. Roy, and V.R. Edgerton, *Myonuclear domains in muscle adaptation and disease.* Muscle Nerve, 1999. **22**(10): p. 1350-60.
26. Lieber, R.L., *Skeletal muscle structure, function, and plasticity*. 2002: Lippincott Williams & Wilkins.
27. Ottenheijm, C.A., L.M. Heunks, and R.P. Dekhuijzen, *Diaphragm adaptations in patients with COPD.* Respir Res, 2008. **9**(1): p. 12.
28. Peter, A.K., et al., *The costamere bridges sarcomeres to the sarcolemma in striated muscle.* Prog Pediatr Cardiol, 2011. **31**(2): p. 83-88.
29. Huxley, A.F. and R. Niedergerke, *Structural changes in muscle during contraction; interference microscopy of living muscle fibres.* Nature, 1954. **173**(4412): p. 971-3.
30. Jayasinghe, I.D. and B.S. Launikonis, *Three-dimensional reconstruction and analysis of the tubular system of vertebrate skeletal muscle.* J Cell Sci, 2013. **126**(Pt 17): p. 4048-58.
31. Edwards, J.N. and B.S. Launikonis, *The accessibility and interconnectivity of the tubular system network in toad skeletal muscle.* J Physiol, 2008. **586**(21): p. 5077-89.
32. Beard, N.A., D.R. Laver, and A.F. Dulhunty, *Calsequestrin and the calcium release channel of skeletal and cardiac muscle.* Prog Biophys Mol Biol, 2004. **85**(1): p. 33-69.
33. Murphy, R.M., et al., *Calsequestrin content and SERCA determine normal and maximal Ca2+ storage levels in sarcoplasmic reticulum of fast- and slow-twitch fibres of rat.* J Physiol, 2009. **587**(2): p. 443-60.
34. Franzini-Armstrong, C. and F. Protasi, *Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions.* Physiol Rev, 1997. **77**(3): p. 699-729.
35. Lamboley, C.R., et al., *Sarcoplasmic reticulum Ca2+ uptake and leak properties, and SERCA isoform expression, in type I and type II fibres of human skeletal muscle.* J Physiol, 2014. **592**(6): p. 1381-95.
36. Kayar, S.R., et al., *Mitochondrial size and shape in equine skeletal muscle: a three-dimensional reconstruction study.* Anat Rec, 1988. **222**(4): p. 333-9.
37. Cisternas, P., et al., *Wnt signaling in skeletal muscle dynamics: myogenesis, neuromuscular synapse and fibrosis.* Mol Neurobiol, 2014. **49**(1): p. 574-89.
38. Guarino, S.R., A. Canciani, and F. Forneris, *Dissecting the Extracellular Complexity of Neuromuscular Junction Organizers.* Front Mol Biosci, 2019. **6**(156): p. 156.

39. Galpin, A.J., et al., *Human skeletal muscle fiber type specific protein content.* Anal Biochem, 2012. **425**(2): p. 175-82.
40. Schiaffino, S. and C. Reggiani, *Fiber types in mammalian skeletal muscles.* Physiol Rev, 2011. **91**(4): p. 1447-531.
41. Sahlin, K., *Muscle energetics during explosive activities and potential effects of nutrition and training.* Sports Med, 2014. **44 Suppl 2**(2): p. S167-73.
42. Weibel, E.R., *The structural conditions for oxygen supply to muscle cells: the Krogh cylinder model.* J Exp Biol, 2013. **216**(Pt 22): p. 4135-7.
43. Westerblad, H., J.D. Bruton, and A. Katz, *Skeletal muscle: energy metabolism, fiber types, fatigue and adaptability.* Exp Cell Res, 2010. **316**(18): p. 3093-9.
44. Spriet, L.L. and M.J. Watt, *Regulatory mechanisms in the interaction between carbohydrate and lipid oxidation during exercise.* Acta Physiol Scand, 2003. **178**(4): p. 443-52.
45. Maeda, H., et al., *Fucoxanthin from edible seaweed, Undaria pinnatifida, shows antiobesity effect through UCP1 expression in white adipose tissues.* Biochemical and Biophysical Research Communications, 2005. **332**(2): p. 392-397.
46. Seca, A.M.L. and D. Pinto, *Overview on the Antihypertensive and Anti-Obesity Effects of Secondary Metabolites from Seaweeds.* Mar Drugs, 2018. **16**(7): p. 237.
47. Lu, Y.A., et al., *Anti-obesity effects of red seaweed, Plocamium telfairiae, in C57BL/6 mice fed a high-fat diet.* Food Funct, 2020. **11**(3): p. 2299-2308.
48. Kang, M.C., et al., *Anti-obesity effects of seaweeds of Jeju Island on the differentiation of 3T3-L1 preadipocytes and obese mice fed a high-fat diet.* Food Chem Toxicol, 2016. **90**: p. 36-44.
49. Suchkov, S., et al., *Ketogenic Diet Is Good for Aging-Related Sarcopenic Obesity*, in *Ketogenic Diet*. 2021, IntechOpen.
50. Yokoyama, Y., et al., *Association of nutrient-derived dietary patterns with sarcopenia and its components in community-dwelling older Japanese: a cross-sectional study.* Nutr J, 2021. **20**(1): p. 7.
51. Kim, S.A., et al., *The Association between Major Dietary Pattern and Low Muscle Mass in Korean Middle-Aged and Elderly Populations: Based on the Korea National Health and Nutrition Examination Survey.* Nutrients, 2020. **12**(11): p. 3543.
52. Kim, S.Y., et al., *Diphlorethohydroxycarmalol (DPHC) Isolated from the Brown Alga Ishige okamurae Acts on Inflammatory Myopathy as an Inhibitory Agent of TNF-alpha.* Mar Drugs, 2020. **18**(11): p. 529.
53. Yang, H.W., et al., *Ca(2+)-Dependent Glucose Transport in Skeletal Muscle by Diphlorethohydroxycarmalol, an Alga Phlorotannin: In Vitro and In Vivo Study.* Oxid Med Cell Longev, 2021. **2021**: p. 8893679.
54. Yang, H.-W., et al., *Ishige okamurae reduces blood glucose levels in high-fat diet mice and improves glucose metabolism in the skeletal muscle and pancreas.* Fisheries and Aquatic Sciences, 2020. **23**(1): p. 24.
55. Rosenberg, I.H., *Sarcopenia: origins and clinical relevance.* J Nutr, 1997. **127**(5 Suppl): p. 990S-991S.
56. Narici, M.V. and N. Maffulli, *Sarcopenia: characteristics, mechanisms and functional significance.* Br Med Bull, 2010. **95**(1): p. 139-59.
57. Lexell, J. and D.Y. Downham, *The occurrence of fibre-type grouping in healthy human muscle: a quantitative study of cross-sections of whole vastus lateralis from men between 15 and 83 years.* Acta Neuropathol, 1991. **81**(4): p. 377-81.

58. D'Antona, G., et al., *The effect of ageing and immobilization on structure and function of human skeletal muscle fibres.* J Physiol, 2003. **552**(Pt 2): p. 499-511.
59. Macaluso, A. and G. De Vito, *Muscle strength, power and adaptations to resistance training in older people.* Eur J Appl Physiol, 2004. **91**(4): p. 450-72.
60. Berger, M.J. and T.J. Doherty, *Sarcopenia: prevalence, mechanisms, and functional consequences*, in *Body composition and aging*. 2010, Karger Publishers. p. 94-114.
61. Haus, J.M., et al., *Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle.* J Appl Physiol (1985), 2007. **103**(6): p. 2068-76.
62. Reeves, N.D., C.N. Maganaris, and M.V. Narici, *Effect of strength training on human patella tendon mechanical properties of older individuals.* J Physiol, 2003. **548**(Pt 3): p. 971-81.
63. Maganaris, C.N., M.V. Narici, and N.D. Reeves, *In vivo human tendon mechanical properties: effect of resistance training in old age.* J Musculoskelet Neuronal Interact, 2004. **4**(2): p. 204-8.
64. Montano-Loza, A.J., et al., *Sarcopenic obesity and myosteatosis are associated with higher mortality in patients with cirrhosis.* J Cachexia Sarcopenia Muscle, 2016. **7**(2): p. 126-35.
65. Zamboni, M., S. Rubele, and A.P. Rossi, *Sarcopenia and obesity.* Curr Opin Clin Nutr Metab Care, 2019. **22**(1): p. 13-19.
66. Kalinkovich, A. and G. Livshits, *Sarcopenic obesity or obese sarcopenia: A cross talk between age-associated adipose tissue and skeletal muscle inflammation as a main mechanism of the pathogenesis.* Ageing Res Rev, 2017. **35**: p. 200-221.
67. Dube, J. and B.H. Goodpaster, *Assessment of intramuscular triglycerides: contribution to metabolic abnormalities.* Curr Opin Clin Nutr Metab Care, 2006. **9**(5): p. 553-9.
68. Kohara, K., et al., *Leptin in sarcopenic visceral obesity: possible link between adipocytes and myocytes.* PLoS One, 2011. **6**(9): p. e24633.
69. Hawke, T.J. and D.J. Garry, *Myogenic satellite cells: physiology to molecular biology.* J Appl Physiol (1985), 2001. **91**(2): p. 534-51.
70. Gallegly, J.C., et al., *Satellite cell regulation of muscle mass is altered at old age.* J Appl Physiol (1985), 2004. **97**(3): p. 1082-90.
71. Le Grand, F. and M.A. Rudnicki, *Skeletal muscle satellite cells and adult myogenesis.* Curr Opin Cell Biol, 2007. **19**(6): p. 628-33.
72. Carlson, M.E., et al., *Molecular aging and rejuvenation of human muscle stem cells.* EMBO Mol Med, 2009. **1**(8-9): p. 381-91.
73. Edstrom, E., et al., *Factors contributing to neuromuscular impairment and sarcopenia during aging.* Physiol Behav, 2007. **92**(1-2): p. 129-35.
74. Ramirez-Leon, V., et al., *Increased glutathione levels in neurochemically identified fibre systems in the aged rat lumbar motor nuclei.* Eur J Neurosci, 1999. **11**(8): p. 2935-48.
75. Kung, T.A., et al., *Motor unit changes seen with skeletal muscle sarcopenia in oldest old rats.* J Gerontol A Biol Sci Med Sci, 2014. **69**(6): p. 657-65.
76. Larsson, L., *Motor units: remodeling in aged animals.* J Gerontol A Biol Sci Med Sci, 1995. **50 Spec No**(Special_Issue): p. 91-5.
77. Deschenes, M.R., et al., *The neuromuscular junction. Muscle fibre type differences, plasticity and adaptability to increased and decreased activity.* Sports Med, 1994. **17**(6): p. 358-72.

78. Jang, Y.C. and H. Van Remmen, *Age-associated alterations of the neuromuscular junction.* Exp Gerontol, 2011. **46**(2-3): p. 193-8.
79. Young, V.R., *Amino acids and proteins in relation to the nutrition of elderly people.* Age Ageing, 1990. **19**(4): p. S10-24.
80. Campbell, W.W., et al., *Increased protein requirements in elderly people: new data and retrospective reassessments.* Am J Clin Nutr, 1994. **60**(4): p. 501-9.
81. Kim, J.S., J.M. Wilson, and S.R. Lee, *Dietary implications on mechanisms of sarcopenia: roles of protein, amino acids and antioxidants.* J Nutr Biochem, 2010. **21**(1): p. 1-13.
82. Volpi, E., R. Nazemi, and S. Fujita, *Muscle tissue changes with aging.* Curr Opin Clin Nutr Metab Care, 2004. **7**(4): p. 405-10.
83. Cuthbertson, D., et al., *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.* FASEB J, 2005. **19**(3): p. 422-4.
84. Thomson, D.M. and S.E. Gordon, *Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation.* J Appl Physiol (1985), 2005. **98**(2): p. 557-64.
85. Hakkinen, K. and A. Pakarinen, *Serum hormones and strength development during strength training in middle-aged and elderly males and females.* Acta Physiol Scand, 1994. **150**(2): p. 211-9.
86. Meng, S.J. and L.J. Yu, *Oxidative stress, molecular inflammation and sarcopenia.* Int J Mol Sci, 2010. **11**(4): p. 1509-26.
87. Wanagat, J., et al., *Mitochondrial DNA deletion mutations colocalize with segmental electron transport system abnormalities, muscle fiber atrophy, fiber splitting, and oxidative damage in sarcopenia.* FASEB J, 2001. **15**(2): p. 322-32.
88. Lee, C.M., et al., *Multiple mitochondrial DNA deletions associated with age in skeletal muscle of rhesus monkeys.* J Gerontol, 1993. **48**(6): p. B201-5.
89. Wang, Y., et al., *Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4022-7.
90. Hiona, A. and C. Leeuwenburgh, *The role of mitochondrial DNA mutations in aging and sarcopenia: implications for the mitochondrial vicious cycle theory of aging.* Exp Gerontol, 2008. **43**(1): p. 24-33.
91. Trifunovic, A., et al., *Premature ageing in mice expressing defective mitochondrial DNA polymerase.* Nature, 2004. **429**(6990): p. 417-23.
92. Jang, Y.C., et al., *Increased superoxide in vivo accelerates age-associated muscle atrophy through mitochondrial dysfunction and neuromuscular junction degeneration.* FASEB J, 2010. **24**(5): p. 1376-90.
93. Tchkonia, T., et al., *Cellular senescence and the senescent secretory phenotype: therapeutic opportunities.* J Clin Invest, 2013. **123**(3): p. 966-72.
94. Baylis, D., et al., *Immune-endocrine biomarkers as predictors of frailty and mortality: a 10-year longitudinal study in community-dwelling older people.* Age (Dordr), 2013. **35**(3): p. 963-71.
95. Sartorelli, V. and M. Fulco, *Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy.* Sci STKE, 2004. **2004**(244): p. re11.
96. Mavalli, M.D., et al., *Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice.* J Clin Invest, 2010. **120**(11): p. 4007-20.
97. Risson, V., et al., *Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy.* J Cell Biol, 2009. **187**(6): p. 859-74.

98. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 21-35.
99. Ohanna, M., et al., *Atrophy of S6K1(-/-) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control.* Nat Cell Biol, 2005. **7**(3): p. 286-94.
100. Latres, E., et al., *Insulin-like growth factor-1 (IGF-1) inversely regulates atrophy-induced genes via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway.* J Biol Chem, 2005. **280**(4): p. 2737-44.
101. Sandri, M., et al., *Signalling pathways regulating muscle mass in ageing skeletal muscle: the role of the IGF1-Akt-mTOR-FoxO pathway.* Biogerontology, 2013. **14**(3): p. 303-23.
102. Markofski, M.M., et al., *Effect of age on basal muscle protein synthesis and mTORC1 signaling in a large cohort of young and older men and women.* Exp Gerontol, 2015. **65**: p. 1-7.
103. McPherron, A.C., A.M. Lawler, and S.J. Lee, *Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member.* Nature, 1997. **387**(6628): p. 83-90.
104. Grobet, L., et al., *A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle.* Nat Genet, 1997. **17**(1): p. 71-4.
105. Lee, S.J., *Regulation of muscle mass by myostatin.* Annu Rev Cell Dev Biol, 2004. **20**(1): p. 61-86.
106. McCroskery, S., et al., *Myostatin negatively regulates satellite cell activation and self-renewal.* J Cell Biol, 2003. **162**(6): p. 1135-47.
107. Joulia, D., et al., *Mechanisms involved in the inhibition of myoblast proliferation and differentiation by myostatin.* Exp Cell Res, 2003. **286**(2): p. 263-75.
108. Megeney, L.A., et al., *MyoD is required for myogenic stem cell function in adult skeletal muscle.* Genes Dev, 1996. **10**(10): p. 1173-83.
109. Siriett, V., et al., *Antagonism of myostatin enhances muscle regeneration during sarcopenia.* Mol Ther, 2007. **15**(8): p. 1463-70.
110. McFarlane, C., et al., *Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism.* J Cell Physiol, 2006. **209**(2): p. 501-14.
111. Sartori, R., et al., *Smad2 and 3 transcription factors control muscle mass in adulthood.* Am J Physiol Cell Physiol, 2009. **296**(6): p. C1248-57.
112. Carlson, M.E., M. Hsu, and I.M. Conboy, *Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells.* Nature, 2008. **454**(7203): p. 528-32.
113. Welle, S., et al., *Gene expression profile of aging in human muscle.* Physiol Genomics, 2003. **14**(2): p. 149-59.
114. Ratkevicius, A., et al., *Serum concentrations of myostatin and myostatin-interacting proteins do not differ between young and sarcopenic elderly men.* J Gerontol A Biol Sci Med Sci, 2011. **66**(6): p. 620-6.
115. Passmore, L.A. and D. Barford, *Getting into position: the catalytic mechanisms of protein ubiquitylation.* Biochem J, 2004. **379**(Pt 3): p. 513-25.
116. Lecker, S.H., A.L. Goldberg, and W.E. Mitch, *Protein degradation by the ubiquitin-proteasome pathway in normal and disease states.* J Am Soc Nephrol, 2006. **17**(7): p. 1807-19.
117. Lecker, S.H., et al., *Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression.* FASEB J, 2004. **18**(1): p. 39-51.

118. Cong, H., et al., *Inhibition of atrogin-1/MAFbx expression by adenovirus-delivered small hairpin RNAs attenuates muscle atrophy in fasting mice.* Hum Gene Ther, 2011. **22**(3): p. 313-24.
119. Baehr, L.M., J.D. Furlow, and S.C. Bodine, *Muscle sparing in muscle RING finger 1 null mice: response to synthetic glucocorticoids.* J Physiol, 2011. **589**(Pt 19): p. 4759-76.
120. Tintignac, L.A., et al., *Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase.* J Biol Chem, 2005. **280**(4): p. 2847-56.
121. Lagirand-Cantaloube, J., et al., *The initiation factor eIF3-f is a major target for atrogin1/MAFbx function in skeletal muscle atrophy.* EMBO J, 2008. **27**(8): p. 1266-76.
122. Lokireddy, S., et al., *Myostatin induces degradation of sarcomeric proteins through a Smad3 signaling mechanism during skeletal muscle wasting.* Mol Endocrinol, 2011. **25**(11): p. 1936-49.
123. Rom, O. and A.Z. Reznick, *The role of E3 ubiquitin-ligases MuRF-1 and MAFbx in loss of skeletal muscle mass.* Free Radic Biol Med, 2016. **98**: p. 218-230.
124. Foletta, V.C., et al., *The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy.* Pflugers Arch, 2011. **461**(3): p. 325-35.
125. Stitt, T.N., et al., *The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors.* Mol Cell, 2004. **14**(3): p. 395-403.
126. Brunet, A., et al., *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.* Cell, 1999. **96**(6): p. 857-68.
127. Fuentes, E.N., et al., *The TORC1/P70S6K and TORC1/4EBP1 signaling pathways have a stronger contribution on skeletal muscle growth than MAPK/ERK in an early vertebrate: Differential involvement of the IGF system and atrogenes.* Gen Comp Endocrinol, 2015. **210**: p. 96-106.
128. Cai, D., et al., *Ubiquitin expression is up-regulated in human and rat skeletal muscles during aging.* Arch Biochem Biophys, 2004. **425**(1): p. 42-50.
129. Raue, U., et al., *Proteolytic gene expression differs at rest and after resistance exercise between young and old women.* J Gerontol A Biol Sci Med Sci, 2007. **62**(12): p. 1407-12.
130. Neel, B.A., Y. Lin, and J.E. Pessin, *Skeletal muscle autophagy: a new metabolic regulator.* Trends Endocrinol Metab, 2013. **24**(12): p. 635-43.
131. Mammucari, C., et al., *FoxO3 controls autophagy in skeletal muscle in vivo.* Cell Metab, 2007. **6**(6): p. 458-71.
132. Jung, C.H., et al., *mTOR regulation of autophagy.* FEBS Lett, 2010. **584**(7): p. 1287-95.
133. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-41.
134. Mammucari, C., S. Schiaffino, and M. Sandri, *Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle.* Autophagy, 2008. **4**(4): p. 524-6.
135. Masiero, E. and M. Sandri, *Autophagy inhibition induces atrophy and myopathy in adult skeletal muscles.* Autophagy, 2010. **6**(2): p. 307-9.
136. Nygard, L.A.K., et al., *Nutrition and physical performance in older people-effects of marine protein hydrolysates to prevent decline in physical performance: a randomised controlled trial protocol.* BMJ Open, 2018. **8**(10): p. e023845.

137. Sidhu, K.S., *Health benefits and potential risks related to consumption of fish or fish oil.* Regul Toxicol Pharmacol, 2003. **38**(3): p. 336-44.
138. Hosomi, R., M. Yoshida, and K. Fukunaga, *Seafood consumption and components for health.* Glob J Health Sci, 2012. **4**(3): p. 72-86.
139. Rodriguez-Martin, C., et al., *The Relationship of the Atlantic Diet with Cardiovascular Risk Factors and Markers of Arterial Stiffness in Adults without Cardiovascular Disease.* Nutrients, 2019. **11**(4): p. 742.
140. Gonzalez, C.A. and E. Riboli, *Diet and cancer prevention: Where we are, where we are going.* Nutrition and Cancer-an International Journal, 2006. **56**(2): p. 225-231.
141. Alexandrov, N.V., et al., *Dietary Protein Sources and Muscle Mass over the Life Course: The Lifelines Cohort Study.* Nutrients, 2018. **10**(10): p. 1471.
142. Gray, S.R. and B. Mittendorfer, *Fish oil-derived n-3 polyunsaturated fatty acids for the prevention and treatment of sarcopenia.* Curr Opin Clin Nutr Metab Care, 2018. **21**(2): p. 104-109.
143. Granic, A., et al., *Myoprotective Whole Foods, Muscle Health and Sarcopenia: A Systematic Review of Observational and Intervention Studies in Older Adults.* Nutrients, 2020. **12**(8): p. 2257.
144. Morishima, T., et al., *Muscular endurance and muscle metabolic responses to 8 weeks of omega-3 polyunsaturated fatty acids supplementation.* 2020. **8**(16): p. e14546.
145. Yoshino, J., et al., *Effect of dietary n-3 PUFA supplementation on the muscle transcriptome in older adults.* Physiol Rep, 2016. **4**(11): p. e12785.
146. Petermann-Rocha, F., et al., *Vegetarians, fish, poultry, and meat-eaters: who has higher risk of cardiovascular disease incidence and mortality? A prospective study from UK Biobank.* European Heart Journal, 2020. **42**(12): p. 1136-1143.
147. Jiang, L., et al., *Intake of Fish and Marine n-3 Polyunsaturated Fatty Acids and Risk of Cardiovascular Disease Mortality: A Meta-Analysis of Prospective Cohort Studies.* Nutrients, 2021. **13**(7): p. 2342.
148. Cacabelos, R., et al., *Basic and Clinical Studies With Marine LipoFishins and Vegetal Favalins in Neurodegeneration and Age-Related Disorders*, in *Studies in Natural Products Chemistry*, R. Atta ur, Editor. 2018, Elsevier. p. 195-225.
149. Kerstetter, J.E., K.O. O'Brien, and K.L. Insogna, *Low protein intake: the impact on calcium and bone homeostasis in humans.* J Nutr, 2003. **133**(3): p. 855S-861S.
150. Rondanelli, M., et al., *Novel insights on intake of meat and prevention of sarcopenia: all reasons for an adequate consumption.* Nutricion Hospitalaria, 2015. **32**(5): p. 2136-2143.
151. Zhang, J., et al., *Dietary inclusion of salmon, herring and pompano as oily fish reduces CVD risk markers in dyslipidaemic middle-aged and elderly Chinese women.* Br J Nutr, 2012. **108**(8): p. 1455-65.
152. Daczkowska-Kozon, E.G. and B. Sun Pan, *Environmental Effects on Seafood Availability, Safety, and Quality*. 2016: CRC Press.
153. Domingo, J.L., et al., *Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants.* Toxicology, 2007. **230**(2-3): p. 219-26.
154. Rondanelli, M., et al., *Novel Insights on Intake of Fish and Prevention of Sarcopenia: All Reasons for an Adequate Consumption.* Nutrients, 2020. **12**(2): p. 307.
155. Hernandez, E. and M. Hosokawa, *Omega-3 Oils*. 2011: Elsevier.

156. Schaap, L.A., et al., *Inflammatory markers and loss of muscle mass (sarcopenia) and strength.* Am J Med, 2006. **119**(6): p. 526 e9-17.
157. Bian, A.L., et al., *A study on relationship between elderly sarcopenia and inflammatory factors IL-6 and TNF-alpha.* Eur J Med Res, 2017. **22**(1): p. 25.
158. Burdge, G.C., A.E. Jones, and S.A. Wootton, *Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*.* Br J Nutr, 2002. **88**(4): p. 355-63.
159. Custodero, C., et al., *Evidence-based nutritional and pharmacological interventions targeting chronic low-grade inflammation in middle-age and older adults: A systematic review and meta-analysis.* Ageing Res Rev, 2018. **46**: p. 42-59.
160. Tan, A., et al., *Supplementation with eicosapentaenoic acid and docosahexaenoic acid reduces high levels of circulating proinflammatory cytokines in aging adults: A randomized, controlled study.* Prostaglandins Leukot Essent Fatty Acids, 2018. **132**: p. 23-29.
161. Smith, G.I., et al., *Fish oil-derived n-3 PUFA therapy increases muscle mass and function in healthy older adults.* Am J Clin Nutr, 2015. **102**(1): p. 115-22.
162. Albert, B.B., et al., *Higher omega-3 index is associated with increased insulin sensitivity and more favourable metabolic profile in middle-aged overweight men.* Sci Rep, 2014. **4**(1): p. 6697.
163. Wolfe, R.R., S.L. Miller, and K.B. Miller, *Optimal protein intake in the elderly.* Clin Nutr, 2008. **27**(5): p. 675-84.
164. Calvani, R., et al., *Current nutritional recommendations and novel dietary strategies to manage sarcopenia.* J Frailty Aging, 2013. **2**(1): p. 38-53.
165. Laviano, A., C. Gori, and S. Rianda, *Chapter Three - Sarcopenia and Nutrition*, in *Advances in Food and Nutrition Research*, J. Henry, Editor. 2014, Academic Press. p. 101-136.
166. Tahergorabi, R., S.V. Hosseini, and J. Jaczynski, *Seafood proteins*, in *Handbook of Food Proteins*, G.O. Phillips and P.A. Williams, Editors. 2011, Woodhead Publishing. p. 116-149.
167. Fleurence, J., M. Morançais, and J. Dumay, *Seaweed proteins*, in *Proteins in Food Processing*, R.Y. Yada, Editor. 2018, Woodhead Publishing. p. 245-262.
168. Kawabata, F., et al., *Fish protein intake induces fast-muscle hypertrophy and reduces liver lipids and serum glucose levels in rats.* Biosci Biotechnol Biochem, 2015. **79**(1): p. 109-16.
169. Wu, G., et al., *Dietary requirements of "nutritionally non-essential amino acids" by animals and humans.* Amino Acids, 2013. **44**(4): p. 1107-13.
170. Koopman, R., et al., *Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein.* Am J Clin Nutr, 2009. **90**(1): p. 106-15.
171. Deutz, N.E., et al., *Effect of beta-hydroxy-beta-methylbutyrate (HMB) on lean body mass during 10 days of bed rest in older adults.* Clin Nutr, 2013. **32**(5): p. 704-12.
172. Pasantes-Morales, H., et al., *Taurine content in foods.* Nutr Rep Int, 1989. **40**: p. 793-801.
173. Renganathan, M., M.L. Messi, and O. Delbono, *Dihydropyridine receptor-ryanodine receptor uncoupling in aged skeletal muscle.* J Membr Biol, 1997. **157**(3): p. 247-53.

174. Dutka, T.L., et al., *Acute effects of taurine on sarcoplasmic reticulum Ca2+ accumulation and contractility in human type I and type II skeletal muscle fibers.* J Appl Physiol (1985), 2014. **117**(7): p. 797-805.
175. Puzianowska-Kuznicka, M. and J. Kuznicki, *The ER and ageing II: calcium homeostasis.* Ageing Res Rev, 2009. **8**(3): p. 160-72.
176. Huxtable, R.J., *Physiological actions of taurine.* Physiol Rev, 1992. **72**(1): p. 101-63.
177. Charge, S.B. and M.A. Rudnicki, *Cellular and molecular regulation of muscle regeneration.* Physiol Rev, 2004. **84**(1): p. 209-38.
178. Scicchitano, B.M. and G. Sica, *The Beneficial Effects of Taurine to Counteract Sarcopenia.* Curr Protein Pept Sci, 2018. **19**(7): p. 673-680.
179. Kim, S.-Y., et al., *Enzymatic Hydrolysates of Hippocampus abdominalis regulates the skeletal muscle growth in C2C12 Cells and Zebrafish Model.* 2019. **28**(3): p. 264-274.
180. Muthuramalingam, K., et al., *Bigbelly seahorse (Hippocampus abdominalis)-derived peptides enhance skeletal muscle differentiation and endurance performance via activated P38MAPK/AKT signalling pathway: An in vitro and in vivo analysis.* Journal of Functional Foods, 2019. **52**: p. 147-155.
181. Seo, M.H., et al., *The association between daily calcium intake and sarcopenia in older, non-obese Korean adults: the fourth Korea National Health and Nutrition Examination Survey (KNHANES IV) 2009.* Endocr J, 2013. **60**(5): p. 679-86.
182. Berchtold, M.W., H. Brinkmeier, and M. Muntener, *Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease.* Physiol Rev, 2000. **80**(3): p. 1215-65.
183. Cinar, V., et al., *Effects of magnesium supplementation on testosterone levels of athletes and sedentary subjects at rest and after exhaustion.* Biol Trace Elem Res, 2011. **140**(1): p. 18-23.
184. Hayhoe, R.P.G., et al., *Cross-sectional associations of dietary and circulating magnesium with skeletal muscle mass in the EPIC-Norfolk cohort.* Clin Nutr, 2019. **38**(1): p. 317-323.
185. Orndahl, G., A. Rindby, and E. Selin, *Myotonic dystrophy and selenium.* Acta Med Scand, 1982. **211**(6): p. 493-9.
186. Balsom, P.D., et al., *Skeletal muscle metabolism during short duration high-intensity exercise: influence of creatine supplementation.* Acta Physiol Scand, 1995. **154**(3): p. 303-10.
187. Derave, W., et al., *Combined creatine and protein supplementation in conjunction with resistance training promotes muscle GLUT-4 content and glucose tolerance in humans.* J Appl Physiol (1985), 2003. **94**(5): p. 1910-6.
188. Kley, R.A., M.A. Tarnopolsky, and M. Vorgerd, *Creatine for treating muscle disorders.* Cochrane Database Syst Rev, 2013(6): p. CD004760.
189. Santos, R.V., et al., *The effect of creatine supplementation upon inflammatory and muscle soreness markers after a 30km race.* Life Sci, 2004. **75**(16): p. 1917-24.
190. Penalver, R., et al., *Seaweeds as a Functional Ingredient for a Healthy Diet.* Mar Drugs, 2020. **18**(6): p. 301.
191. Ganapathy, A. and J.W. Nieves, *Nutrition and Sarcopenia-What Do We Know?* Nutrients, 2020. **12**(6): p. 1755.

192. Liu, P.H., et al., *The astaxanthin-induced improvement in lipid metabolism during exercise is mediated by a PGC-1alpha increase in skeletal muscle.* J Clin Biochem Nutr, 2014. **54**(2): p. 86-9.
193. Vilter, R.W., et al., *The effect of vitamin B6 deficiency induced by desoxypyridoxine in human beings.* J Lab Clin Med, 1953. **42**(3): p. 335-57.
194. Bjornsen, T., et al., *Vitamin C and E supplementation blunts increases in total lean body mass in elderly men after strength training.* Scand J Med Sci Sports, 2016. **26**(7): p. 755-63.
195. Bischoff, H.A., et al., *Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial.* J Bone Miner Res, 2003. **18**(2): p. 343-51.
196. Oh, J.H., et al., *The level of vitamin D in the serum correlates with fatty degeneration of the muscles of the rotator cuff.* J Bone Joint Surg Br, 2009. **91**(12): p. 1587-93.
197. Bischoff-Ferrari, H.A., et al., *Vitamin D receptor expression in human muscle tissue decreases with age.* J Bone Miner Res, 2004. **19**(2): p. 265-9.
198. Kim, S.-Y., et al., *Diphlorethohydroxycarmalol (DPHC) Isolated from the Brown Alga Ishige okamurae Acts on Inflammatory Myopathy as an Inhibitory Agent of TNF-α.* 2020. **18**(11): p. 529.
199. Yang, H.-W., et al., *Ca²⁺-Dependent Glucose Transport in Skeletal Muscle by Diphlorethohydroxycarmalol, an Alga Phlorotannin: In Vitro and In Vivo Study*. Oxidative Medicine and Cellular Longevity, 2021. **2021**: p. 8893679.

Part II: *Ishige okamurae*, diphloroethohydroxycarmalol, and ishophloroglucin A inhibit palmitic acid-impaired skeletal myogenesis and improve muscle regenerative potential

2.1 Introduction

Sarcopenia, defined as the loss of muscle mass and strength with increasing age, leads to disability, frailty, morbidity, and mortality. Anorexia and cachexia lead to the loss of muscle mass. Besides decreased strength, the loss of muscle mass also negatively affects aerobic capacity and metabolic rate, ultimately reducing function and quality of life [1, 2]. Obesity, another health concern, is defined as abnormal and excessive body fat. In recent years, the prevalence of obese individuals in both middle and old age has increased. Similar to sarcopenia, the risk of obesity increases with age [3]. The emerging and challenging epidemiological trends in developing countries include sarcopenia and obesity. The prevalence of obesity combined with sarcopenia is referred to as sarcopenic obesity/ obese-sarcopenia, which exhibits synergistic complications of both syndromes [4].

Obesity causes substantial changes in fat metabolism, leading to fat deposition in non-adipose tissues such as skeletal tissue [5]. The development of obesity is associated with high-lipid diets, predominantly saturated fatty acids such as palmitic acid (16:0, PA). PA is the most common saturated fatty acid in the human body [6]. Excess fatty acid infiltration into skeletal tissues alters metabolic signaling. Moreover, growth molecular signaling and catabolic pathways that support the proper functioning of skeletal cells are also affected.

Obese-sarcopenia is a silent, progressive condition that is intrinsically related to aging. While the pathological condition is linked to a decline in quality of life. However, the condition is largely undiagnosed, which has negative consequences [7]. Obese-sarcopenia is a growing concern and a challenging pathogenesis condition among aging populations as a result of modern-day lifestyle and food habits. Obese-sarcopenia

management is considered complicated due to its multifactorial nature. Given their physical disability and unwillingness to change their lifestyle, age-related diseases in the elderly are difficult to reverse. Thus, preventing obese-sarcopenia is unquestionably more rational than treating it. Proper diet and exercise are the cornerstones of obese-sarcopenia prevention measurements. These measures are expected to reduce the prevalence of obese-sarcopenia [8]. Individuals with obese-sarcopenia have greater risks of metabolic disorders compared to those for sarcopenia or obesity alone. Strong and healthy muscles are vital for the handling of overload created by obesity. Skeletal muscle myogenesis is of the utmost importance in maintaining muscle mass and regenerative capacity. However, lipid overload disturbs skeletal myogenesis. Recent research by Paixao et al. demonstrated that PA impaired myogenesis and changed microRNA expression in C2C12 myoblasts [9].

Plant species that grow in the marine environment; namely, seaweeds or sea vegetables, have become an emerging sustainable nutritional source and an essential part of healthy human diets, mostly in the East Asian region. They are a good source of vitamins and minerals, fiber, and polysaccharides that support low caloric intake [10]. Furthermore, seaweeds contain pigments, proteins, polyunsaturated fatty acids (PUFAs), and phenolic compounds. Bioactive components such as fucoxanthin have potential anti-obesity effects. Meada et al. described *Undaria pinatifida*, an edible brown seaweed, as a source of fucoxanthin with anti-obesity effects through the expression of mitochondrial uncoupling protein 1 white adipose tissue [11]. Polyphenols are an important component of marine algae (brown algae) that provide functional food and pharmaceutical significance. However, studies reporting their use as interventions in muscle-related research are limited. Among them, diphloroethohydoxycarmalol (DPHC) isolated from *Ishige okamurae* reportedly showed the potential to inhibit inflammatory myopathy [12].

Moreover, DPHC promoted cytosolic Ca^{2+}, resulting in increased glucose transport to myotubes to modulate blood glucose levels [13]. Ishophloroglucin A (IPA) is a phlorotannin purified from marine brown alga *Ishige okamurae*, reported lately to serve as a natural product intervention for disorders such as glucose homeostasis in muscle high fat diet [14], high glucose induced angiogenesis [15], and peripheral metabolism [16]. However, its association with IGF-1R driven PI3K/Akt/mTOR pathway in C2C12 myotubes remains unclear. The study evaluates association of PA in the myotubes formed via *in vitro* serum deprivation and the negative effects of fatty acids on skeletal muscle. The myocellular mechanisms in skeletal myogenesis are researched to understand its implications. Current research broadened the knowledge horizon to fill the gaps of lipid induced-sarcopenia understanding and to compete against it with prospective functional supplements through the sustainable utilization of marine natural products specifically brown alga *Ishige okamurae*, and its bioactive components IO, DPHC, and IPA.

2.2 Material and methods

2.2.1 Reagents

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Growth media (Dulbecco's modified Eagle's medium; DMEM) for cell culture was obtained from GIBCO, Inc. (Grand Island, NY, USA). C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, VA, USA). The growth medium was supplemented with serum (fetal bovine serum [FBS]/horse serum [HS]) and antibiotics (penicillin and streptomycin) from GIBCO, Inc. Primary and secondary antibodies used for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG H&L (Alexa Fluor ® 488; ab150113) and goat anti-rabbit IgG H&L (Alexa Fluor ® 647; ab150079) were obtained from Abcam (Cambridge, UK). Primers for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were obtained from Bioneer (Daejeon, Korea). A Millipore (Burlington, MA, USA) system was used to measure cell proliferation.

2.2.2 Obtaining 70% ethanol extract from Ishige okamurae

Marine algae species *Ishige okamurae, Sargassum thunbergii, Ecklonia cava, Sargassum autumnale,* and *Padina arborescens* were collected from the shores of Jeju Island in April 2020. The samples were immediately washed with running water to remove epiphytes, lyophilized, and ground into a powder. A 100 g portion of each powder was extracted into 70% ethanol under shaking kinetics and repeated three times. The filtrates were collected at each time point and evaporated under vacuum to obtain *I. okamurae* (IO), *S. thunbergii* (ST), *E. cava* (EC), *S. autumnale* (SA), and *P. arborescens* (PAr) 70% ethanol extracts.

2.2.3 The isolated compounds from Ishige okamurae

DPHC was purified as previously described [17]. The identification and structural elucidation were performed as described by Fernando et al. (2018) [18]. IPA was purified as previously described [15]. The identification and structural elucidation were performed as described by Ryu et al. (2018) [19].

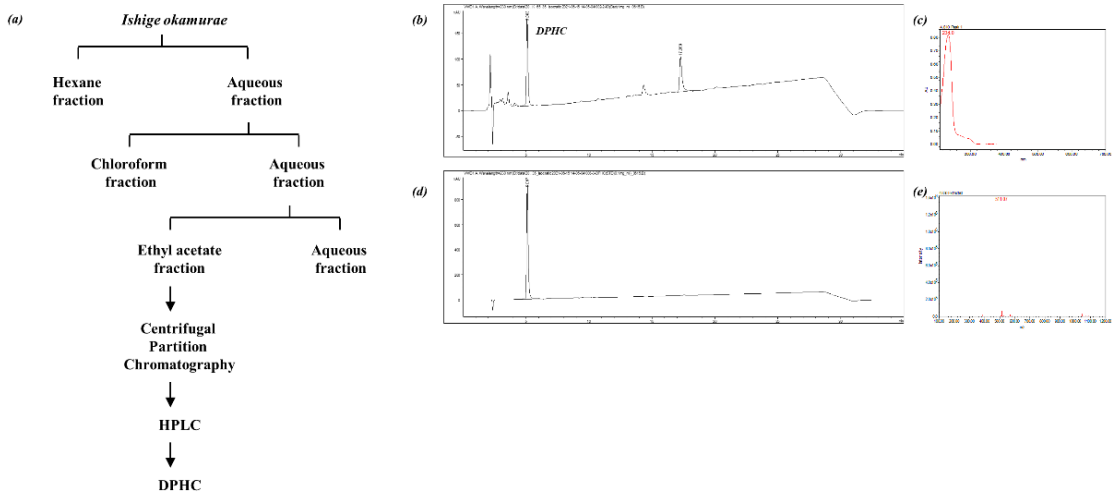

Figure 2- 1. *Ishige okamurae ethanol extract (IOE) and purified diphloroethohydoxycarmalol (DPHC) analysis data. (**a**) Sketch diagram for DPHC purification procedure. (**b**) HPLC chromatogram at 230 nm of IOE, (**c**) UV spectrum for DPHC peak, (**d**) HPLC chromatogram at 230 nm of DPHC, and (**e**) mass spectrum of DPHC. The system was equipped with a Poroshell column (120 C18, 4.6*100 mm, 4µm). Mobile phase: A; DW (+0.1% Formic acid), B; ACN (+0.1% Formic acid). Method: 0min A; 80% B; 20%, 0-25min A; 60% B; 40%, 25-27min A; 80% B; 20%, 27-35min A; 80% B; 20%, injection volume: 10 µL, flow rate: 0.5 ml/min.*

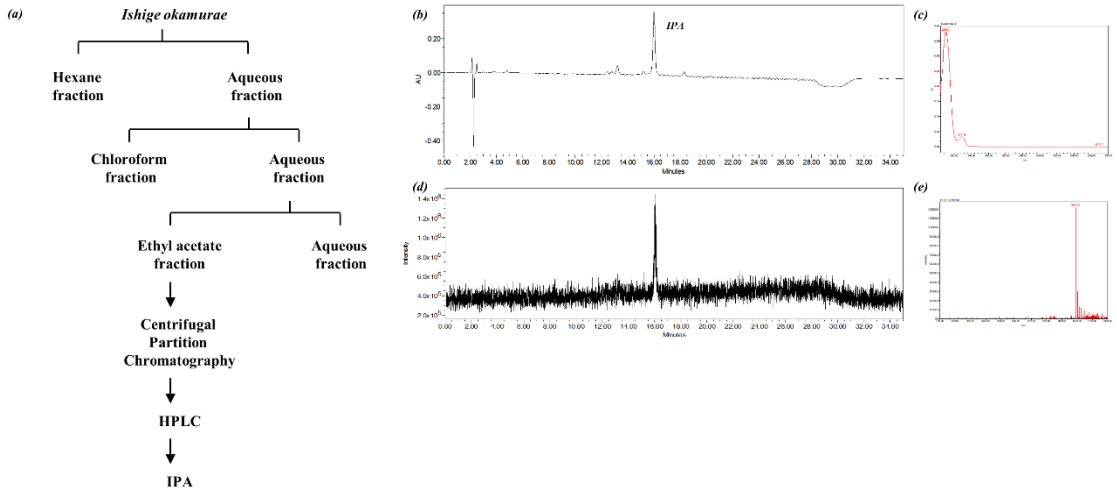

Figure 2- 2. *Ishige okamurae ethanol extract (IOE) and purified ishophloroglucin (IPA) analysis data. (a) Sketch diagram for IPA purification procedure. (b) HPLC chromatogram at 230 nm of IPA, (c) UV spectrum for IPA peak, (d) HPLC chromatogram QDA negative TIC, and (e) mass spectrum of IPA. The system was equipped with a Poroshell column (120 C18, 4.6*100 mm, 4µm). Mobile phase: A; DW (+0.1% Formic acid), B; ACN (+0.1% Formic acid). Method: 0min A; 80% B; 20% , 0-25min A; 60% B; 40%, 25-27min A; 80% B; 20%, 27-35min A; 80% B; 20%, injection volume: 10 µL, flow rate: 0.5 ml/min.*

2.2.4 Muscle cell culture and myotube formation

Myoblasts were cultured and plated in DMEM supplemented with 10% FBS and antibiotics. Differentiation was initiated once the cells had reached 80% confluence by changing the medium to DMEM containing 2% HS and antibiotics. The experimental models were designed, and the cells were stimulated at each required timeline, as appropriate. Myotubes were observed 3–4 days post-differentiation. The cells were maintained in a Sanyo incubator (Sanyo MCO-18AIC; Moriguchi, Japan) at 37°C in a humidified atmosphere with 5% CO_2.

2.2.5 Experimental treatments

IO, DPHC, and IPA were first dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and diluted in serum-free DMEM to obtain working standards. Palmitic acid was dissolved in 2-propanol and diluted in serum-free DMEM containing 2% BSA (FA-free) [20]. These were treated according to the experimental plan at different time points. Palmitic acid stimulation was conducted for up to 16–24 h, where the IO extract and DPHC, IPA were added every other day following media changes.

2.2.6 Determining optimal palmitate and compound concentrations

The cytotoxicity of each sample and palmitic acid was assessed in myoblasts using the MTT assay [21]. First, 96-well plates were seeded at a cell concentration of 1×10^5 cells mL^{-1}. The samples were treated after a 24-hour incubation period and further incubated for 24 hours. The 70% ethanol extracts and DPHC, IPA of each sample were considered in multiple ranges and treated in the wells. The assay results were obtained at an optical

density of 540 nm [22]. Palmitic acid was used at concentrations of 0.1, 0.3, 0.5, and 0.75 mM.

The 5-bromo-2´-deoxyuridine (BrdU) assay was used to evaluate cell proliferation effects of the samples on C2C12 myoblasts and their transition to myotubes. The cells were plated in 48-well plates at 1×10^5 cells·mL^{-1}. Differentiation was induced by switching media to 2% HS in DMEM and changing the media every other day until day six. Each time the media was changed, the samples were treated. Cell proliferation was determined at each time point by BrdU assay according to the manufacturer's instructions. Briefly, the cells were fixed and washed. A BrdU monoclonal antibody was added to each well, followed by incubation and washing. Goat anti-mouse IgG was added and incubated. The TMB substrate was then attached to this substrate and a stop solution was added to terminate the reaction. The readings were obtained at 450 nm and the control group was compared to the treated groups [23, 24]. Based on the results obtained, subsequent experiments were planned.

2.2.7 Western blotting

Cells were harvested depending on the protein analysis and the effect of the sample. Briefly, the cells were harvested, lysed, and the proteins were measured by BCA protein assay. The normalized proteins were separated by electrophoresis on 8% or 10% sodium dodecyl sulfate-polyacrylamide gels. The proteins were then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with skim milk and then probed with primary antibodies overnight at 4°C. Secondary antibody incubation was conducted, and membranes were developed with enhanced chemiluminescence (ECL) substrate (Cyanagen Srl, Bologna, Italy). Images were

captured using a FUSION SOLO Vilber Lourmat system (Paris, France). Quantification was performed using ImageJ software (version 1.50i, NIH, USA) [25, 26].

2.2.8 Immunofluorescence

Gelatin-coated coverslips were used to grow C2C12 myoblasts. The samples were treated, and the media was changed accordingly to proceed with differentiation. The cells were fixed and stained at different times, depending on the experimental model. Paraformaldehyde (4%) was used to fix the cells. The cells were then permeabilized with 0.1% Triton X-100. The cells were blocked with 10% donkey serum and incubated with primary antibody (1:100 in 10% donkey serum) for 1 h at ambient temperature. Alexa Fluor ® 488 and/or Alexa Fluor ® 647 conjugated secondary antibodies were used, followed by 4′,6-diamidino-2-phenylindole (DAPI) staining. The stained coverslips were mounted onto glass slides with Dako Faramount Aqueous Mounting Media (Agilent Technologies, Santa Clara, CA, USA). Images were captured using a confocal microscope (Carl Zeiss, Oberkochen, Germany). Myotube lengths were calculated using ImageJ (version 1.50i, NIH, USA), the averages of multiple randomly chosen fields from three independent experiments [27, 28]. The fusion index was calculated as the percentage of nuclei inside the myotubes compared to the total number of nuclei. Only myotubes with at least three nuclei inside a continuous cell membrane were considered.

2.2.9 RT-qPCR

The cells were seeded, the media was changed, the samples were treated, and stimulation was conducted as described above. The cells were harvested and the total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer’s

instructions. RNA content was measured using a μDrop plate (Thermo Scientific, Waltham, MA, USA), and 2 μg of RNA was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (TaKaRa, Shiga, Japan). The primers used in the experiment are listed in Table 1. The PCR cycling conditions were as described by [29]. Livak and Schmittgen (2001) implemented a scheme to quantify the relative gene expression [30]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the relative gene expression levels.

2.2.10 Statistical analysis

The experiments were performed in triplicate to obtain the final data. Data are expressed as means ± standard deviation. One-way analysis of variance (ANOVA) using Tukey's test, was used to evaluate statistical significance. Two-tailed Student's *t*-tests were performed when two conditions were compared. The significance level was defined as $p < 0.05$ ($p<0.05$). GraphPad Prism 7 (GraphPad, CA, USA) was used to perform the statistical analyses. Furthermore, each figure legend contains the statistical conditions.

2.3 Results

2.3.1 Potential of IO extract

Sample extracts (70% EtOH) of multiple marine algae were used to treat C2C12 myoblasts. Cell viability was measured via MTT assay after 48 h. As shown, in Figure 2-3a, C2C12 myoblasts samples treated at concentrations of ≤25 μg/mL did not show cytotoxicity. However, the proliferation analysis via BrdU assay in differentiated myoblasts showed a significant effect in the IO sample compared to the other samples (Figure 2-3b). Both ST and SA exhibited negative behavior in the lower concentration ranges, while ST increased proliferation only at its highest concentration. Thus, IO (25 μg/mL) was chosen for subsequent experiments according to its toxicity and proliferation potential results.

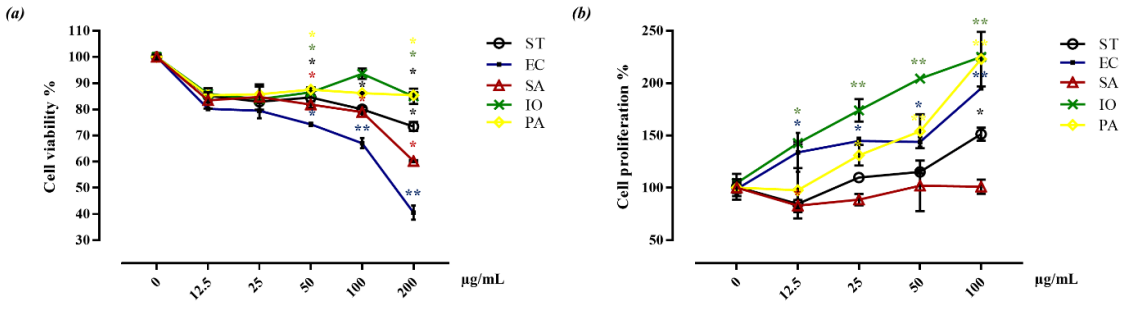

Figure 2- 3. *Assessment of 70% EtOH extracts. (a) Cell viability analysis (48 h) of C2C12 myoblasts with the treatment of 70% EtOH extracts of multiple brown algae species by MTT assay. (b) Cell proliferation analysis via BrdU assay in differentiated myotubes treated with samples extracts. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Each color code denotes individual sample. Sargassum thunbergii (ST), Ecklonia cava (EC), Sargassum autumnale (SA), Ishige okamurae (IO), Padina arborescens (PAr)*

2.3.2 Palmitate inhibits skeletal muscle differentiation and myogenic regulatory factor expression

Palmitic acid is the most common saturated fatty acid found in animals, plants, and microorganisms. It has long been negatively depicted for its detrimental health effects related to multiple crucial physiological activities. Palmitic acid accounts for 20–30% of total fatty acids in the human body and can be provided in the diet or synthesized endogenously [6]. The cytotoxicity of palmitic acid was evaluated via MTT assay after 48 hrs for a range of concentrations. The results showed a half-maximal inhibitory concentration (IC50) of 0.55 mM. Thus, a concentration of 0.1 mM (100 μM) was used in subsequent experiments based on the evidence that PA did not impair myoblast survival (Figure 2-4a).

2.3.3 Effect of palmitate on myoblast proliferation

The effect of palmitate on myoblast proliferation was assessed on days 0–6 alongside the IO and co-treated samples. IO increased C2C12 myoblast proliferation, whereas PA inhibited it. However, IO co-treatment ameliorated the negative effects of PA (Figure 2-4b).

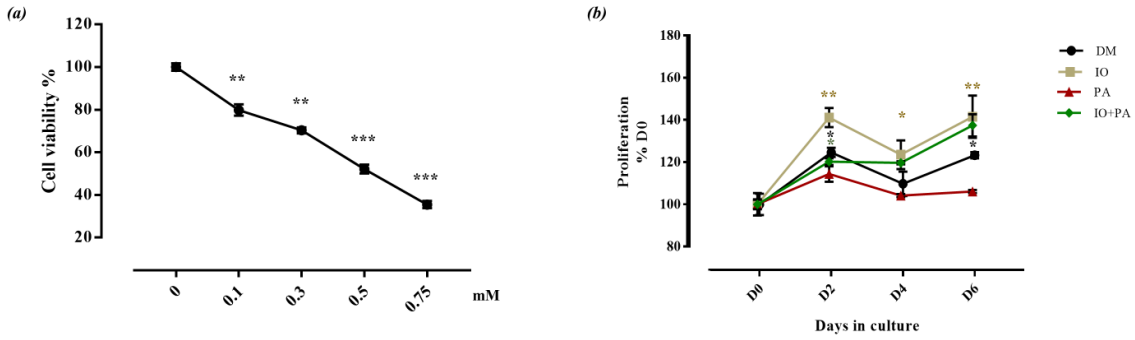

Figure 2- 4. *Assessment of palmitic acid (PA) in myoblasts/myotubes. (a) Cell viability of C2C12 myoblasts with different concentrations of palmitic acid. (b) Proliferation rate in days in culture. C2C12 cells were treated with 70% EtOH extract of IO, palmitic acid, and co-treatment, evaluating its proliferating potential. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Each color code denotes individual sample.*

2.3.4 Palmitate affects skeletal muscle differentiation

We evaluated myogenic markers to confirm that palmitate altered the myogenesis process. As shown in Figure 2-5a-c, myogenin expression was assessed by immunofluorescence after treating the myoblasts with palmitic acid. Palmitic acid reduced the percentage of myogenin-positive cells in differentiating myotubes, altering their differentiation lineage. Consistent with these results, analysis of myosin heavy chain (MyHC) and myoblast determination protein 1 (MyoD) in the differentiated myotubes revealed significantly lower levels in the palmitic acid-treated group compared to the non-treated group. However, the effect of palmitic acid was successfully overcome by co-treatment with IO.

Further, IO was a potent stimulator of myogenesis and an effective inhibitor of the negative effects of palmitic acid. As shown in Figure 2-6a, palmitic acid inhibited the relative expression of MyHC (Figure 2-6b) and palmitic acid significantly downregulated MyoD (Figure 2-6c), a central regulator of myogenesis. IO treatment successfully restored the expression of myogenic regulators. As shown in Figure 2-5d, IO treatment enhanced the fusion index. The increased number of nuclei inside the myotube explained the potential improvement in myogenesis. The fusion index value of the palmitic acid-treated group was significantly lower than that of the control group, indicating that palmitic acid altered myogenesis and, therefore, inhibited the expression of myogenic markers such as MyHC and MyoD.

Moreover, myogenic marker levels were assessed by western blotting using a time-course (Figure 2-7). Myogenin (Figure 2-7a, b), MyoD (Figure 2-7c, d), and MyHC (Figure 2-7e, f) expression was inhibited by palmitic acid treatment on each day of analysis, while IO showed a potential positive regulatory action compared to the non-treated group. Hence, myoblasts induced to differentiate with serum deprivation were impaired by

palmitic acid due to its potential to alter myogenic marker levels. Overall, these data indicated that palmitic acid impaired myoblast differentiation to myotubes, while IO improved myogenesis.

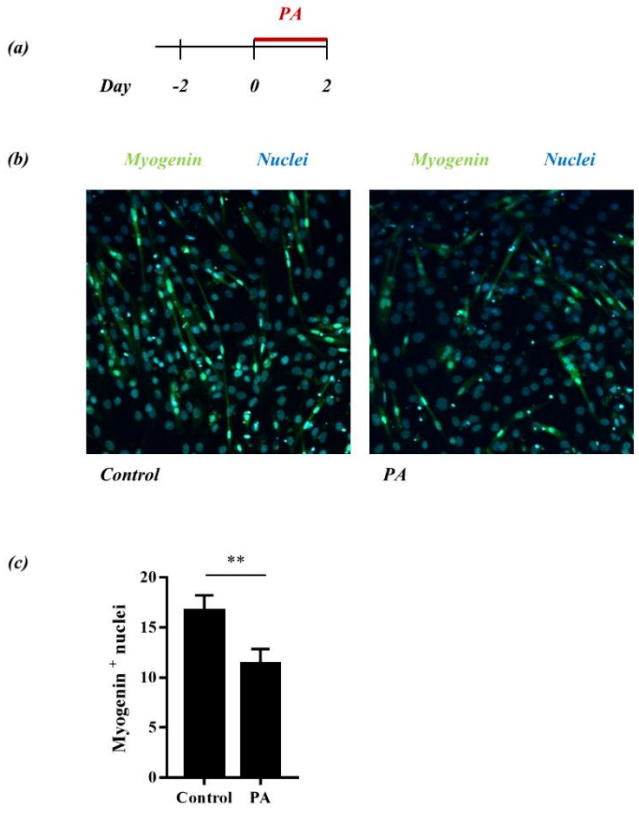

Figure 2- 5. *Myogenin expression in myoblasts. (a) Experimental model involved in myoblast seeding and PA treatment. (b) representative immunofluorescence analysis of C2C12 myoblasts after 48 h of PA treatment in differentiation medium shows nuclear localization of myogenin. (c) Quantitative expression of myogenin positive nuclei. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$.*

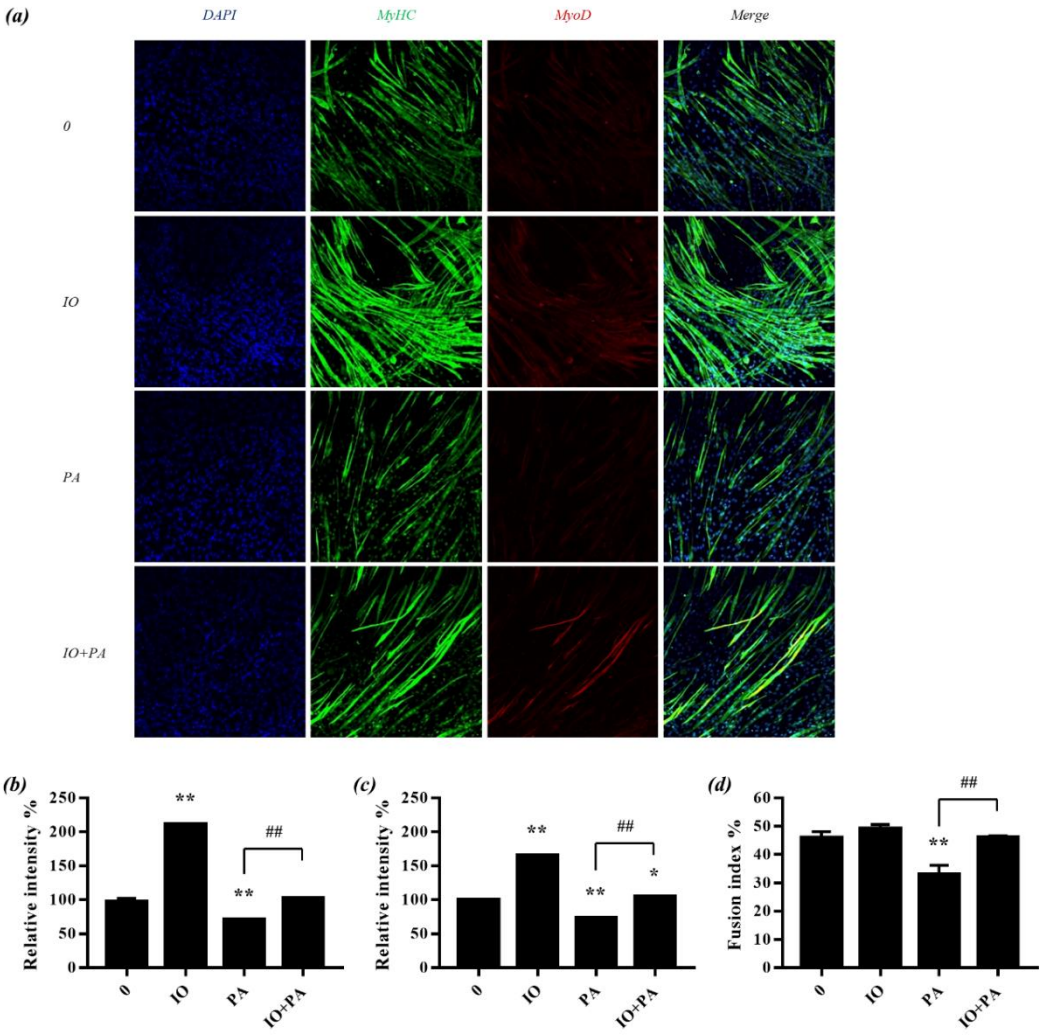

Figure 2- 6. *Immunofluorescence analysis of selected proteins. (a) Immunofluorescence microscopy for the expression of myogenic markers (b) Myosin heavy chain (MyHC) and (c) MyoD in day 6. (d) Fusion index, represented as the % nuclei inside myotubes compared to the total number of nuclei. Cells were induced to differentiate via serum deprivation in differentiation medium. IO, PA and co-treatment of IO+PA was conducted. Relative intensities of each protein expression and fusion index were analyzed by ImageJ software. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance among PA and IO+PA is denoted as # $p < 0.05$, ## $p < 0.01$.*

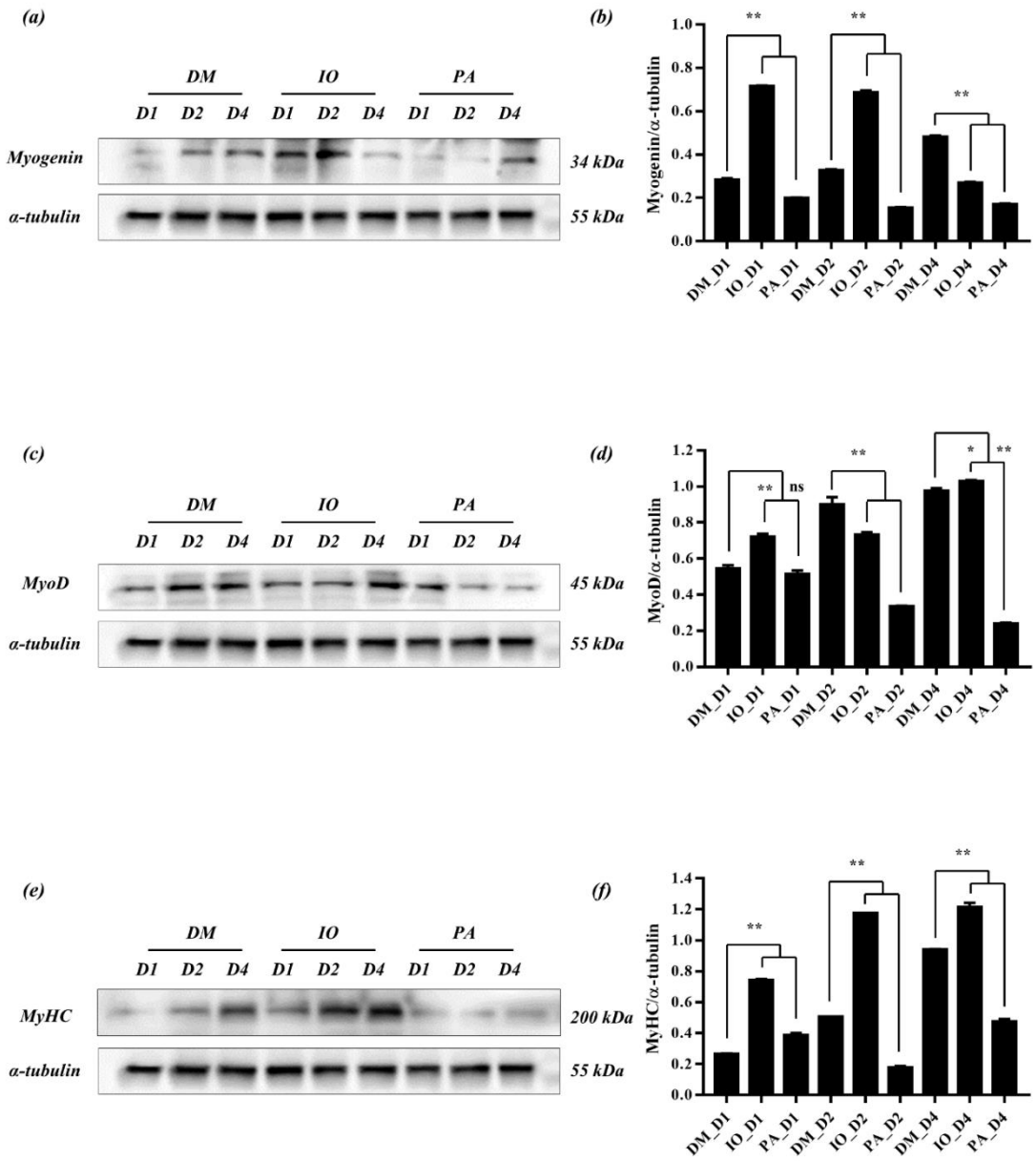

Figure 2- 7. *Evaluation of myogenic markers in differentiating myoblasts through myotubes. Western blotting analysis of (a) MyoD, (c) Myogenin, and (c) MyHC expression. Cells were induced to differentiate via serum deprivation in differentiation medium. IO and/or PA treatments were conducted. ImageJ software was used to analyze intensities and respective quantitative results are showed in (b), (d), and (f) . The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control of each day analyzed. * $p < 0.05$, ** $p < 0.01$.*

2.3.5 DPHC, IPA effects on myoblast and myotubes

Given the potential of IO to ameliorate the negative effects of palmitic acid, we assessed the cytotoxic effects of DPHC, an isolated purified component of IO. DPHC showed toxicity at concentrations >25 µg/mL (Figure 2-8a). Cell proliferation examined by BrdU assay revealed that DPHC exhibited significant potential (Figure 2-8b). As shown in Figure 2-8c, DPHC improved PA-altered proliferation of C2C12 myoblasts compared to the non-treated group. IPA expressed similar effects against PA stimulation though the optimum concentration was different from DPHC (Figure 2-9). Thus, DPHC (25 µM) and IPA (12.5 µM) were assessed for its potential to improve myogenesis and overcome the negative effects of PA.

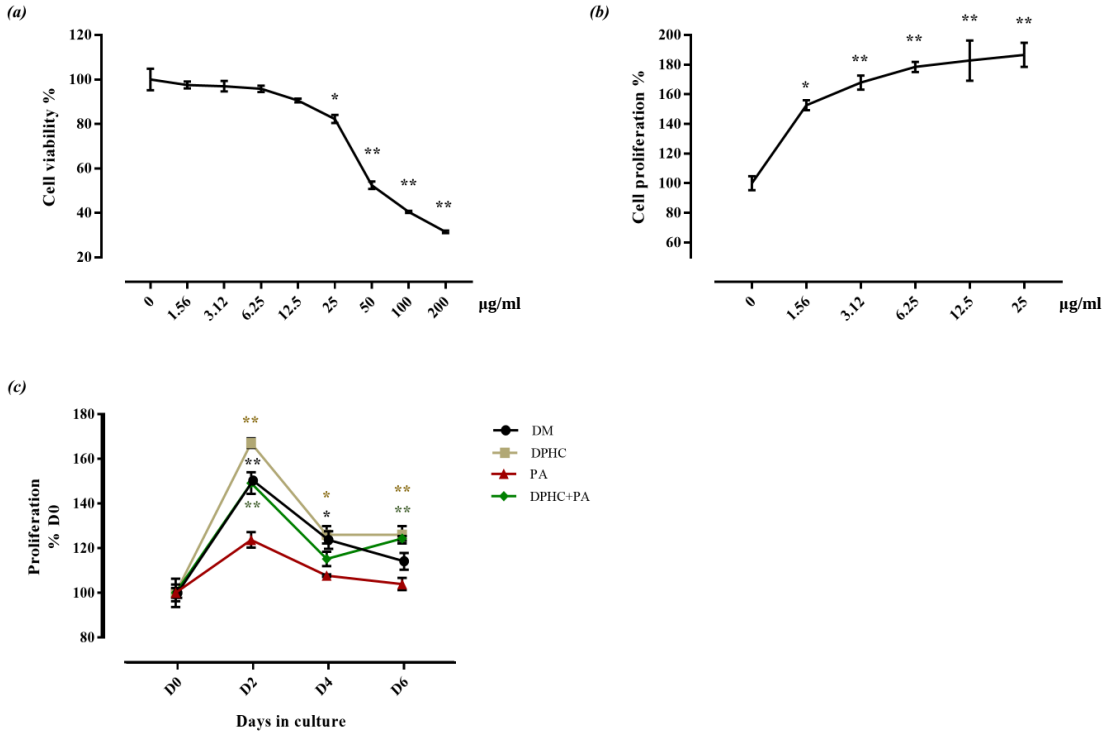

Figure 2- 8. *Assessment of DPHC in C2C12 myoblasts and myotubes. (a) Cell viability analysis (48 h) of C2C12 myoblasts with the treatment of DPHC by MTT assay. (b) Cell proliferation analysis via BrdU assay in differentiated myotubes treated with samples. (c) Proliferation rate in days in culture; C2C12 cells were treated with DPHC, palmitic acid, and co-treatment, evaluating its proliferating potential. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Each color code denotes individual sample.*

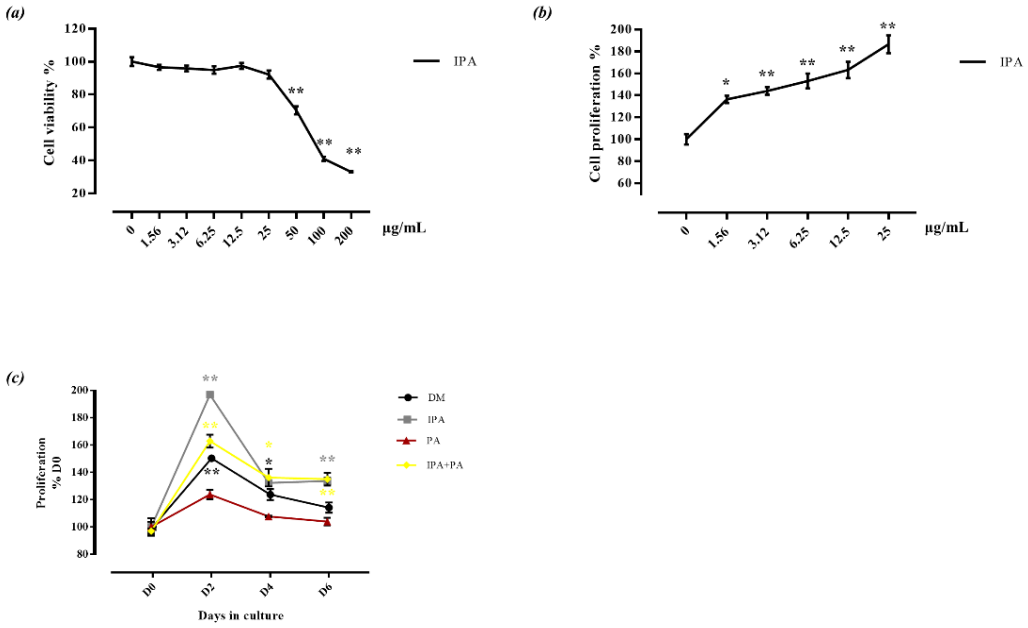

Figure 2- 9. *Assessment of IPA in C2C12 myoblasts and myotubes. (**a**) Cell viability analysis (48 h) of C2C12 myoblasts with the treatment of IPA by MTT assay. (**b**) Cell proliferation analysis via BrdU assay in differentiated myotubes treated with samples. (**c**) Proliferation rate in days in culture; C2C12 cells were treated with IPA, palmitic acid, and co-treatment, evaluating its proliferating potential. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Each color code denotes individual sample.*

2.3.6 DPHC, IPA, and PA behavior in myotubes

The expression levels of myogenic marker genes were evaluated in C2C12 myotubes to assess the effects of palmitic acid, DPHC, and IPA. Myostatin, a negative regulator of myogenesis, was upregulated with palmitic acid treatment, whereas co-treatment with DPHC successfully repressed this effect. Myostatin levels did not change significantly compared to the control group with DPHC treatment alone (Figure 2-10a). Although MyoD (Figure 2-10b) and myogenin (Figure 2-10c) showed similar trends, their expression levels differed in each group. MyoD levels differed significantly between the control and palmitic acid-treated groups. In contrast, while myogenin levels declined due to palmitic acid treatment, the difference was not statistically significant. Thus, palmitic acid should work through MyoD, not myogenin, under the given conditions.

In contrast, DPHC significantly improved myogenin expression (**) compared to MyoD. Nevertheless, DPHC treatment overcame the effects of palmitic acid. As phosphorylated Smad3 inhibits MyoD transcription in the nucleus, Smad3 phosphorylation was evaluated. It exhibited stimulatory behavior following palmitic acid treatment (Figure 2-10d). DPHC alone suppressed protein phosphorylation. DPHC downregulated the effect of palmitic acid. Similar trends were observed with the IPA (Figure 2-11).

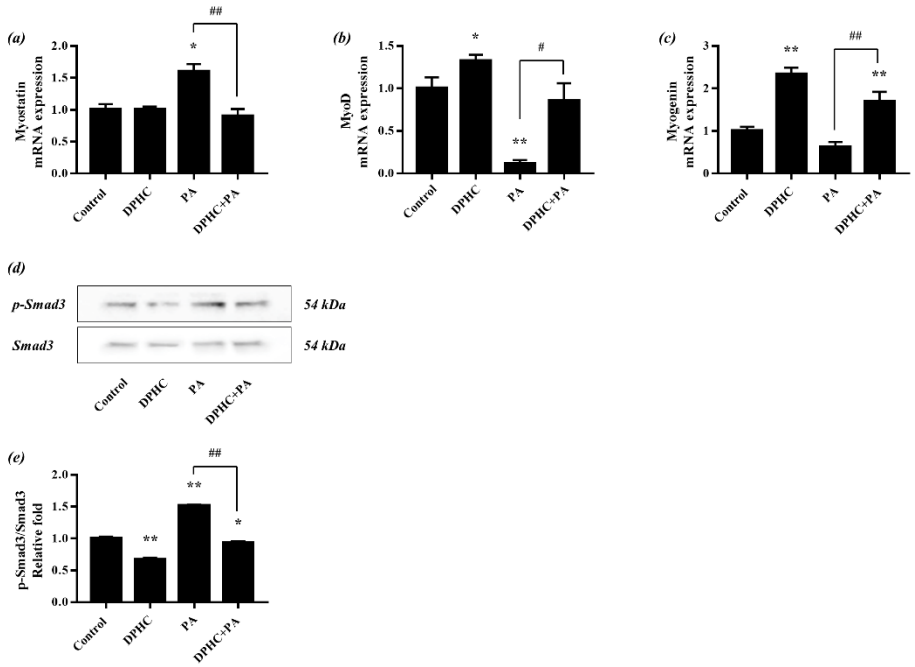

Figure 2- 10. *Assessment of DPHC on myogenic regulators. Expression of mRNA levels of (a) Myostatin, (b) MyoD, and (c) Myogenin via RT-qPCR analysis. Western blotting results of (d) p-Smad3; respective quantification data are showed in figure (e). The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * p < 0.05, ** p < 0.01.*

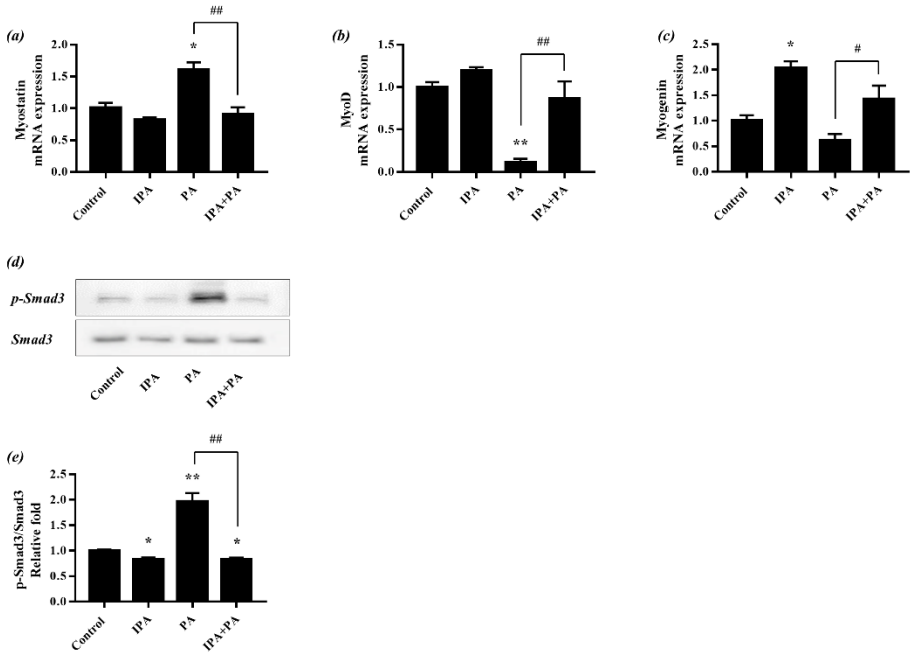

Figure 2- 11. *Assessment of IPA on myogenic regulators. Expression of mRNA levels of (**a**) Myostatin, (**b**) MyoD, and (**c**) Myogenin via RT-qPCR analysis. Western blotting results of (**d**) p-Smad3; respective quantification data are showed in figure (**e**). The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

2.3.7 Palmitate improves adipogenic character in muscle cells

Adipogenic markers were assessed in differentiated myotubes following treatment with palmitic acid, DPHC, and IPA. Myogenic differentiation showed a shift in early myotubes in the adipogenic direction, expressing adipogenic characteristics. Adipogenic markers, such as peroxisome proliferator-activated receptor-gamma (PPARγ) (Figure 2-12a) and CCAAT/enhancer-binding protein alpha (c/EBPα) (Figure 2-12b) were improved by palmitic acid treatment. Further, western blotting of adipocyte fatty acid-binding protein (A-FABP) (Figure 2-12c, e) revealed its involvement in palmitic acid stimulation via its significant upregulation compared to the non-treated group. The levels of the myogenic marker MyHC significantly declined with palmitic acid treatment (Figure 2-12d, f). DPHC repressed the effects of palmitic acid on the adipogenic markers and improved myogenic marker levels. IPA exhibited similar results against the detrimental effects of PA (Figure 2-13). These data suggest that palmitic acid is responsible for the expression of adipogenic characteristics, while DPHC and IPA inhibit it and restore the myogenic characteristics. These data demonstrated that palmitic acid inhibited myogenesis and improved adipogenic characteristics in the late stages of myogenic differentiation.

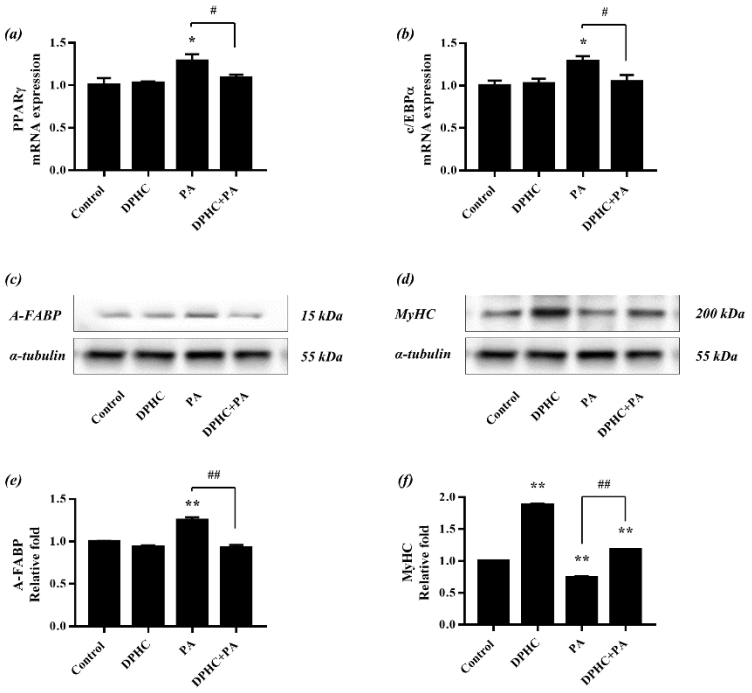

Figure 2- 12. *Assessment of DPHC on adipogenic regulators. Expression of mRNA levels of (a) PPARγ and (b) c/EBPα via RT-qPCR analysis. Western blotting results of (c) A-FABP and myogenic marker (d) MyHC; respective quantification data are showed in figure (e), and (f). The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$.*

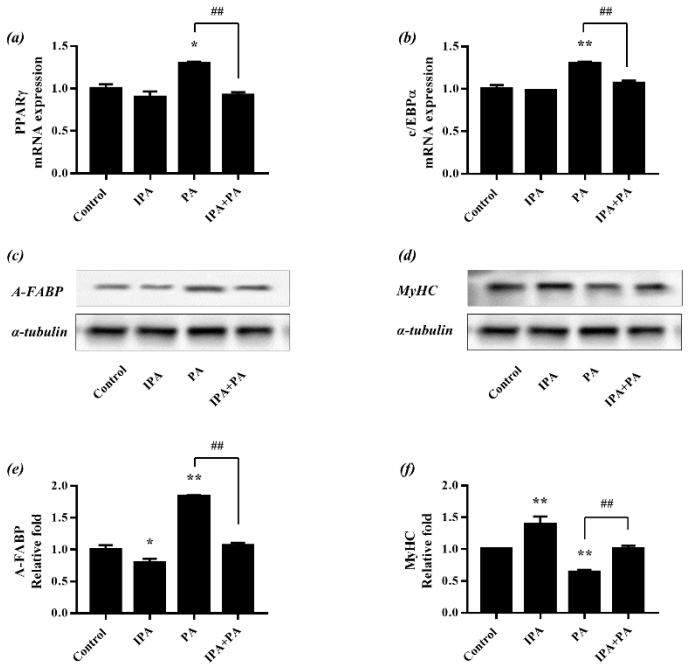

Figure 2- 13. *Assessment of IPA on adipogenic regulators. Expression of mRNA levels of (**a**) PPARγ and (**b**) c/EBPα via RT-qPCR analysis. Western blotting results of (**c**) A-FABP and myogenic marker (**d**) MyHC; respective quantification data are showed in figure (e), and (f). The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

2.3.8 Effects of palmitate, DPHC, and IPA on the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) growth regulatory axis

The growth regulatory axis is essential for proper muscle function and regeneration. This axis directs the anabolic pathway to produce proteins. Thus, the phosphorylation levels of the PI3K/Akt/mTOR axis were assessed in cells treated with palmitic acid, DPHC, and both (Figure 2-14a–f). DPHC significantly enhanced protein phosphorylation. A substantial downregulation of phosphorylation was observed with palmitic acid treatment, which was reversed with DPHC co-treatment. IPA was expressing similar trends with DPHC (Figure 2-15). These results suggest that DPHC and IPA restore palmitic acid-impaired myogenesis and promotes anabolic cell processes, ultimately assisting myogenesis.

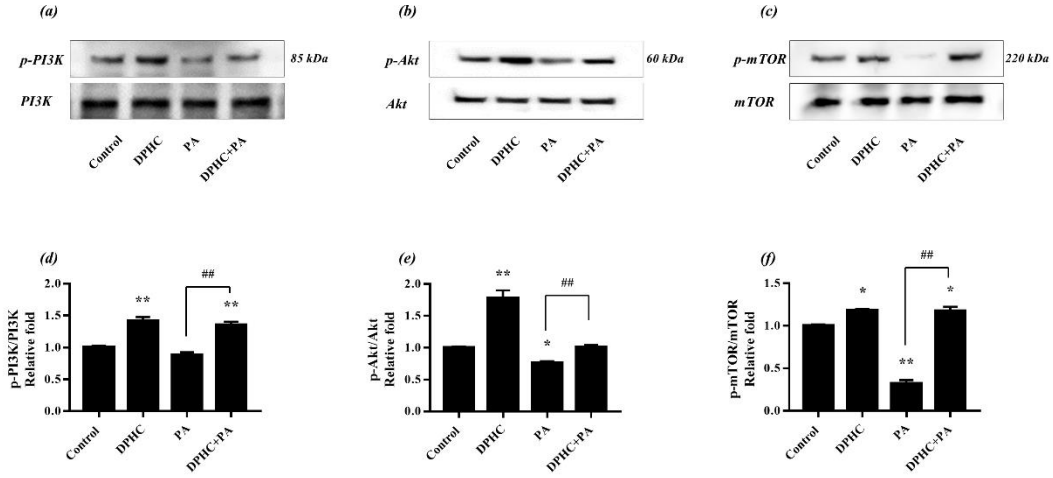

Figure 2- 14. *Growth regulators assessment. Protein expression of (a) p-PI3K, (b) p-Akt, (c) p-mTOR via western blotting analysis. Figures (e), (f), and (g) exhibit the quantitative data. ImageJ program was used in the quantification of relative intensities. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$.*

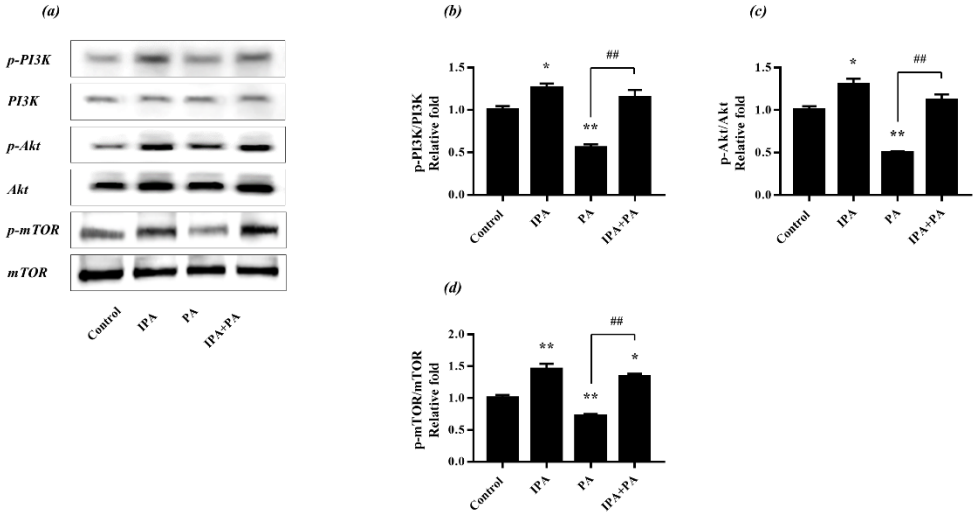

Figure 2- 15. *Growth regulators assessment. Protein expression of (**a**) p-PI3K, p-Akt, and p-mTOR via western blotting analysis. Figures (**b**), (**c**), and (**d**) exhibit the quantitative data. ImageJ program was used in the quantification of relative intensities. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

2.3.9 IPA ameliorates atrophic signaling in PA-induced myotube weakening

To determine if IPA exerted myogenic effects during atrophic conditions caused by a PA, we evaluated expression of IGF-1 in PA-treated myotubes with and without IPA. PA treatment resulted in decreased phosphorylation of IGF-1R, indicating reduced receptor activity. In IPA-treated myotubes, PA had no significant effect on IGF-1R activity (Figure 2-16a). To investigate the suppressive effect of IPA on PA-mediated atrophic signaling, we assessed expression of the muscle-specific ubiquitin ligases (atrogenes), atrogin-1 and MuRF-1, and the transcriptional activity of FoxO3, a critical mediator of atrogenes. PA treatment increased activation of FoxO3, as evidenced by decreased phosphorylation (p-FoxO3) and increased atrogin-1 and MuRF-1 expression. In contrast, IPA treatment of myotubes resulted in decreased atrogene signaling and slightly higher FoxO3 phosphorylation and decreased MuRF1 expression. When IPA was co-treated to PA-induced myotubes, the decreased in p-FoxO3 with PA treatment was abolished, and p-FoxO3 was close to control levels. The increased atrogene expression due to PA was also significantly lower, suggesting attenuation of ubiquitin-mediated atrophic signaling (Figure 2-16a-g).

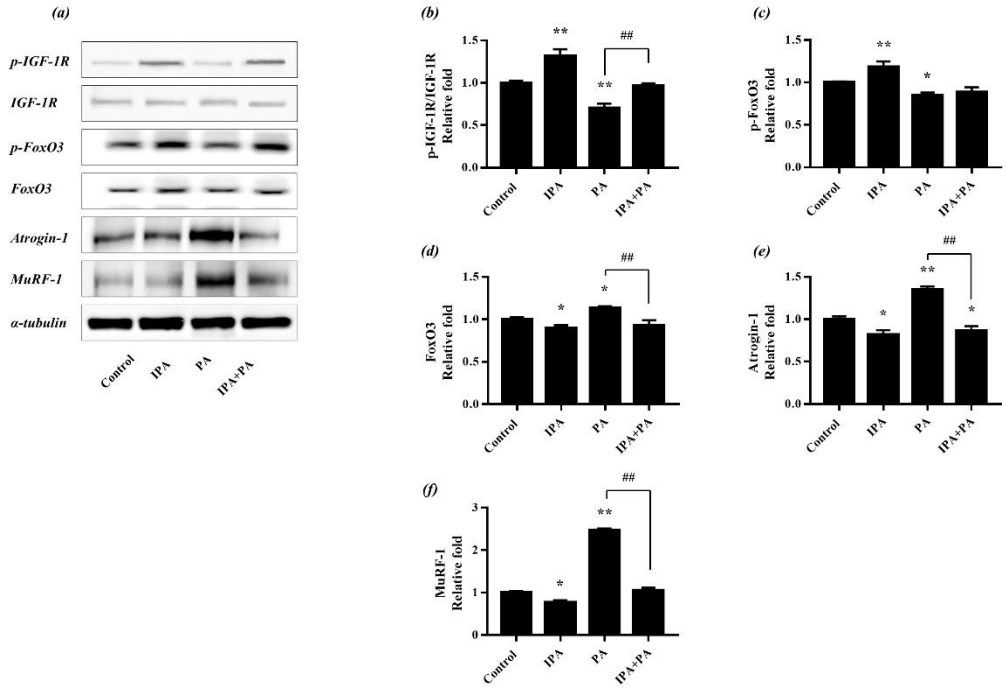

Figure 2- 16. *IPA ameliorates IGF-1 signaling transduction and attenuates FoxO-mediated expression of muscle-specific ubiquitin ligases in PA-induced myotube atrophy. Protein expression of (**a**) western blotting analysis. Quantitative data (**b**) p-IGF-1R/IGF-1R, (**c**) p-FoxO3, (**d**) FoxO3, (**e**) Atrogin-1, and (f) MuRF-1. ImageJ program was used in the quantification of relative intensities. The experiments were triplicated and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

2.4 Discussion

The health-promoting properties of natural products have increased market demand above that for their synthetic counterparts due to their reduced side effects. Marine brown algae comprise of a wide variety of phloroglucinol based polyphenols. Phenolic compounds have long been studied for their healing properties such as antioxidant, antiobesity and antimutagenic effects [31] [32]. A recent study by Kim et al. (2021) reported 6 polyphenols from Ecklonia cava out of which dieckol (DK), 2,7″-phloroglucinol-6,6′-bieckol (PHB) to improve proliferation and differentiation potential of skeletal muscle cells implementing an in vitro system of C2C12 cells. Further, these compounds were observed to regulate myogenesis via Smad signaling. The in-silico studies conducted in this report revealed that DK and PHB had higher potential to enhance IGF-1 a positive regulator and to inhibit myostatin which is a negative regulator [33]. *I. okamurae* is an edible brown seaweed that grows along the coasts of Korea, Japan, and China [34]. In vitro and in vivo studies have evaluated *I. okamurae* as a source of bioactive components. Diphloroethohydoxycarmalol (DPHC) is a purified compound derived from *I. okamurae* and is among the most widely studied phlorotannins. Wang et al. reported the protective effects of DPHC isolated from *I. okamurae* against UVB-induced cell damage [35], while Fernando et al. evaluated glucose-induced angiogenesis [18]. Lu et al. assessed the vasodilatory effects of DPHC via calcium signaling pathways, suggesting the use of this bioactive component to prevent endothelium-dependent cardiovascular diseases [17]. However, the associations of *I. okamurae* and DPHC with skeletal muscles have been scarcely reported. Yang et al. proposed the potential of brown algae to improve glucose metabolism in skeletal muscle in a murine high-fat diet (HFD) model [36]. DPHC associated with inflammatory myopathy was assessed in detail as an inhibitory agent of tumor necrosis factor (TNF)-α, demonstrating its action through the

NF-κB and MAPK pathways [12]. These studies attracted attention to *I. okamurae* as a material in the cosmeceutical, nutraceutical, and functional food sectors. However, the effects of *I. okamurae* and DPHC on lipid-induced skeletal myogenesis have not yet been evaluated. Thus, this study attempted to fill the gap between *I. okamurae* as a functional food source and lipid-impaired skeletal myogenesis in the context of sarcopenic obesity.

Skeletal muscle maintenance and function are essential for whole-body homeostasis. Obesity is associated with saturated fatty acids, which are enriched in high-fat diets. The impact of palmitic acid (PA), an abundant circulating free fatty acid, on myotube formation without inducing cell death was evaluated (Figure 2-4a). Exposure of skeletal muscle to PA may affect the expression of multiple genes, ultimately contributing to impaired skeletal myogenesis.

This study evaluated 70% EtOH extracts of five brown algal species for their potential to improve skeletal muscle cell proliferation. The non-cytotoxic concentration range of IO showed significant potential to improve skeletal muscle cell proliferation (Figure 2-3a, b). Therefore, IO was selected for further experiments. DPHC and IPA were assessed for its potential in a similar manner (Figure 2-8a, b and Figure 2-9a, b). Our results showed the potential of both for skeletal muscle cell proliferation. The selected concentration of PA deregulated proliferation, where IO (Figure 2-4b), DPHC (Figure 2-8c), and IPA (Figure 2-9c) reversed the negative effect. Myoblast proliferation is an important step in the highly coordinated and complex process of skeletal myogenesis. The amplified cell number due to cell division is termed cell proliferation. This is a firmly controlled and an intricate process. Toxic substances cause severe cell injury reflected by the absence of compensatory cell division or ultimate cell failure and death. Momentarily, the response against would be induction of healthy cells to divide and to restore tissue structure and

function. The potential of the cell to enter and progress through the cell cycle against toxic injury declines with age manifesting the fact that obesity enhances sarcopenic character with older age. Thus, myoblast proliferation can be singled out as a vital process combines with the differentiation to develop the muscle health. The proper maintenance of skeletal muscle cell proliferation and differentiation is crucial during the embryogenesis and postnatal skeletal muscle regeneration that is essential for muscle repair following injury or exercise [37, 38].

Previous studies have shown that myoblast over-proliferation leads to skeletal myogenesis, altering cell cycle exit and myoblast fusion. Conteras et al. reported that nilotinib, a second-generation tyrosine kinase inhibitor, compromised skeletal myogenesis by increasing myoblast proliferation [39]. However, our results showed that both IO and DPHC improved myoblast proliferation but did not affect skeletal myogenesis at the selected concentrations. In contrast, PA impaired differentiation by altering the expression of myogenic regulators. MyoD was repressed by PA, whereas IO restored expression levels (Figure 2-6a, c). This resulted in a reduced myotube number in the PA-treated group, ultimately resulting in a lower fusion index (Figure 2-6d). MyHC was used as a marker gene to evaluate the level of differentiation (Figure 2-6b, 2-7e-f). The decreased MyHC expression suggested debilitated myotube formation, indicating declining muscle regeneration and strength. As shown in Figure 2-7a–d, myogenic markers were inhibited on each day of the *in vitro* differentiation with PA treatment.

MyHC expression was consistent with the behavior of myogenic markers (Figure 2-7e, f). IO alone was effective in increasing the expression of these genes, suggesting its potential to improve skeletal myogenesis. Myoblasts are highly proliferative and differentiate fusing with neighboring cells to form multinucleated cells (myotubes) that

establish muscle fibers. The differentiation of skeletal muscle is accompanied by the transcriptional activation of muscle-specific genes, including MyoD, myogenin, Myf5, and MRFs, which are members of the MyoD family of basic helix-loop-helix (bHLH) transcription factors. Sequential activation of these genes can initiate muscle differentiation, with MyoD as the master regulator [40]. Similar results were observed for DPHC treatment (Figure 2-10b and c, and 2-12d and f) and for IPA treatment (Figure 2-11b and c, and 2-13d and f), further indicating the potential of DPHC as a myogenic stimulator and palmitic acid inhibitor.

Using MyoD as the central and principal transcription factor that maintains and coordinates the myogenesis process, we assessed negative factors associated with its altered expression. The transforming growth factor-beta (TGF-β)/Smad pathway is a major pathway that negatively regulates myogenesis. Myostatin, the best-known member of the TGF-β family, inhibits differentiation and fusion, thereby preventing myotube formation. The signal is downstream through Smad3, where it can suppress MyoD transcription by binding to the bHLH region [41-43]. Our results demonstrated that DPHC inhibited palmitic-acid-enhanced myostatin levels (Figure 2-10a).

Phosphorylated Smad3 levels were restored by DPHC treatment (Figure 2-10d, e). Accordingly, the negative regulation mechanism of PA was mediated through Smad3, which impairs myogenesis. Hence, DPHC was a significant therapeutic intervention acting through the myostatin/Smad3 pathway, supporting its role in the proper maintenance of muscle regeneration.

PPARγ and c/EBPα are prominent members involved in the growth arrest and activation of adipose-related genes to induce adipocyte differentiation [44]. To address the influence of PA on the determination and alteration of the molecular basis of the terminal

differentiation lineage, we examined PPARγ and c/EBPα expression in myotubes. Our data revealed that the expression of both these genes impaired the myogenesis process stimulated by serum deprivation (Figure 2-12a, b). Furthermore, DPHC did not influence the expression of these adipogenic markers, which significantly inhibited their PA-driven expression. In the presence of the adipogenic markers stimulated via PA, MyoD, and myogenin, myogenic markers were repressed, resulting in decreased MyHC expression. He et al. (2017) reported that PPARγ overexpression played a critical role in myogenic differentiation by mediating crosstalk among multiple pathways and transcription factors [45]. A-FABP is an intracellular lipid chaperone that is strongly associated with metabolic pathways. The regulation of A-FABP is transcriptionally controlled by fatty acids and PPARγ agonists, including c/EBPα, during adipogenic differentiation [46, 47]. The results demonstrated that PA stimulated A-FABP expression (Figure 2-12c, e), explaining the observed PPARγ and c/EBPα levels. Nevertheless, DPHC successfully repressed the effect of palmitic acid.

MAFbx/Atrogin-1 and MuRF1 are collectively termed atrogens (E3 ubiquitin ligases). These were observed to be increased during muscle atrophy induced by denervation and considered as sensitive markers of protein degradation [48]. Intriguingly, atrogin-1 knockdown resulted in muscle loss prevention during fasting [49], where dexamethasone-induced muscle atrophy resistance was reported in the absence of MuRF1 [50]. Growth-related and survival pathway proteins appear to be the substrates of atrogin-1. MyoD degradation is enhanced by atrogin-1, inhibiting myotube formation [51]. An important activator of protein synthesis, eukaryotic translation factor 3 subunit 5 (eIF3-f) ubiquitination is promoted by atrogin-1 [52]. Atrogin-1 interacts with sarcomere proteins, enzymes related to glycolysis, and mitochondrial proteins linking it to both structural and functional proteins [53]. In contrast, target substrates of MuRF-1

are structural protein titin, troponin1 including myosin heavy chain, further including proteins associated with glycolysis and glycogen metabolism [54, 55]. The atrogenes are related to the FoxO transcriptional factors [56]. Phosphorylated Akt suppresses protein degradation via promoting FoxO phosphorylation other than its involvement in the mTORC1 mechanisms. The dephosphorylated form of FoxO has the potential to translocate to the nucleus, hence initiate the transcription of target genes supporting catabolic roles (MuRF1 and atrogin-1) [56, 57]. With regard, the activity of Akt on FoxO could alleviate muscle atrophy. Atrogene transcription is further stimulated via oxidative stress, pro-inflammatory cytokines, and glucocorticoids.

Muscle regrowth after injury is crucial to restore muscle function. As palmitic acid impaired myogenesis, we examined its effects on growth regulators. The growth regulatory axis contains PI3K/Akt/mTOR mediators, which are responsible for protein synthesis. Myostatin, which was evaluated earlier, compromises the growth regulators, ultimately resulting in the poor healing capacity of injured muscles associated with PA induction. The results demonstrated reduced phosphorylation levels of PI3K/Akt/mTOR mediators, reflecting suppressed growth (Figure 2-14a-f). Akt, in particular, was associated with the hypertrophic response in muscle cells, where it acts as a crossing point between IGF-1 and myostatin signaling. The literature provides evidence of the inverse proportionality between myostatin and Akt. McFarlane et al. reported that myostatin reversed Akt phosphorylation, thereby improving atrophy-related genes [58]. However, DPHC was potent enough to overcome the negative effects of palmitic acid. Furthermore, DPHC alone demonstrated an ability to improve growth regulation.

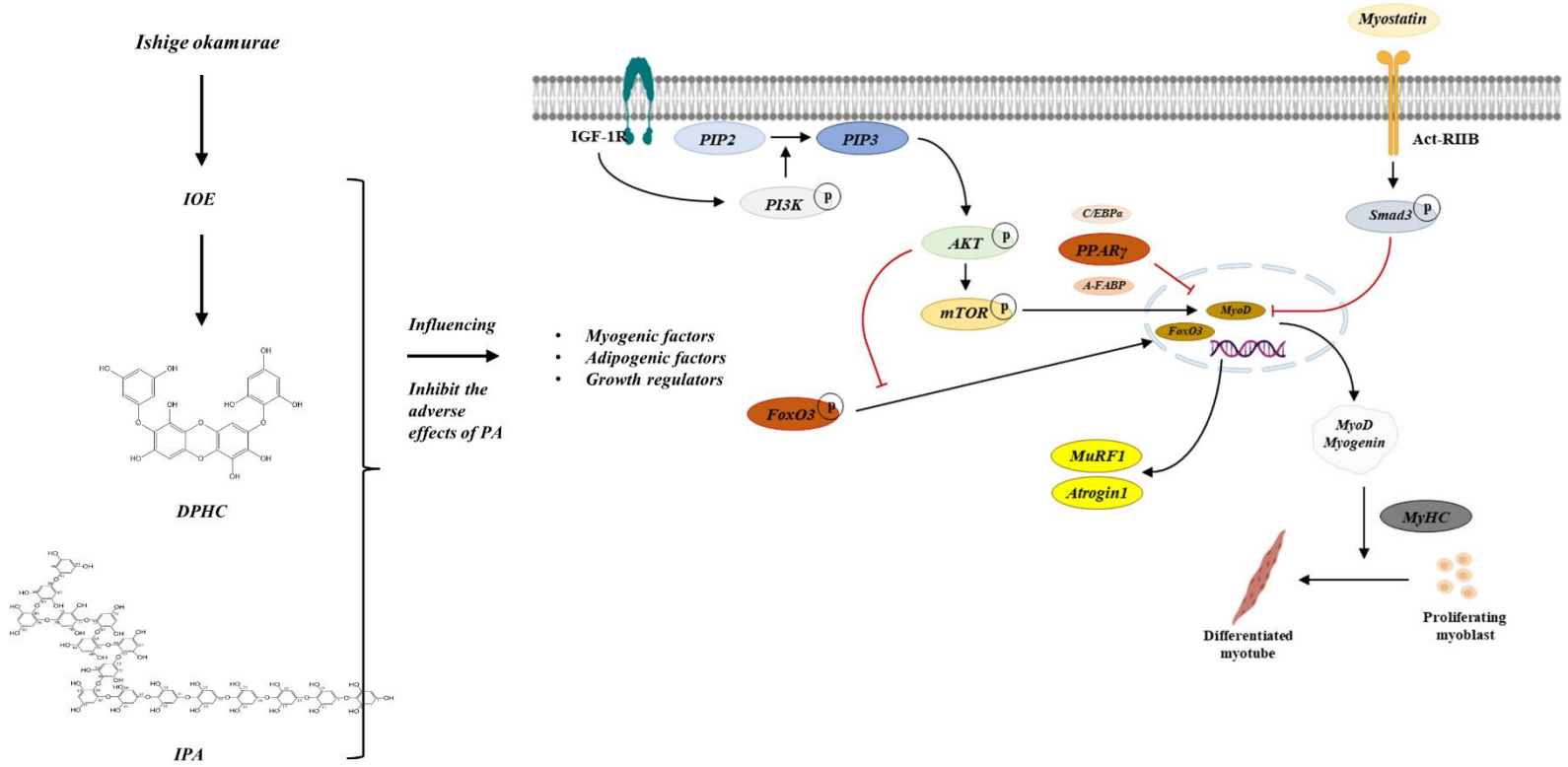

Figure 2- 17. *Graphical representation of the study pathway analyzed.*

Sarcopenia, obesity, and their combined presence, known as obese-sarcopenia, are distinct public health concerns that are expected to increase in prevalence as the human population ages. Nonetheless, despite extensive efforts, the research community is still far from fully understanding the governing molecular mechanism of obese-sarcopenia due to its complexity. Untangling these mechanisms is critical for laying the groundwork for preventive and therapeutic interventions. This study provides mechanistic insights into the effect of PA on skeletal myogenesis and the involvement of adipogenic factors. The findings further explain the vitality of regenerative capacity through growth regulators. These results can inform efforts to minimize palmitic acid levels, including appropriate diets and the use of drugs to normalize palmitic acid levels. The results of the present study combine these approaches by suggesting the usefulness of an ethanol extract of the edible brown seaweed *I. okamurae* (as a dietary tool) as well as its purified phlorotannin DPHC and IPA (as a drug) to reduce the effects of palmitic acid.

2.5 Conclusion

A better understanding and of the molecular pathways of sarcopenic obesity can have profound implications in the development of therapeutic interventions. Thus, the results of this study suggest the potential for treatments based on the bioactive components IO, DPHC, and IPA with the sustainable utilization of the *I. okamurae* marine algae.

References

1. von Haehling, S., J.E. Morley, and S.D. Anker, *An overview of sarcopenia: facts and numbers on prevalence and clinical impact.* J Cachexia Sarcopenia Muscle, 2010. **1**(2): p. 129-133.
2. Morley, J.E., et al., *Sarcopenia.* J Lab Clin Med, 2001. **137**(4): p. 231-43.
3. Atkins, J.L. and S.G. Wannamathee, *Sarcopenic obesity in ageing: cardiovascular outcomes and mortality.* Br J Nutr, 2020. **124**(10): p. 1102-1113.
4. Biolo, G., T. Cederholm, and M. Muscaritoli, *Muscle contractile and metabolic dysfunction is a common feature of sarcopenia of aging and chronic diseases: from sarcopenic obesity to cachexia.* Clin Nutr, 2014. **33**(5): p. 737-48.
5. Tchkonia, T., et al., *Fat tissue, aging, and cellular senescence.* Aging Cell, 2010. **9**(5): p. 667-84.
6. Carta, G., et al., *Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications.* Front Physiol, 2017. **8**(902): p. 902.
7. Cho, Y., S.Y. Shin, and M.J. Shin, *Sarcopenic obesity is associated with lower indicators of psychological health and quality of life in Koreans.* Nutr Res, 2015. **35**(5): p. 384-92.
8. Polyzos, S.A. and A.N. Margioris, *Sarcopenic obesity.* Hormones (Athens), 2018. **17**(3): p. 321-331.
9. da Paixao, A.O., et al., *Palmitic Acid Impairs Myogenesis and Alters Temporal Expression of miR-133a and miR-206 in C2C12 Myoblasts.* Int J Mol Sci, 2021. **22**(5): p. 2748.
10. Shirosaki, M. and T. Koyama, *Chapter 15 - Laminaria japonica as a Food for the Prevention of Obesity and Diabetes*, in *Advances in Food and Nutrition Research*, S.-K. Kim, Editor. 2011, Academic Press. p. 199-212.
11. Maeda, H., et al., *Fucoxanthin from edible seaweed, Undaria pinnatifida, shows antiobesity effect through UCP1 expression in white adipose tissues.* Biochem Biophys Res Commun, 2005. **332**(2): p. 392-7.
12. Kim, S.Y., et al., *Diphlorethohydroxycarmalol (DPHC) Isolated from the Brown Alga Ishige okamurae Acts on Inflammatory Myopathy as an Inhibitory Agent of TNF-alpha.* Mar Drugs, 2020. **18**(11): p. 529.
13. Yang, H.W., et al., *Ca(2+)-Dependent Glucose Transport in Skeletal Muscle by Diphlorethohydroxycarmalol, an Alga Phlorotannin: In Vitro and In Vivo Study.* Oxid Med Cell Longev, 2021. **2021**: p. 8893679.
14. Yang, H.-W., et al., *Effect of Ishophloroglucin A, A Component of Ishige okamurae, on Glucose Homeostasis in the Pancreas and Muscle of High Fat Diet-Fed Mice.* 2019. **17**(11): p. 608.
15. Fernando, K.H.N., et al., *Ishige okamurae Extract and Its Constituent Ishophloroglucin A Attenuated In Vitro and In Vivo High Glucose-Induced Angiogenesis.* 2019. **20**(22): p. 5542.
16. Kang, N., et al., *Ishophloroglucin A, derived from Ishige okamurae, regulates high-fat-diet-induced fat accumulation via the leptin signaling pathway, associated with peripheral metabolism.* Algal Research, 2020. **50**: p. 101974.
17. Lu, Y.A., et al., *Diphlorethohydroxycarmalol Isolated from Ishige okamurae Exerts Vasodilatory Effects via Calcium Signaling and PI3K/Akt/eNOS Pathway.* Int J Mol Sci, 2021. **22**(4): p. 1610.

18. Fernando, K.H.N., et al., *Diphlorethohydroxycarmalol Isolated from Ishige okamurae Represses High Glucose-Induced Angiogenesis In Vitro and In Vivo.* Mar Drugs, 2018. **16**(10): p. 375.
19. Ryu, B., et al., *Ishophloroglucin A, a Novel Phlorotannin for Standardizing the Anti-α-Glucosidase Activity of Ishige okamurae.* 2018. **16**(11): p. 436.
20. Malhi, H., et al., *Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis.* J Biol Chem, 2006. **281**(17): p. 12093-101.
21. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
22. Fernando, I.P.S., et al., *3β-Hydroxy-Δ5-steroidal congeners from a column fraction of Dendronephthya puetteri attenuate LPS-induced inflammatory responses in RAW 264.7 macrophages and zebrafish embryo model.* RSC Advances, 2018. **8**(33): p. 18626-18634.
23. Kang, N., et al., *Anti-fatigue activity of a mixture of seahorse (Hippocampus abdominalis) hydrolysate and red ginseng.* Fisheries and Aquatic Sciences, 2017. **20**(1): p. 3.
24. Lamon, S., et al., *EPO-receptor is present in mouse C2C12 and human primary skeletal muscle cells but EPO does not influence myogenesis.* Physiol Rep, 2014. **2**(3): p. e00256.
25. Jayawardena, T.U., et al., *Particulate Matter-Induced Inflammation/Oxidative Stress in Macrophages: Fucosterol from Padina boryana as a Potent Protector, Activated via NF-kappaB/MAPK Pathways and Nrf2/HO-1 Involvement.* Mar Drugs, 2020. **18**(12): p. 628.
26. Jayawardena, T.U., et al., *Alginic Acid from Padina boryana Abate Particulate Matter-Induced Inflammatory Responses in Keratinocytes and Dermal Fibroblasts.* Molecules, 2020. **25**(23): p. 5746.
27. Molagoda, I.M.N., et al., *Anthocyanins from Hibiscus syriacus L. Inhibit NLRP3 Inflammasome in BV2 Microglia Cells by Alleviating NF-kappaB- and ER Stress-Induced Ca(2+) Accumulation and Mitochondrial ROS Production.* Oxid Med Cell Longev, 2021. **2021**: p. 1246491.
28. Jayawardena, T.U., et al., *Anti-Inflammatory Effects of Sulfated Polysaccharide from Sargassum Swartzii in Macrophages via Blocking TLR/NF-Kappab Signal Transduction.* Mar Drugs, 2020. **18**(12): p. 601.
29. Jayawardena, T.U., et al., *Sargassum horneri and isolated 6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one (HTT); LPS-induced inflammation attenuation via suppressing NF-κB, MAPK and oxidative stress through Nrf2/HO-1 pathways in RAW 264.7 macrophages.* Algal Research, 2019. **40**: p. 101513.
30. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
31. Wijesekara, I., N.Y. Yoon, and S.K. Kim, *Phlorotannins from Ecklonia cava (Phaeophyceae): biological activities and potential health benefits.* Biofactors, 2010. **36**(6): p. 408-14.
32. Li, Y.X., et al., *Phlorotannins as bioactive agents from brown algae.* Process Biochemistry, 2011. **46**(12): p. 2219-2224.

33. Kim, S.Y., et al., *The Effects of Marine Algal Polyphenols, Phlorotannins, on Skeletal Muscle Growth in C2C12 Muscle Cells via Smad and IGF-1 Signaling Pathways.* Mar Drugs, 2021. **19**(5): p. 266.
34. Asanka Sanjeewa, K.K., et al., *Exploiting biological activities of brown seaweed Ishige okamurae Yendo for potential industrial applications: a review.* Journal of Applied Phycology, 2017. **29**(6): p. 3109-3119.
35. Wang, L., et al., *Protective effect of diphlorethohydroxycarmalol isolated from Ishige okamurae against UVB-induced damage in vitro in human dermal fibroblasts and in vivo in zebrafish.* Food Chem Toxicol, 2020. **136**: p. 110963.
36. Yang, H.-W., et al., *Ishige okamurae reduces blood glucose levels in high-fat diet mice and improves glucose metabolism in the skeletal muscle and pancreas.* Fisheries and Aquatic Sciences, 2020. **23**(1): p. 24.
37. Yang, N., S.D. Ray, and K. Krafts, *Cell Proliferation*, in *Encyclopedia of Toxicology*, P. Wexler, Editor. 2014, Academic Press: Oxford. p. 761-765.
38. Langlois, S. and K.N. Cowan, *Regulation of Skeletal Muscle Myoblast Differentiation and Proliferation by Pannexins.* Adv Exp Med Biol, 2017. **925**: p. 57-73.
39. Contreras, O., M. Villarreal, and E. Brandan, *Nilotinib impairs skeletal myogenesis by increasing myoblast proliferation.* Skelet Muscle, 2018. **8**(1): p. 5.
40. Tapscott, S.J., *The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription.* Development, 2005. **132**(12): p. 2685-95.
41. Liu, D., B.L. Black, and R. Derynck, *TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3.* Genes Dev, 2001. **15**(22): p. 2950-66.
42. McFarlane, C., et al., *Human myostatin negatively regulates human myoblast growth and differentiation.* Am J Physiol Cell Physiol, 2011. **301**(1): p. C195-203.
43. Langley, B., et al., *Myostatin inhibits myoblast differentiation by down-regulating MyoD expression.* J Biol Chem, 2002. **277**(51): p. 49831-40.
44. Rosen, E.D. and O.A. MacDougald, *Adipocyte differentiation from the inside out.* Nat Rev Mol Cell Biol, 2006. **7**(12): p. 885-96.
45. He, K., et al., *A transcriptomic study of myogenic differentiation under the overexpression of PPARgamma by RNA-Seq.* Sci Rep, 2017. **7**(1): p. 15308.
46. Furuhashi, M. and G.S. Hotamisligil, *Fatty acid-binding proteins: role in metabolic diseases and potential as drug targets.* Nat Rev Drug Discov, 2008. **7**(6): p. 489-503.
47. Yang, X., et al., *The role of BAMBI in regulating adipogenesis and myogenesis and the association between its polymorphisms and growth traits in cattle.* Mol Biol Rep, 2020. **47**(8): p. 5963-5974.
48. Lecker, S.H., et al., *Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression.* FASEB J, 2004. **18**(1): p. 39-51.
49. Cong, H., et al., *Inhibition of atrogin-1/MAFbx expression by adenovirus-delivered small hairpin RNAs attenuates muscle atrophy in fasting mice.* Hum Gene Ther, 2011. **22**(3): p. 313-24.
50. Baehr, L.M., J.D. Furlow, and S.C. Bodine, *Muscle sparing in muscle RING finger 1 null mice: response to synthetic glucocorticoids.* J Physiol, 2011. **589**(Pt 19): p. 4759-76.
51. Tintignac, L.A., et al., *Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase.* J Biol Chem, 2005. **280**(4): p. 2847-56.

52. Lagirand-Cantaloube, J., et al., *The initiation factor eIF3-f is a major target for atrogin1/MAFbx function in skeletal muscle atrophy.* EMBO J, 2008. **27**(8): p. 1266-76.
53. Lokireddy, S., et al., *Myostatin induces degradation of sarcomeric proteins through a Smad3 signaling mechanism during skeletal muscle wasting.* Mol Endocrinol, 2011. **25**(11): p. 1936-49.
54. Rom, O. and A.Z. Reznick, *The role of E3 ubiquitin-ligases MuRF-1 and MAFbx in loss of skeletal muscle mass.* Free Radic Biol Med, 2016. **98**: p. 218-230.
55. Foletta, V.C., et al., *The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy.* Pflugers Arch, 2011. **461**(3): p. 325-35.
56. Stitt, T.N., et al., *The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors.* Mol Cell, 2004. **14**(3): p. 395-403.
57. Brunet, A., et al., *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.* Cell, 1999. **96**(6): p. 857-68.
58. McFarlane, C., et al., *Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism.* J Cell Physiol, 2006. **209**(2): p. 501-14.

Part III: Mitochondrial ROS/Nlrp3 inflammasome axis contribute PA-induced skeletal muscle wasting and *Ishige okamurae* purified DPHC as a potent intervention

3.1 Introduction

Sarcopenia, characterized by the loss of muscle mass and function leads to lower quality of life and inclined morbidity and mortality [1]. Though, this is an age-related process associated with older community, sarcopenia is improved by other factors such as genetic factors and lifestyle. The field has received the focus of intense research with interests in the development of drugs to augment the issues, concerns in the interventions through nutritional factors, and via resistance exercise [1, 2]. Another age-related prominent disorder is obesity, which is an imminent risk factor. Inclined body fat and declined skeletal muscle mass is a key age associated body composition alteration. This would intricate the conventional pathophysiological mechanism leading to biased levels of regulatory protein, hormone, and other vital component expressions. The reciprocal influence of sarcopenia and obesity paves the way to a vicious cycle. Reduced physical activity due to sarcopenia take the lead to lower energy expenditure thus, contributing to improved risk of obesity. Vise versa, factors accumulated through obesity such that pro-inflammatory factors contribute towards the development of sarcopenia [3].

Generation of reactive oxygen species (ROS) is closely related with the ectopic lipid accumulation due to improved flux of energy substrate such as excessive dietary fat, thus influence cellular dysfunction. Pathogenesis of diabetes and linked sarcopenia is associated with the activation of pathways related to oxidative stress and muscular mitochondrial dysfunction [4, 5]. Further, mitochondria function as a signaling platform for multiple biological responses including immunity and metabolism. Literature provides evidence on the function of mitochondrial ROS (mtROS) in the activation of nucleoside oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) inflammasomes and the regulation of innate signaling pathways [6,

7]. Controlled levels of mtROS are vital for the innate host defense against pathogens, though unrestrained amounts may lead to pathologies including chronic inflammation [8]. The Nlrp3 inflammasome consisting of apoptotic speck-containing protein with a CARD (ASC) and inactive pro-caspase-1 conveys inflammatory signals downstream by cleaving pro-IL-1β to IL-1β. The secreted IL-1β and IL-18 delivers and continues inflammatory signaling. Palmitate an abundant, saturated, free fatty acid in the serum produces ceramides and diacylglycerols (DAGs). Palmitate influences the Nlrp3 inflammasome, mtROS as well as pattern recognition receptors (PRRs) [9, 10]. Ultimately, this could lead to apoptosis which in myocellular environment is a tissue weakening.

Plant species that grow in the marine environment; namely, seaweeds or sea vegetables, have become an emerging sustainable nutritional source and an essential part of healthy human diets, mostly in the East Asian region. They are a good source of vitamins and minerals, fiber, and polysaccharides that support low caloric intake [11]. Furthermore, seaweeds contain pigments, proteins, polyunsaturated fatty acids (PUFAs), and phenolic compounds. Bioactive components such as fucoxanthin have potential anti-obesity effects. Meada et al. described *Undaria pinatifida*, an edible brown seaweed, as a source of fucoxanthin with anti-obesity effects through the expression of mitochondrial uncoupling protein 1 white adipose tissue [12]. Polyphenols are an important component of marine algae (brown algae) that provide functional food and pharmaceutical significance. However, studies reporting their use as interventions in muscle-related research are limited. Among them, diphloroethohydoxycarmalol (DPHC) isolated from *Ishige okamurae* reportedly showed the potential to inhibit inflammatory myopathy [13]. Moreover, DPHC promoted cytosolic Ca^{2+}, resulting in increased glucose transport to myotubes to modulate blood glucose levels [14].

It is reported that mitochondrial dysfunction is implicated in muscle wasting [15, 16]. Though the relationship between mtROS and Nlrp3 inflammasome was not discussed previously according to the best of our knowledge. Further, this study investigates the influence of NF-κB in the lipid induced muscle atrophy as well as the mitochondria mediated caspase driven muscle wasting. *Ishige okamurae* and purified DPHC are implemented as sustainable sources of marine algal resources in the fight against obesity influenced sarcopenia.

3.2 Material and methods

3.2.1 Reagents

The required cells (C2C12 myoblasts) were obtained from the American Type Culture Collection (ATCC, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO, Inc. (Grand Island, NY, USA). Growth medium supplements (fetal bovine serum [FBS], horse serum [HS]) and antibiotics (penicillin and streptomycin) were obtained from GIBCO, Inc. Primary and secondary antibodies used for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG H&L (Alexa Fluor ® 488; ab150113) and goat anti-rabbit IgG H&L (Alexa Fluor ® 647; ab150079) were obtained from Abcam (Cambridge, UK). Mito-Tempo from MedChemExpress (NJ, USA) and MCC950 from AbMole (TX, USA) were purchased. All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

3.2.2 Cell culture

Myoblasts were cultured and plated in DMEM supplemented with 10% FBS and antibiotics. Differentiation was initiated once the cells had reached 80% confluence by changing the medium to DMEM containing 2% HS and antibiotics. The experimental models were designed, and the cells were stimulated at each required timeline, as appropriate. Myotubes were observed 3–4 days post-differentiation. The cells were maintained in a Sanyo incubator (Sanyo MCO-18AIC; Moriguchi, Japan) at 37°C in a humidified atmosphere with 5% CO_2.

3.2.3 Experimental treatments

IO and DPHC were first dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and diluted in serum-free DMEM to obtain working standards. Palmitic acid was dissolved in 2-propanol and diluted in serum-free DMEM containing 2% BSA (FA-free) [17]. These were treated according to the experimental plan at different time points. Palmitic acid stimulation was conducted for up to 16–24 h (unless otherwise specified), where the IO extract and DPHC were added every other day following media changes. The mentioned experimental treatments were applied identically with or without 1 h preincubation of the cell in the presence of Mito-Tempo (10 μM) [18], MCC950 (1 μM) [19], prior to stimulation by PA.

3.2.4 Determining optimal palmitate and compound concentrations

The cytotoxicity of each sample and palmitic acid was assessed in myoblasts using the MTT assay [20]. First, 96-well plates were seeded at a cell concentration of 1×10^5 cells mL^{-1}. The samples were treated after a 24-hour incubation period and further incubated for 24 hours. The 70% ethanol extracts and DPHC of each sample were considered in multiple ranges and treated in the wells. The assay results were obtained at an optical density of 540 nm [21]. Palmitic acid was used at concentrations of 0.1, 0.3, 0.5, and 0.75 mM.

3.2.5 Analysis of mitochondrial membrane potential by flowcytometer

The mitochondrial membrane potential was assessed following a 6 h incubation from PA treatment. A MitoProbe JC-1 detection kit (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide; Thermo Fisher Scientific, Waltham, MA,

USA) was utilized in the process of flowcytometric analysis (BD LSRFortessa, Franklin Lakes, NJ, USA) following the manufactures instructions. Cultured cells were differentiated to myotubes and were harvested for the assay following sample treatment and stimulation.

3.2.6 ROS detection

The intracellular ROS levels were assessed via 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA) fluorescent dye where molecular probe Dihydrorhodamine 123 (DHR123) was used to evaluate mitochondrial ROS [22]. The differentiated, treated, and stimulated myotubes were stained with DCFH2-DA and DHR123 for 30 min at 37°C. The cells were washed with PBS and fluorescent images were captured with a laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). The images were analyzed assisting the ImageJ software (version 1.50i, NIH, USA).

3.2.7 Immunofluorescence

Gelatin-coated coverslips were used to grow C2C12 myoblasts. The samples were treated, and the media was changed accordingly to proceed with differentiation. The cells were fixed and stained at different times, depending on the experimental model. Paraformaldehyde (4%) was used to fix the cells. The cells were then permeabilized with 0.1% Triton X-100. The cells were blocked with 10% donkey serum and incubated with primary antibody (1:100 in 10% donkey serum) for 1 h at ambient temperature. Alexa Fluor ® 488 and/or Alexa Fluor ® 647 conjugated secondary antibodies were used, followed by 4′,6-diamidino-2-phenylindole (DAPI) staining. The stained coverslips were mounted onto glass slides with Dako Faramount Aqueous Mounting Media (Agilent

Technologies, Santa Clara, CA, USA). Images were captured using a confocal microscope (Carl Zeiss, Oberkochen, Germany) [23, 24].

3.2.8 Western blot analysis

Cells were harvested depending on the protein analysis and the effect of the sample. Briefly, the cells were harvested, lysed, and the proteins were measured by BCA protein assay. The normalized proteins were separated by electrophoresis on 8% or 10% sodium dodecyl sulfate-polyacrylamide gels. The proteins were then electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with skim milk and then probed with primary antibodies overnight at 4°C. Secondary antibody incubation was conducted, and membranes were developed with enhanced chemiluminescence (ECL) substrate (Cyanagen Srl, Bologna, Italy). Images were captured using a FUSION SOLO Vilber Lourmat system (Paris, France). Quantification was performed using ImageJ software (version 1.50i, NIH, USA) [25, 26].

3.2.9 ELISA detection

The C2C12 myotubes were treated with sample and were PA-induced. The cell supernatants were collected and assayed for IL-1β and IL-18 using commercial ELISA kits following manufactures instructions (Thermo Scientific, Waltham, MA, USA).

3.2.10 Statistical analysis

The experiments were performed in triplicate to obtain the final data. Data are expressed as means ± standard deviation. One-way analysis of variance (ANOVA) using Tukey's test, was used to evaluate statistical significance. Two-tailed Student's *t*-tests were

performed when two conditions were compared. The significance level was defined as p <0.05 ($p<0.05$). GraphPad Prism 7 (GraphPad, CA, USA) was used to perform the statistical analyses. Furthermore, each figure legend contains the statistical conditions.

3.3 Results

3.3.1 Palmitate affects myoblast cell viability

The cytotoxicity of palmitic acid was evaluated via MTT assay after 48 hrs for a range of concentrations. The results showed a half-maximal inhibitory concentration (IC50) of 0.55 mM. Thus, a concentration of 0.5 mM was used in subsequent experiments based on the evidence that PA did impair myoblast survival (Figure 3-1a).

3.3.2 Effect of IO extract and DPHC on myoblast survival

Ishige okamurae extract (70% EtOH) and DPHC were assessed for its impact on myoblast survival using MTT assay. This revealed IO extract sample concentrations ≤25 μg/mL did not show cytotoxicity (Figure 3-1b). DPHC sample concentrations ≤12.5 μg/mL were not altering cell viability significantly (Figure 3-1c). Thus, IO (25 μg/mL) and DPHC (12.5 μg/mL) were chosen for subsequent experiments. The pre-treatment of IO/ DPHC substantially improved the cell survival against PA stimulation of C2C12 myoblasts (Figure 3-2a, b).

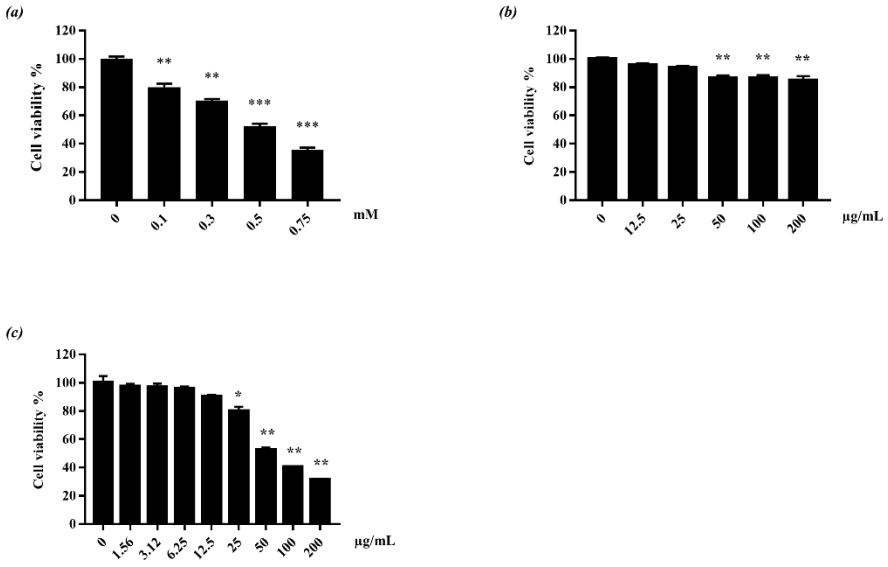

Figure 3- 1. *Assessment of cell viability in C2C12 myoblasts. (**a**) Cytotoxicity of palmitic acid by MTT assay. (**b**) Cell viability against 70% EtOH extract of Ishige okamurae and (**c**) DPHC via MTT assay. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$.*

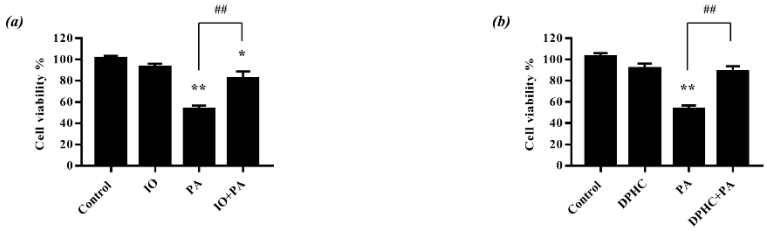

Figure 3- 2. *Cell viability assessed in selected concentrations of (a) IO and (b) DPHC under the stimulation of PA. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.3 IO extract inhibits PA induced ROS generation

Images from confocal microscope revealed that PA exposed myotubes to express significant fluorescence intensity when stained with DCFH2-DA. This correlates to the ROS production. Though, the ROS levels were successfully constrained by the IO extract treatment (Figure 3-3a, b). The results were further confirmed by the JC-1 assay by flowcytometry. This directly measured the mitochondrial membrane potential through the JC-1 probe, where it unveiled the palmitates potential to develop unhealthy mitochondria leading to declined myotube survival (Figure 3-4a, b). JC-1 is a mitochondrial monitoring probe to assess the potential dependent behavior. Lower membrane potentials correlate unhealthy mitochondria yielding green fluorescence, whereas at higher membrane potential is reflected as red fluorescence. However, IO extract recovered the myotubes mitochondrial membrane potentials significantly.

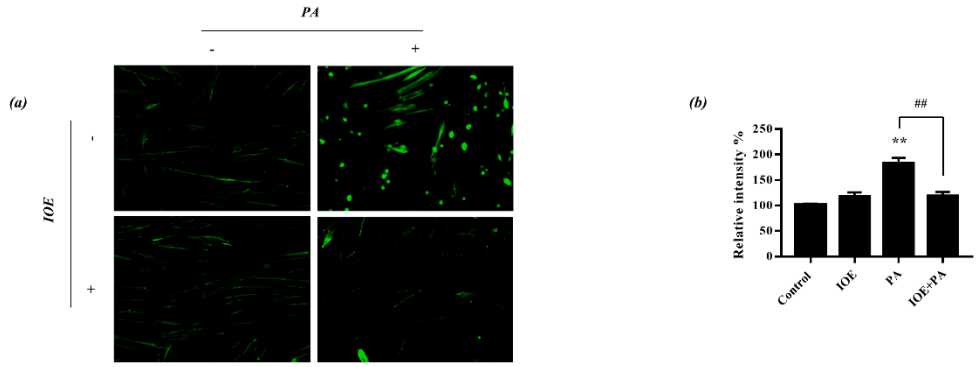

Figure 3- 3. *The effects of IOE on PA-induced ROS production in C2C12 myotubes.* ***(a)*** *Fluorescence microscopic images,* ***(b)*** *quantitative expression of fluorescence intensities. The cells were pre-incubated in IOE, and cells were stimulated with PA for 24 h. The cells were stained with DCFH-DA, incubated, and subjected to fluorescence analysis. The relative fluorescence levels were measured by ImageJ software. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

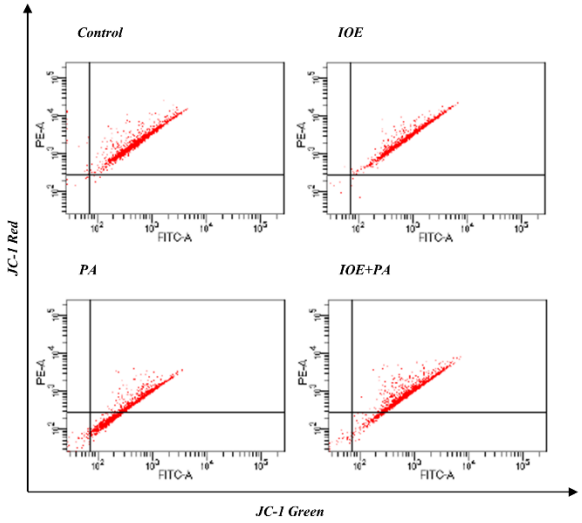

Figure 3- 4. *Ishige okamurae ethanol extract protects C2C12 myotubes from PA induced mitochondrial ROS. Flowcytometry showed the mitochondrial membrane potential with JC-1 probe reduced from red fluorescence to green fluorescence with PA treatment.*

3.3.4 Palmitate induced mitochondrial damage/depolarization is weakened by DPHC, alleviating mitochondrial ROS production

The depolarized mitochondrial membrane potential indicated by green fluorescence in PA stimulation was suppressed by DPHC treatment as shown in Figure 3-5a, b. Mitochondrial status was further examined using DHR123 as a ROS indicator. An overexpression of mitochondrial ROS (mtROS) was indicated by fluorescence intensities in the PA induced group. Pre-treatment with DPHC significantly reduced the effect caused by PA (Figure 3-6a, b). DPHC stabilized mitochondrial membrane integrity leading to declined levels of mtROS production. Mitochondrial membrane potential is closely related to apoptosis via caspase associated proteins.

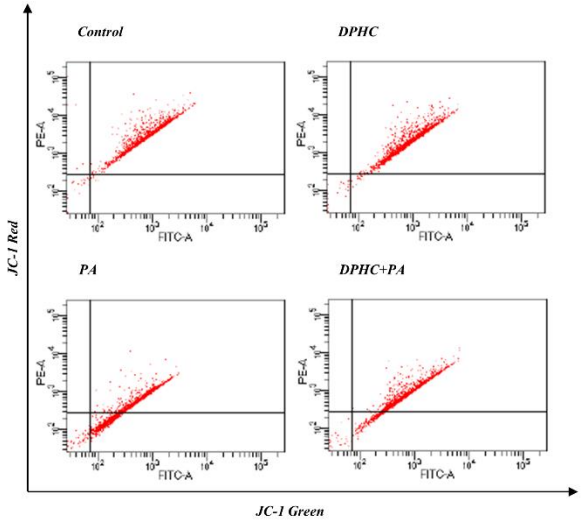

Figure 3- 5. *Effect of DPHC on suppressing disruption of mitochondria membrane potential in PA-stimulated C2C12 myotubes. Investigation of the loss of mitochondrial inner transmembrane potential after PA stimulation via JC-1 assay.*

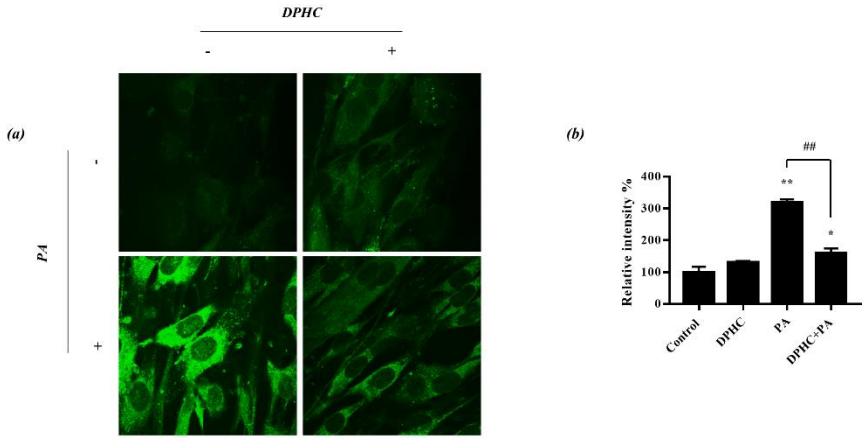

Figure 3- 6. *DPHC reduced mitochondrial reactive oxygen species (ROS) generation in PA-induced C2C12 myotubes. (a) Mitochondrial ROS was assessed using confocal microscopy after staining with dihydrorhodamine 123 (DHR123) (10 μM). (b) Relative intensity assessed via ImageJ software. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.5 DPHC alleviated the expression and formation of Nlrp3 inflammasome in C2C12 myotubes stimulated by PA

Western blotting analysis was performed to analyze the expression of Nlrp3, ASC, and caspase-1, to address the contribution of PA in the Nlrp3 inflammasome. It was evident that components of Nlrp3 inflammasome were expressed with the stimulation of PA (Figure 3-7a-e). The effect of DPHC pre-treatment to alleviate the negative effects were then verified. It was observed, DPHC could repress the expression of the Nlrp3 inflammasome substantially. Further, the IL-1β and IL-18 secretion with PA induction was also detected through ELISA analysis (Figure 3-8a, b). This was significantly altered with the DPHC treatment. Additionally, Nlrp3 complex formation (Nlrp3/ASC) was verified through immunofluorescence staining (Figure 3-9a, b). It was found that PA considerably increased the inflammasome formation compared to the control group. Treatment with DPHC markedly inhibited inflammasome formation. The myotubes were pre-treated with MCC950, a Nlrp3 inhibitor to confirm the significance of Nlrp3 inflammasome formation in IL-1β and IL-18 release. It was observed that the Nlrp3 inflammasome inhibition led to the downregulation of the expression of PA stimulated IL-1β and IL-18 (Figure 3-10a-c). This confirms the vital role of Nlrp3 inflammasome in the release of IL-1β and IL-18. Thus, the results support the potential of PA to induce Nlrp3 inflammasome and to release IL-1β and IL-18. However, DPHC successfully hampered the negative effects caused by PA where it would lead to a healthy cellular environment.

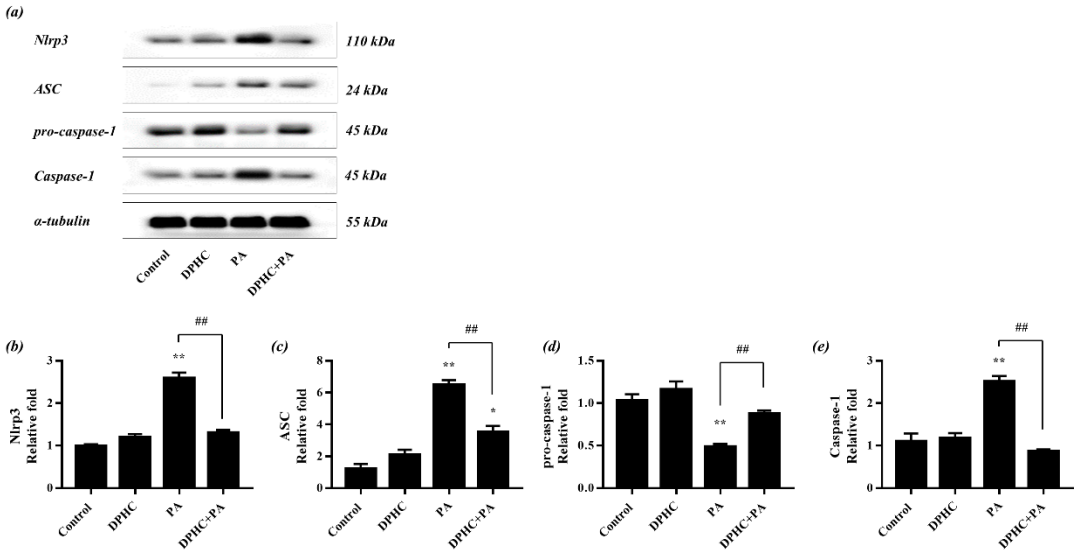

Figure 3- 7. *DPHC inhibits PA-induced Nlrp3 inflammasome activation. (a) Western blotting of Nlrp3 inflammasome components. The cells were treated with DPHC and stimulated with PA prior harvesting C2C12 myotubes. Quantitative representation of (b) Nlrp3, (c) ASC, (d) pro-caspase-1, and (e) Caspase-1. Data are represented as mean ± SE of three independent experiments. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

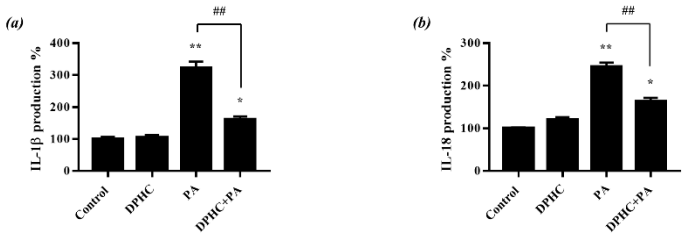

Figure 3- 8. *Potential of DPHC to reduce inflammatory cytokine release stimulated by PA due to Nlrp3 inflammasome activation. (a) IL-1β, (b) IL-18. The ELISA experiments were conducted using C2C12 myotube supernatants following manufactures guidelines. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

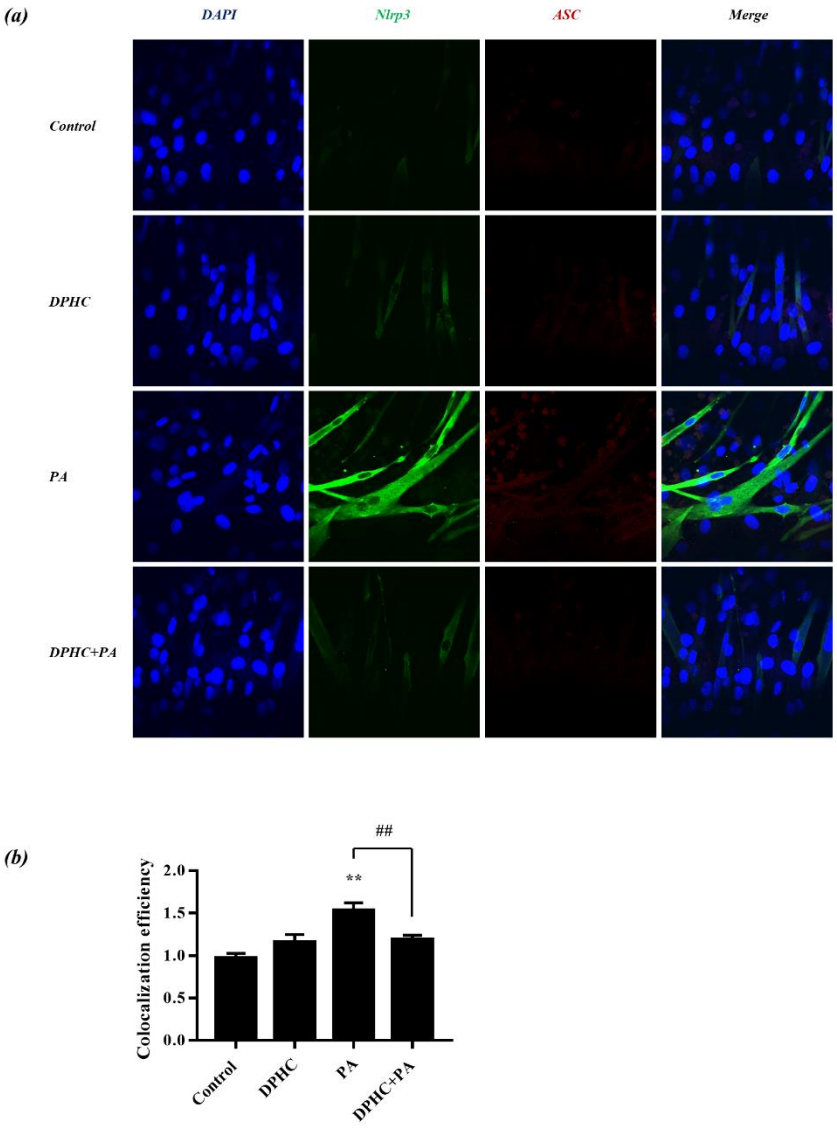

Figure 3- 9. *The effect of DPHC on ameliorating PA-induced formation of Nlrp3 inflammasome. (**a**) Representative fluorescent microscopic images showing the colocalization of Nlrp3/ASC. (**b**) Summarized data expressing colocalization efficiency for Nlrp3/ASC. Data are represented as mean ± SE of three independent experiments. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

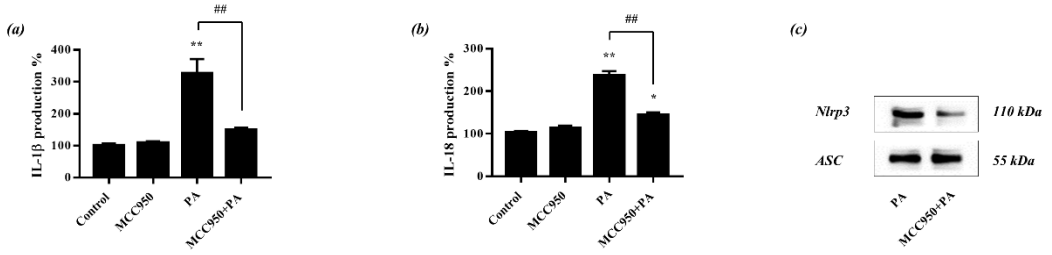

Figure 3- 10. *A Nlrp3 inhibitor, MCC950 blocks the effects caused by PA. The cells were treated with MCC950 prior to the stimulation with PA. ELISA analysis was conducted to evaluate (**a**) IL-1β, (**b**) IL-18 levels in the myotube supernatants. (**c**) Western blotting for PA and MCC950 treated myotubes. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.6 Palmitate activates Nlrp3 inflammasome through mitochondrial ROS production

It was determined whether ROS mediate PA-induced mitochondrial dysfunction. Thus, the C2C12 myotubes were pre-incubated with Mito-Tempo, a mitochondrial targeted superoxide scavenger. The mitochondrial ROS was then measured with DHR123. Accordingly, mtROS scavenger suppressed the basal level of ROS production (Figure 3-11a, b). Further, Mito-Tempo significantly reversed the PA induced mitochondrial ROS production. Simultaneously, the implication of mtROS in the activation of Nlrp3 inflammasome was assessed. As elaborated in Figure 3-12 a-e, mtROS scavenger Mito-Tempo remarkably reduced PA-induced Nlrp3 inflammasome activation. Additionally, PA-stimulated production of IL-1β and IL-18 were as well inhibited with the Mito-Tempo treatment (Figure 3-13a, b). Taken together, the results implicate mtROS production induced by PA alters cellular dynamics and plays a central role in the Nlrp3 inflammasome mediated IL-1β and IL-18 expression. Collectively, DPHC is a potent alleviator of mtROS where it reverses mitochondrial dynamics thus providing mitigation of inflammasome activation.

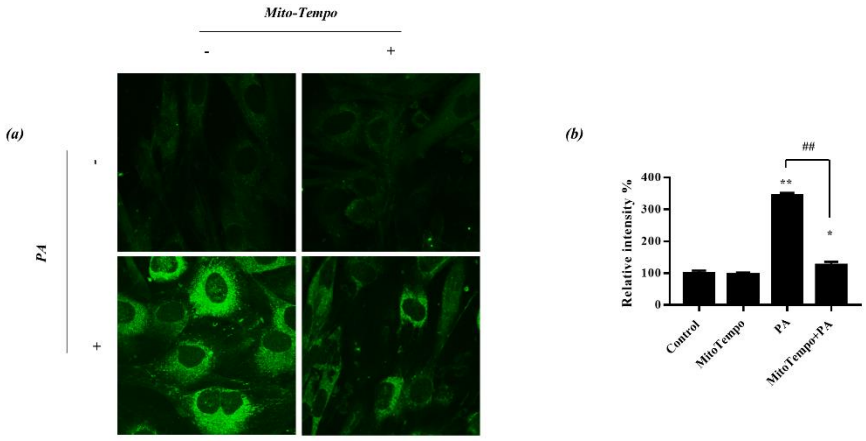

Figure 3- 11. *Mito-Tempo reduced mitochondrial reactive oxygen species (ROS) generation in PA-induced C2C12 myotubes. (**a**) Mitochondrial ROS was assessed using confocal microscopy after staining with dihydrorhodamine 123 (DHR123) (10 μM). (**b**) Relative intensity assessed via ImageJ software. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

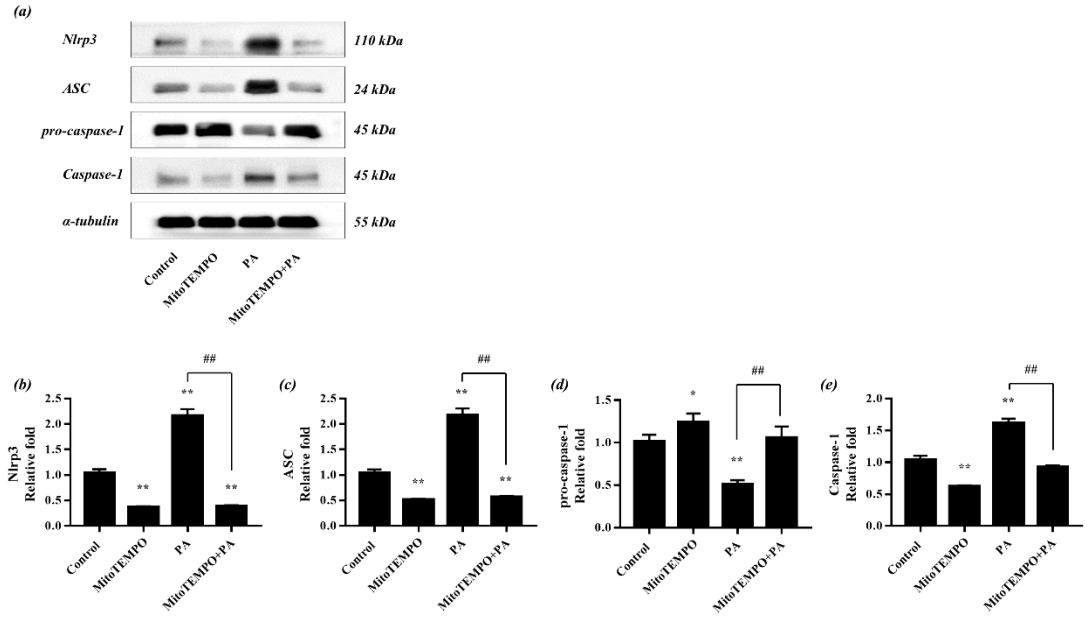

Figure 3- 12. *The effect of MitoTempo on ameliorating PA-induced formation of Nlrp3 inflammasome. MitoTempo (10 μM), a mitochondrial ROS scavenger was treated prior to the stimulation of PA. (**a**) Western blotting of Nlrp3 inflammasome components. Quantitative representation of (**b**) Nlrp3, (**c**) ASC, (**d**) pro-caspase-1, and (**e**) Caspase-1. Data are represented as mean ± SE of three independent experiments. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

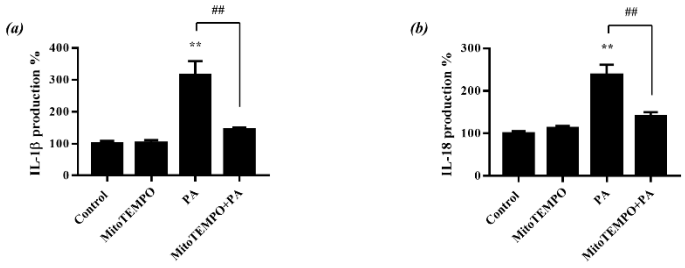

Figure 3- 13. *MitoTempo inhibits Nlrp3 activated pro-inflammatory cytokine release. ELISA evaluation of (**a**) IL-1β and (**b**) IL-18. The C2C12 myotubes were treated with MitoTempo before the PA-induction. The supernatants were collected and subjected to ELISA assay following manufactures guidelines. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.7 DPHC inhibit PA-induced nuclear translocation of NF-κB

Elevated expression of NF-κB signal is a priming stimulus for Nlrp3 inflammasome activation. It was addressed whether PA induce NF-κB, thereby affect Nlrp3 inflammasome. Signaling cascade leading to NF-κB activation was assessed against PA stimulation and subsequently potential of DPHC to attenuate it. It was observed PA markedly enhanced the nuclear translocation increasing nuclear p65 levels (Figure 3-14a, e). The cytosolic p65 phosphorylation was influenced by the PA treatment (Figure 3-14a, d). The signaling reaching from the phosphorylation of IKKα and IκBα were upregulated by PA treatment, that DPHC pre-treatment repressed it bringing back to basal levels (Figure 3-14 a-c). Further, PA-induced p65 nuclear translocation was confirmed by immunofluorescence analysis, where DPHC significantly downregulated it (Figure 3-15).

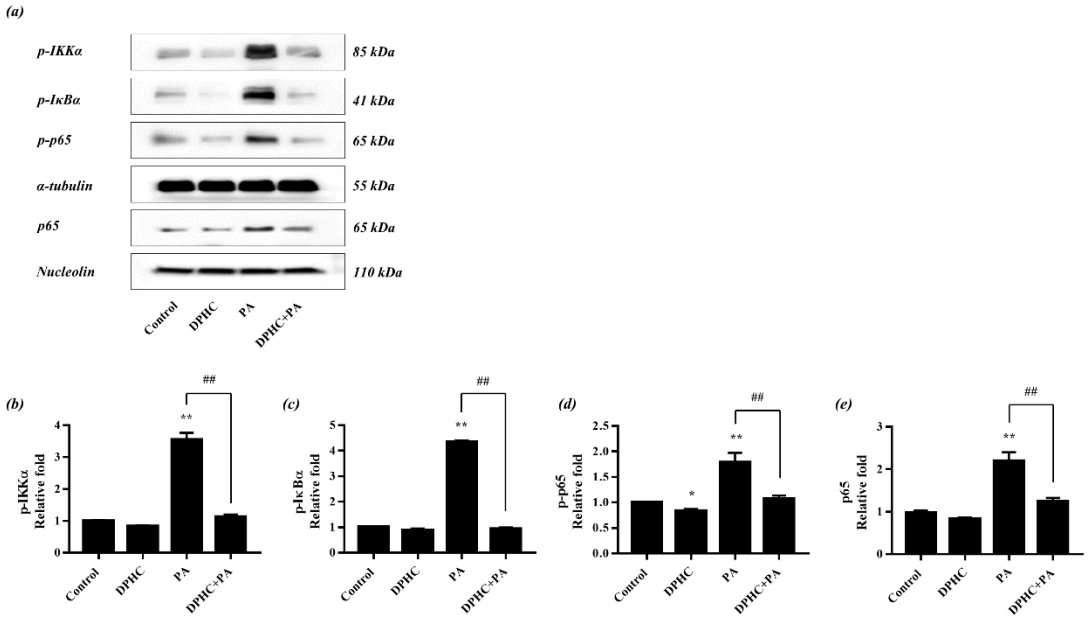

Figure 3- 14. *Palmitate activated NF-κB signaling is attenuated by DPHC. (a) Western blotting analysis of pathway proteins. Quantitative expression of (b) p-IKKα, (c) p-IκBα, (d) cytosolic p-p65, and (e) nuclear p65. The C2C12 myotubes were treated with DPHC and were stimulated with PA. Cells were harvested and subjected to western blotting. Quantitative analysis was assisted by ImageJ software. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

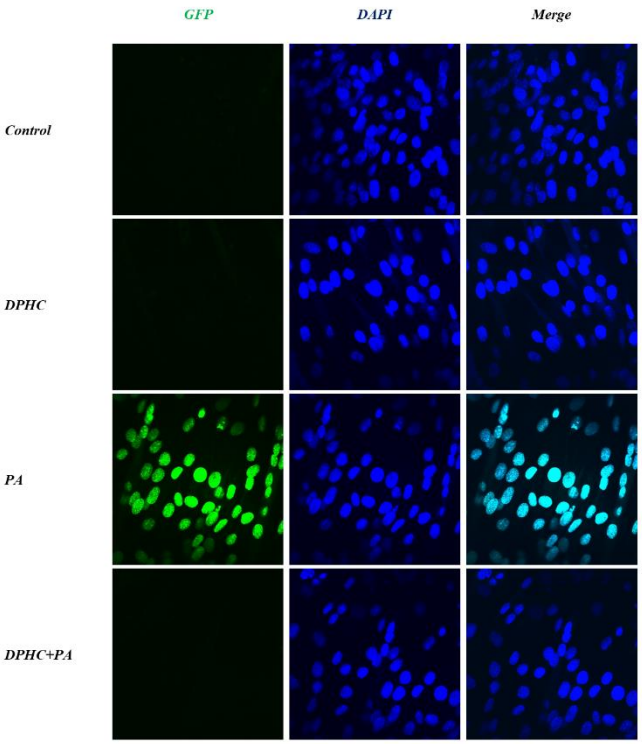

Figure 3- 15. *Influence of DPHC on PA stimulated C2C12 myotubes NF-κB p65 level. Nuclear translocation of NF-κB (p65) through immunofluorescence analysis. Images were captured via confocal microscope.*

3.3.8 DPHC improves PA-altered antioxidant enzyme pathway proteins

Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) play a vital role in the removal of excessive ROS to relieve oxidative stress. Thus, the levels of SOD and CAT in the myotubes were measured under PA stimulation. As illustrated in Figure 3-16 a-c, SOD and CAT levels were improved by DPHC treatment. PA-induced group expressed decreased SOD/ CAT levels leading to increased generation of ROS in mitochondria. Pre-treatment with DPHC could effectively prevent PA-stimulated damage.

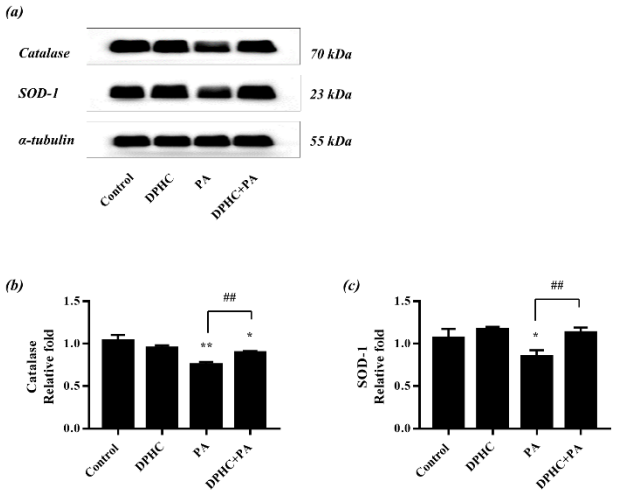

Figure 3- 16. *Potential of DPHC to improve antioxidant activity to protect C2C12 myotubes against PA. Catalase and SOD-1 levels were assessed via (a) Western blotting analysis. (b), (c) Quantitative data analyzed via ImageJ software. Data are represented as mean ± SE of three independent experiments. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.9 DPHC inhibits PA-induced caspase-3/caspase-9 activation and restore the PA-altered Bax/Bcl-2 balance

Apoptosis is closely related with the caspase-3, caspase-9 activation. Palmitate stimulation elevated the caspase-3 and caspase-9 expression significantly (Figure 3-17a-e). This was markedly inhibited by the pre-treatment of DPHC. The activation of caspases linked with apoptosis can be suppressed by the DPHC treatment. Mitochondria membrane confines the Bcl-2 family proteins, where these mediate the downstream mitochondria dependent apoptosis. The mitochondrial membrane potential considerably influences the activation of the particular proteins. Both pro- and anti-apoptotic proteins were evaluated to determine the effects of palmitate related to mitochondria dependent apoptosis. It was evident that DPHC treatment restored the Bcl-2 expression which was declined by the PA-stimulation. Further, Bax expression was increased by PA, where DPHC successfully inhibited it. Thus, the results confirm the potential of DPHC to block PA-mediated alteration of mitochondria mediated apoptosis.

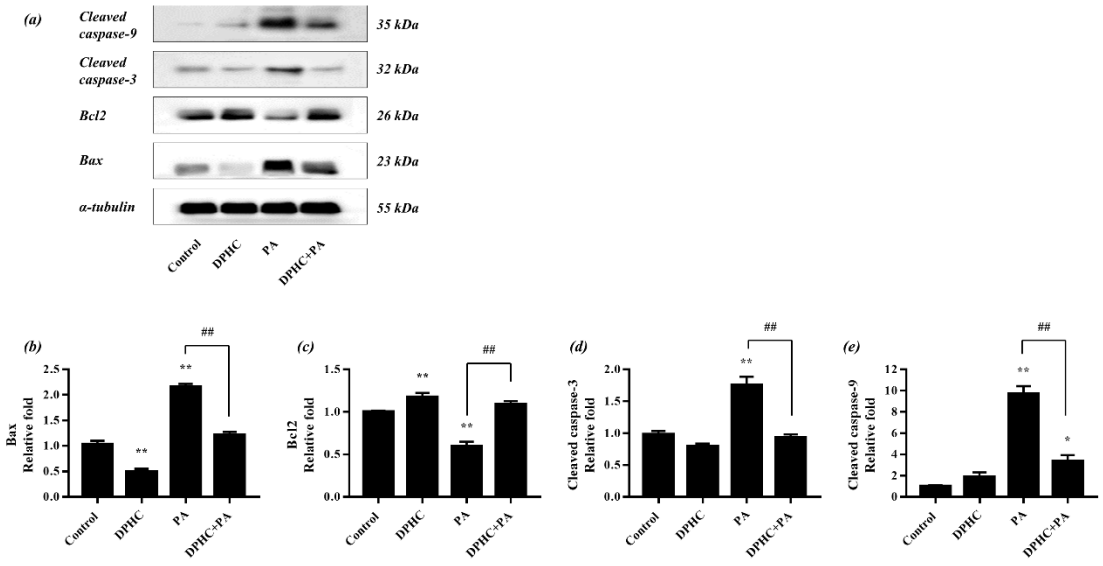

Figure 3- 17. *Effect of DPHC against PA-induced C2C12 myotubes, (a) Western blot analysis of related proteins. Quantitative representation of (b) Bax, (c) Bcl2, (d) Cleaved caspase-3. and (e) cleaved caspase-9 analyzed via ImageJ software. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.3.10 Palmitate leads to muscle wasting in-vitro by influencing on atrogenes

The ubiquitin ligases muscle ring finger-1 (MuRF-1) and muscle atrophy F-box (MAFbx/Atrogin-1) overexpression leads to muscle atrophy. Thus, are direct drug targets against muscle atrophy treatments [27]. NF-κB activation associated with Nlrp3 inflammasome influence the expression of atrogenes. It was observed atrogenes to be overexpressed under the PA-stimulation (Figure 3-18a-c). Though pre-treatment with DPHC significantly, reduced the expression of these.

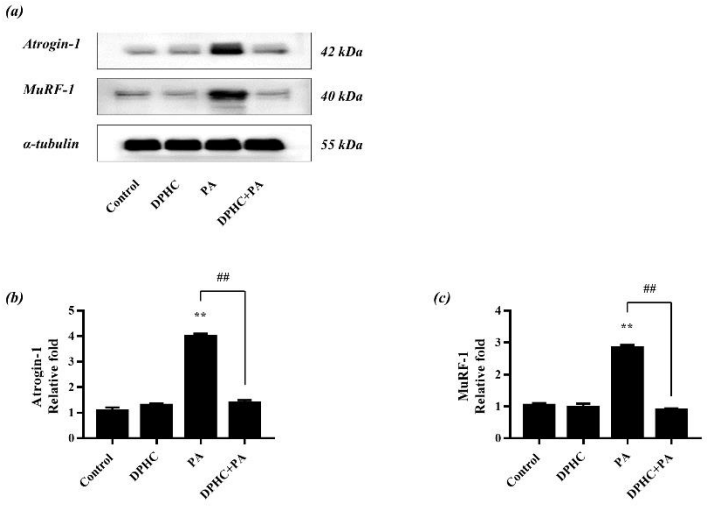

Figure 3- 18. *Effect of DPHC on the expression of atrogenes in PA-induced C2C12 myotubes. (a) Western blot analysis of related proteins. (b) Atrogin-1, (c) MuRF-1 quantitatively represented analyzed via ImageJ software. Data are represented as mean ± SE of three independent experiments. The experiments were triplicated, and data are represented as mean ± SE. Significance is denoted compared to control. * $p < 0.05$, ** $p < 0.01$. Significance compared to PA group is denoted as # $p < 0.05$, ## $p < 0.01$.*

3.4 Discussion

Despite the evidence of free fatty acid (FFA) circulation affect obese-sarcopenia, the underlying mechanisms for fatty acid driven lipotoxicity remains undisclosed. The present study palmitic acid in a substantial cytotoxic concentration is implemented to examine the above. The cell death mediated by PA was further investigated to reach the cellular dynamics. Influence of FFA in the cell damage has been previously reported in brain tumors [28], skeletal muscle cells [29]. It is still vague whether the FFAs directly influence the negative effects, or its metabolites lead the way. A detailed report by Zhang et al. (2019) suggests FFA induced myocardial cell injury to be occurred via Nlrp3 inflammasome activation [9]. The common factor associated with FFA driven cellular damage is noticeable to be its close association with mitochondrial dysfunction. This study appraises the palmitate driven muscular damage to understand the molecular mechanisms of obese-sarcopenia. Lipotoxicity could be rescued by pharmacological inhibitors. Hence, further to the study, we evaluate marine natural products as a tailored strategy to counteract lipotoxicity signaling derived from PA representing obese-sarcopenia in cellular environment.

Reactive oxygen species (ROS) is a byproduct of oxidative phosphorylation in mitochondria. Regular mitochondrial respiration converts ~1–2% of molecular oxygen (O_2) to superoxide and hydroxyl radicals. Further down the chain of respiration combining complexes, molecular oxygen is reduced to water. Due to enzyme activity in the process such as NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxygenases, ROS is generated in the cells [8]. Mitochondrial ROS (mtROS) function as a vital signaling platform for multiple biological responses. These include metabolism, immune functions and inflammation [30]. Moreover, mtROS involve actively in the activation of Nlrp3 inflammasomes. This is critical to defense the pathogenic toxic action.

Though, uncontrolled regulation of mtROS would lead to pathologies. Yet controlled production of mtROS is essential for the induction of efficient immune responses where excessive generation would lead to mitochondrial damage and sustained inflammation [8]. The implemented palmitate concentration reduced the cell viability significantly where treatment with IO and DPHC reinstated it. As per evident by the Figure 3-3, PA substantially improved cellular ROS levels. The fluorescent intensity indicated by the DCFH2-DA staining was overcome by the pre-treatment of IOE. Next, the mitochondrial ROS levels were evaluated by refereeing to its membrane potential. JC-1 probe indicated the unhealthy mitochondria by green fluorescence, where red fluorescence was the indication of healthy mitochondria. Based on these premises, the red/green fluorescence ratio of the dye in the mitochondria can be considered as a direct assessment of the state of mitochondria polarization. A higher mitochondrial polarization is the indication of red shift where depolarized mitochondria are represented by green fluorescence. In the current examination, the flow cytometer was used to detect the accumulation of mitochondria and its status. Initially, IOE recovered the depolarized mitochondria membrane potential due to PA treatment (Figure 3-4). Further, the potential of DPHC was assessed to alleviate mtROS production. Both the JC-1 probe assessment and DHR123 evaluation revealed the ability of DPHC to successfully relieve the mtROS production induced by PA treatment. Gao et al. (2011), published a detailed report on the effect of oxidative damage and mitochondrial dysfunction in C2C12 myotubes [4]. It revealed PA to induce mtROS production, mitochondrial dysfunction, and tetramethylpyrazine (TMP) an active ingredient mitigated the negative effects and improved mitochondrial biogenesis.

Innate immunity involves inflammasomes as a crucial member. Inflammasomes are multiprotein oligomer platforms coupled with caspase and interleukins. Nlrp3

inflammasome is maintained in an inactive state endogenously, where its activation promote assembly with apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC). This process recruits pro-caspase-1, forming a complex ultimately establishing a ring like structure [31]. Activation of inflammasome leads to pyroptosis, a distinctive process of programed cell death via the activation of caspases and downstream signaling of IL-1β and IL-18. Moreover, inclined levels of IL-1β and IL-18 influence inflammatory cascade. ROS is a vital factor of inflammasome activation. Dysfunctional mitochondria accumulation augments the ROS production thus activating inflammasome [6]. Given the importance, inflammasome and related cascades have become a therapeutic drug target for skeletal muscle atrophy. In the present study, Nlrp3 inflammasome behavior was evaluated against PA-stimulation. DPHC was implemented as a potential therapeutic component to alleviate the effects of PA. It was observed, PA to induce Nlrp3 protein complex component protein expression to significant levels compared to control group. The pre-treatment of DPHC successfully deteriorated the effects of PA. This in result affected the secretion of IL-1β and IL-18 levels, which DPHC successfully reduced its levels. The involvement of Nlrp3 was further confirmed via the application of MCC950, a Nlrp3 inhibitor. This revealed, IL-1β and IL-18 expressions to be inhibited with the MCC950 treatment under the stimulation of PA. A comprehensive review article by Peladeau and Sandhu (2021) discuss the Nlrp3 inflammasome activation and association to neuromuscular disease is worth refereeing [31]. Inflammasome upregulation and activation in dysferlin-deficient skeletal muscle was published by Rawat et al. (2010) [32]. They suggest that skeletal muscle is an active contributor of IL-1 and strategies interfere with the given pathway would act therapeutically useful in muscular dystrophy. Adiporon, a small protein adiponectin receptor agonist was reported to alleviate the cardiomyocyte injury caused

by palmitate via suppressing Nlrp3 inflammasome activation [9]. Similar results were reported concerning mtROS production and Nlrp3 inflammasome complex formation. Nlrp3 blocking via siRNA confirmed its involvement in the cardiomyocyte injury. The results of the present study regarding mtROS production and Nlrp3 inflammasome complex formation due to palmitate stimulation well aligns with it. However, present study utilizes marine natural products to intervene the skeletal cell damage due to palmitate stimulation. Additionally, consistent with our results, it was observed that pretreatment with MitoTempo substantially downregulated the Nlrp3 inflammasome activation and resulting IL-1β and IL-18 secretion. This indicated the significance of mtROS in the activation of Nlrp3 inflammasome induced IL-1β and IL-18 release. Nevertheless, the effect of PA on the mtROS driven inflammasome activation was hindered by the DPHC treatment.

Another point of evaluation was the NF-κB pathway signaling. NF-κB component p65 phosphorylation was evident to be improved with the PA treatment. The results demonstrate the upstream signaling (IKKα, IκBα) were delivering pathway for p65 nuclear translocation. Though the membrane signaling was not evaluated in this study, previous reports provide evidence of PA activating TLR2/4. Nicholas et al. (2017), published on the PA stimulated activation of TLR4 receptor in the human dendritic cell secretion of IL-1β [33]. The pathway was further assessed regarding the atrogenes (atrogin-1 and MuRF-1). It was observed atrogenes expression to be improved with the NF-κB, Nlrp3, and IL-1β activity. Fang et al. (2021), elaborates on the LPS-induced skeletal muscle atrophy and the involvement of NF-κB and TNFα. They propose triptolide from *Tripterygium wilfordii* to prevent the LPS-induced inflammation and muscle atrophy ultimately preventing muscle wasting [34]. In the present study atrogene activation due to PA was substantially lowered by DPHC pre-treatment suggesting its

potential in the process of preventing muscle wasting activated through NF-κB and Nlrp3 pathways. Both NF-κB and Nlrp3 could act upon each other to induce mutual pathway leading to production of IL-1β and IL-18. Further, mtROS generation vice versa could be stimulated due to the upregulated myokines, NF-κB signaling, elaborating the character of this vicious cycle/interplay once initiated. Thus, proper modulation of mtROS and Nlrp3 inflammasome is crucial in the good health condition of muscle tissue.

Additionally, apoptotic signaling through mitochondria, the Bax/Bcl-2 pathway in C2C12 skeletal myotubes were assessed to understand the detrimental effects caused by PA. Palmitate improved Bax and decreased Bcl-2 expression where it caused mitochondrial permeabilization [35]. This supports the release of proapoptotic factors such as cytochrome c activating caspase-9 and subsequently caspase-3 leading to macromolecular fragmentation and cell death [36, 37]. However, pre-treatment with DPHC significantly inhibited the pathway proteins and recovered the cellular condition to healthy level.

3.5 Conclusions

The study demonstrates anti-atrophic effect of *Ishige okamurae* and its purified component DPHC in in vitro model of PA induced muscle atrophy. IO and DPHC attenuated PA induced cell injury via inhibiting Nlrp3 activation. The findings suggest DPHC as a potential therapeutic agent for the treatment of skeletal muscle disease from lipotoxicity.

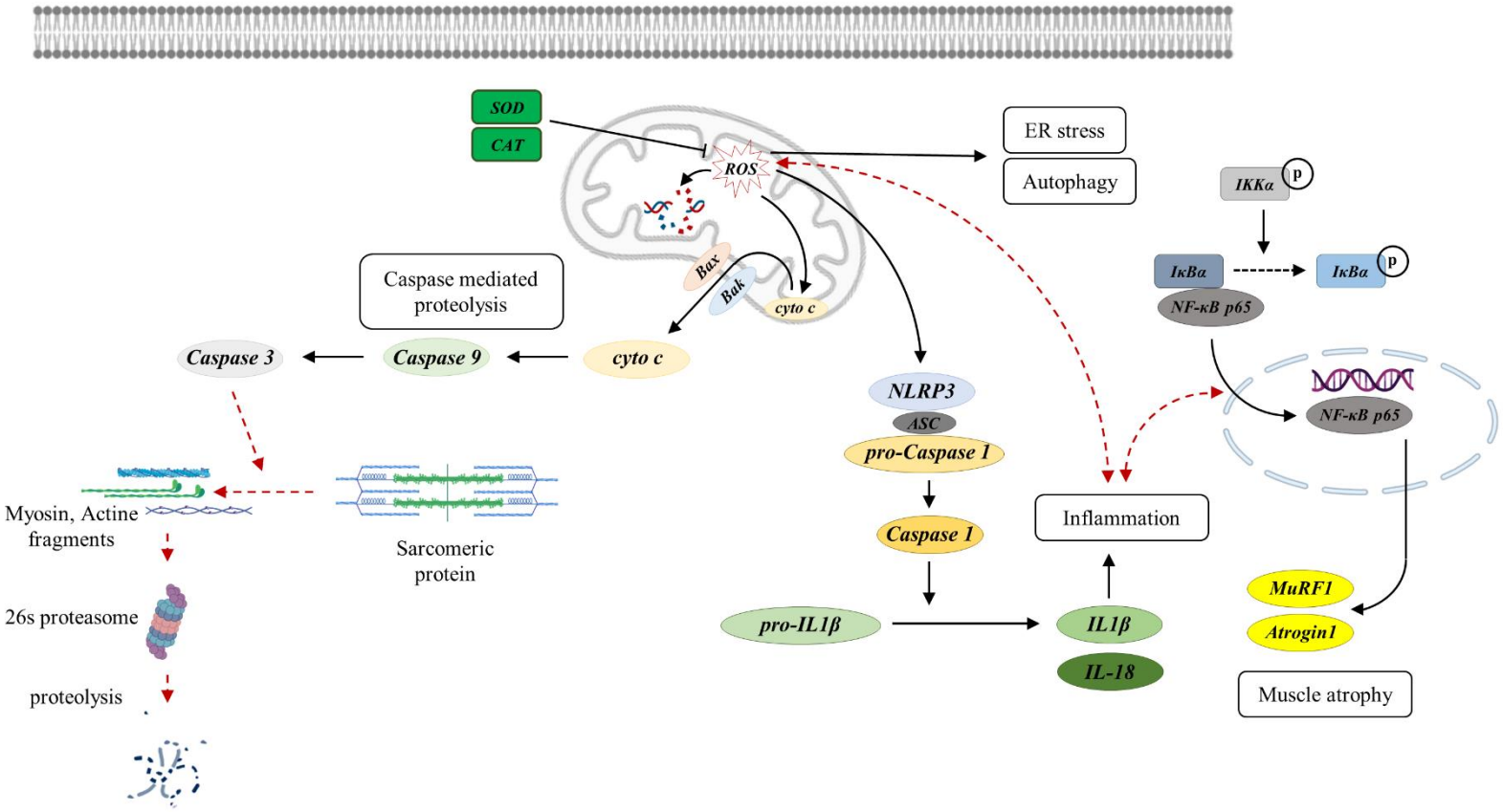

Figure 3- 19. *Graphical representation of the study pathway analyzed.*

References

1. Jayawardena, T.U., S.-Y. Kim, and Y.-J. Jeon, *Sarcopenia; functional concerns, molecular mechanisms involved, and seafood as a nutritional intervention – review article.* Critical Reviews in Food Science and Nutrition, 2021: p. 1-21.
2. Cruz-Jentoft, A.J. and A.A. Sayer, *Sarcopenia.* The Lancet, 2019. **393**(10191): p. 2636-2646.
3. Choi, K.M., *Sarcopenia and sarcopenic obesity.* The Korean journal of internal medicine, 2016. **31**(6): p. 1054-1060.
4. Gao, X., et al., *Tetramethylpyrazine protects palmitate-induced oxidative damage and mitochondrial dysfunction in C2C12 myotubes.* Life Sciences, 2011. **88**(17): p. 803-809.
5. Ceriello, A., *New Insights on Oxidative Stress and Diabetic Complications May Lead to a "Causal" Antioxidant Therapy.* 2003. **26**(5): p. 1589-1596.
6. Harijith, A., D.L. Ebenezer, and V. Natarajan, *Reactive oxygen species at the crossroads of inflammasome and inflammation.* 2014. **5**(352).
7. Abais, J.M., et al., *Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector?* Antioxid Redox Signal, 2015. **22**(13): p. 1111-29.
8. Silwal, P., et al., *Mitochondrial Reactive Oxygen Species: Double-Edged Weapon in Host Defense and Pathological Inflammation During Infection.* 2020. **11**(1649).
9. Zhang, Y.-Z., et al., *AdipoRon Alleviates Free Fatty Acid-Induced Myocardial Cell Injury Via Suppressing Nlrp3 Inflammasome Activation.* Diabetes, metabolic syndrome and obesity : targets and therapy, 2019. **12**: p. 2165-2179.
10. Hwang, D., et al., *Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways.* 2012. **26**(S1): p. 364.8-364.8.
11. Shirosaki, M. and T. Koyama, *Chapter 15 - Laminaria japonica as a Food for the Prevention of Obesity and Diabetes*, in *Advances in Food and Nutrition Research*, S.-K. Kim, Editor. 2011, Academic Press. p. 199-212.
12. Maeda, H., et al., *Fucoxanthin from edible seaweed, Undaria pinnatifida, shows antiobesity effect through UCP1 expression in white adipose tissues.* Biochem Biophys Res Commun, 2005. **332**(2): p. 392-7.
13. Kim, S.Y., et al., *Diphlorethohydroxycarmalol (DPHC) Isolated from the Brown Alga Ishige okamurae Acts on Inflammatory Myopathy as an Inhibitory Agent of TNF-alpha.* Mar Drugs, 2020. **18**(11): p. 529.
14. Yang, H.W., et al., *Ca(2+)-Dependent Glucose Transport in Skeletal Muscle by Diphlorethohydroxycarmalol, an Alga Phlorotannin: In Vitro and In Vivo Study.* Oxid Med Cell Longev, 2021. **2021**: p. 8893679.
15. Argilés, J.M., F.J. López-Soriano, and S. Busquets, *Muscle wasting in cancer: the role of mitochondria.* 2015. **18**(3): p. 221-225.
16. Tabony, A.M., et al., *Protein phosphatase 2C-alpha knockdown reduces angiotensin II-mediated skeletal muscle wasting via restoration of mitochondrial recycling and function.* Skeletal Muscle, 2014. **4**(1): p. 20.
17. Malhi, H., et al., *Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis.* J Biol Chem, 2006. **281**(17): p. 12093-101.
18. Chen, Y., et al., *Histamine H3 Receptor Signaling Regulates the NLRP3 Inflammasome Activation in C2C12 Myocyte During Myogenic Differentiation.* 2021. **12**(1236).
19. Xie, Q., et al., *Chemerin-induced mitochondrial dysfunction in skeletal muscle.* 2015. **19**(5): p. 986-995.

20. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
21. Fernando, I.P.S., et al., *3β-Hydroxy-Δ5-steroidal congeners from a column fraction of Dendronephthya puetteri attenuate LPS-induced inflammatory responses in RAW 264.7 macrophages and zebrafish embryo model.* RSC Advances, 2018. **8**(33): p. 18626-18634.
22. Zhen, A.X., et al., *Diphlorethohydroxycarmalol Attenuates Fine Particulate Matter-Induced Subcellular Skin Dysfunction.* 2019. **17**(2): p. 95.
23. Molagoda, I.M.N., et al., *Anthocyanins from Hibiscus syriacus L. Inhibit NLRP3 Inflammasome in BV2 Microglia Cells by Alleviating NF-kappaB- and ER Stress-Induced Ca(2+) Accumulation and Mitochondrial ROS Production.* Oxid Med Cell Longev, 2021. **2021**: p. 1246491.
24. Jayawardena, T.U., et al., *Anti-Inflammatory Effects of Sulfated Polysaccharide from Sargassum Swartzii in Macrophages via Blocking TLR/NF-Kappab Signal Transduction.* Mar Drugs, 2020. **18**(12): p. 601.
25. Jayawardena, T.U., et al., *Particulate Matter-Induced Inflammation/Oxidative Stress in Macrophages: Fucosterol from Padina boryana as a Potent Protector, Activated via NF-kappaB/MAPK Pathways and Nrf2/HO-1 Involvement.* Mar Drugs, 2020. **18**(12): p. 628.
26. Jayawardena, T.U., et al., *Alginic Acid from Padina boryana Abate Particulate Matter-Induced Inflammatory Responses in Keratinocytes and Dermal Fibroblasts.* Molecules, 2020. **25**(23): p. 5746.
27. Bodine, S.C., et al., *Identification of Ubiquitin Ligases Required for Skeletal Muscle Atrophy.* 2001. **294**(5547): p. 1704-1708.
28. Williams, J.R., et al., *Apoptosis in human primary brain tumours: actions of arachidonic acid.* Prostaglandins, Leukotrienes and Essential Fatty Acids, 1998. **58**(3): p. 193-200.
29. Peterson, J.M., et al., *Bax signaling regulates palmitate-mediated apoptosis in C2C12 myotubes.* 2008. **295**(6): p. E1307-E1314.
30. Sandhir, R., A. Halder, and A. Sunkaria, *Mitochondria as a centrally positioned hub in the innate immune response.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2017. **1863**(5): p. 1090-1097.
31. Péladeau, C. and J.K. Sandhu, *Aberrant NLRP3 Inflammasome Activation Ignites the Fire of Inflammation in Neuromuscular Diseases.* 2021. **22**(11): p. 6068.
32. Rawat, R., et al., *Inflammasome Up-Regulation and Activation in Dysferlin-Deficient Skeletal Muscle.* The American Journal of Pathology, 2010. **176**(6): p. 2891-2900.
33. Nicholas, D.A., et al., *Palmitic acid is a toll-like receptor 4 ligand that induces human dendritic cell secretion of IL-1β.* 2017. **12**(5): p. e0176793.
34. Fang, W.-Y., et al., *Triptolide prevents LPS-induced skeletal muscle atrophy via inhibiting NF-κB/TNF-α and regulating protein synthesis/degradation pathway.* 2021. **178**(15): p. 2998-3016.
35. Zha, H., et al., *Proapoptotic Protein Bax Heterodimerizes with Bcl-2 and Homodimerizes with Bax via a Novel Domain (BH3) Distinct from BH1 and BH2 (*).* 1996. **271**(13): p. 7440-7444.
36. Cai, L., et al., *Hyperglycemia-Induced Apoptosis in Mouse Myocardium.* Mitochondrial Cytochrome c–Mediated Caspase-3 Activation Pathway , 2002. **51**(6): p. 1938-1948.

37. Li, P., et al., *Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade.* Cell, 1997. **91**(4): p. 479-489.

Concluding remarks

Lipid induced sarcopenia is a growing diagnostic and therapeutic challenge because of aging populations, the current obesity epidemic, and changes in lifestyle, especially in the industrialized world. The study of sarcopenia and its associated disease conditions, however, is still in its early stages. The first step towards a more in-depth study of the disease and its consequences should be a consensus on its definition. An important question that remains to be answered is whether the consequences of lipid induced muscle wasting are more severe than those of sarcopenia alone or obesity alone, in other words, whether a synergistic effect of sarcopenia and obesity exists. If sarcopenic obesity does not have a greater impact than sarcopenia alone or obesity alone on long-term consequences and hard endpoints (e.g., CVD and mortality), then even the existence of sarcopenic obesity as a separate entity will be questionable. It should be highlighted that since sarcopenic obesity is a multifactorial disease, its management cannot be based on consideration of a single pathogenetic factor but needs to be multifactorial.

To sum up, sarcopenic obesity is a chronic condition that has metabolic consequences but also impacts physical capacity and possibly the mortality of affected individuals. A consensus on its definition is considered an important step that will greatly facilitate the setting up of mechanistic studies and clinical trials to expand our knowledge, thus improving the management of affected individuals. The current research expected to broaden the knowledge horizon to fill the gaps of understanding of lipid induced muscle wasting, to support fight against it with functional supplements through the sustainable utilization of marine natural products specifically brown alga *Ishige okamurae*, and its bioactive components DPHC and IPA.

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