Investigation on the Effects of Quercetin on Myogenic Differentiation of Human Skeletal Muscle Stem Cells (SkMCs)

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Abstract

This study investigates the effects of Quercetin on the myogenic differentiation of human skeletal muscle stem cells (SkMCs). Skeletal muscles are critical for movement and metabolic processes, and their function declines with age due to conditions like Sarcopenia. This research aims to explore whether Quercetin, a flavonoid known for its antioxidant and anti-inflammatory properties, can enhance the differentiation of SkMCs. The study involved treating SkMCs with varying concentrations of Quercetin and assessing cytotoxicity, differentiation, and microarray analysis. The results were analysed using one-way ANOVA to determine statistical significance. The findings suggest that while Quercetin has a potential impact on SkMCs differentiation, its effects are not statistically significant at the tested concentrations, highlighting the need for further research.

Table of Contents

1. **Introduction:** 8

1.1. Skeletal muscles structure, function., and myogenesis……..……
1.2. Muscle regeneration and role of satellite cells……..……..
1.3. Effects of nutrition on aging muscle.……
1.4. Quercetin supplementation and muscle health……..
1.5. Malnutrition and Sarcopenia……
1.6. Aim…….……..
1.7. Objective……
1.8. Purpose and hypothesis……
2. Methods: ……...
2.1. Skeletal muscle stem cells culture……
2.2. Cells proliferation and counting……
2.3. Nutrient preparation……..
2.4. SkMCs differentiation, nutrient treatment, and cytotoxicity
2.5. SkMCs differentiation, nutrient treatment, and differentiation parameters……
2.6. Differentiation parameters……
2.7. Image J analysis……
2.8. Microarray assay……
2.9. Statistical analysis……
2.10. Ethical consideration
3. Results……....
3.1. Results outline……..
3.2. Cytotoxicity……..
3.3. Differentiation Parameters……
3.4. Microarray Analysis…….
4. Discussion: ……..…….
4.1. Future of the research……..
4.2. Strengths of the research
4.3. Limitations……
5. Conclusion: …….
References

List of Figures:

Figure.1. Structure of skeletal muscles……

Figure.2. Physiology of skeletal muscle, and myogenesis mechanism following injury…

Figure.3. Skeletal muscle regeneration after injury……

Figure.4. Relation between muscle mass and strength to age……

Figure.5. Causes of Sarcopenia……

Figure.6. Haemocytometer and counting chamber……..

Figure.7. ImageJ analysis on immunofluorescent images of myotubes……

Figure.8. Cytotoxicity assay of quercetin treated cells……...

Figure.9. Graphs illustrating changes occurring across three differentiation parameters …..

Figure.10. Immunofluorescent images……

Figure.11. Graphs showing Microarray Analysis……

List of Tables:

Tabe .1. Composition of Growth Media required for SkMCs proliferation……

Table .2. Calculated OD Values for Quercetin treatment at 460nm……...

Table. 3. Components of differentiation media……..

Table. 4. Statistical analysis Growth factors expression……

List of Abbreviations:

SkMCs	Skeletal muscles stem cells
ATP	Adenosine triphosphate
DMD	Duchenne muscular dystrophy
BMD	Becker muscular dystrophy
CTX	Cardiotoxin
SC	Satellite cells
M.A	Myotube area
F.I	Fusion Index
A.R	Aspect Ratio
DPBS	Dulbecco's Phosphate-Buffered Saline (DPBS)
DMSO	Dimethyl sulfoxide
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride

Dissertation Declaration 2024

I Naila Mahmood, hereby declare that this research project titled as "**Investigation on the effects of Quercetin on myogenic differentiation of human skeletal muscle stem cells (SkMCs)"** is solely my work, conducted under the supervision of Dr. Naseer al Shanti at Manchester Metropolitan university.

I affirm that all the data and results presented in this study are original and have been obtained through ethical and standard research methodologies Any outside source or contribution from other researchers have been duly acknowledged and referenced according to academic conventions.

I further certify that this research performed by me has not been submitted before in whole or in part for the award of any degree at any other institute.

This research has been conducted out in conformity with the ethical standards of Manchester Metropolitan University's ethical review board and in accordance with the guidelines provided by the university for research involving human cells.

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MSc Biomedical Sciences

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Manchester Metropolitan university

1. Introduction:

1.1. Skeletal muscle function, structure and myogenesis:

Skeletal muscle accounts for approximately 45-50 % of body mass and is one of the most dynamic and plastic tissues of the human body and is a remarkable component of the locomotion system. (Frontera and Ochala, 2015).They play a vital role in rapid directional movements. Skeletal muscles are attached to the bones through tendons and dominate all the voluntary movements. From evolutionary perspective, skeletal muscles morphology and functions were determined 500-600 million years ago and have been conserved and developed throughout evolution (Wang *et al.*, 2022b).Skeletal muscles are crucial to multiple physiological functions.(Reid and Fielding, 2012). One of the core functions of skeletal muscles is to convert chemical energy into mechanical energy which in returns generates force and power, helping in maintaining the posture, produces movements which help influence social and vocational engagement, sustains or boosts health, and contributes to functional independence. From a metabolic viewpoint, the role of skeletal muscle comprise of a contributor to basal energy metabolism, serving as storage for amino acids and carbohydrates, the production of heat to maintain core temperature, and the consumption of the majority of oxygen and fuel used during physical activity and exercise (Ferrannini *et al.*, 1988). Skeletal muscle also serves as a reservoir of amino acids which are needed by other tissues such as skin, brain, and heart to synthesize organ-specific proteins (Wolfe, 2006).Furthermore, amino acids released by muscle helps in maintaining the blood glucose levels during acute food restrictions and has a highly circadian metabolic profile (Zurlo *et al.*, 1990; DeFronzo *et al.*, 1981; Dyar *et al.*, 2018) .

Skeletal muscle consisting of myofibers or muscle cell which basically are muscle fibres, organized with associated connective tissue. Primarily the size of a muscle is determined by the number and size of these fibres. Muscle fibres are multinucleated and post-mitotic, where each nucleus controls synthesis of protein in its specific region, known as a nuclear domain. Between sarcolemma and basal domain are the satellite cells, which serve as adult stem cells that facilitates muscle growth, repair, and regeneration by proliferating and differentiating into new fibres when activated. Each of the individual muscle is surrounded by a layer of connective tissue called as the epimysium **(Figure 1.**). Within that muscle group of fibres are arrange

themselves in bundles and are surrounded by additional layer of connective tissue known as the perimysium. In addition, complex of various proteins is associated to the sarcolemma which is physically connected to the internal myofilament structure: particularly to a protein actin present in the thin filament. The partial or complete absence or dysfunctions of one of these proteins such as dystrophin may result in damage to the sarcolemma, muscle weakness, and atrophy such as Duchenne and Becker muscular dystrophies (DMD OR BMD) (Thomas, 2013).

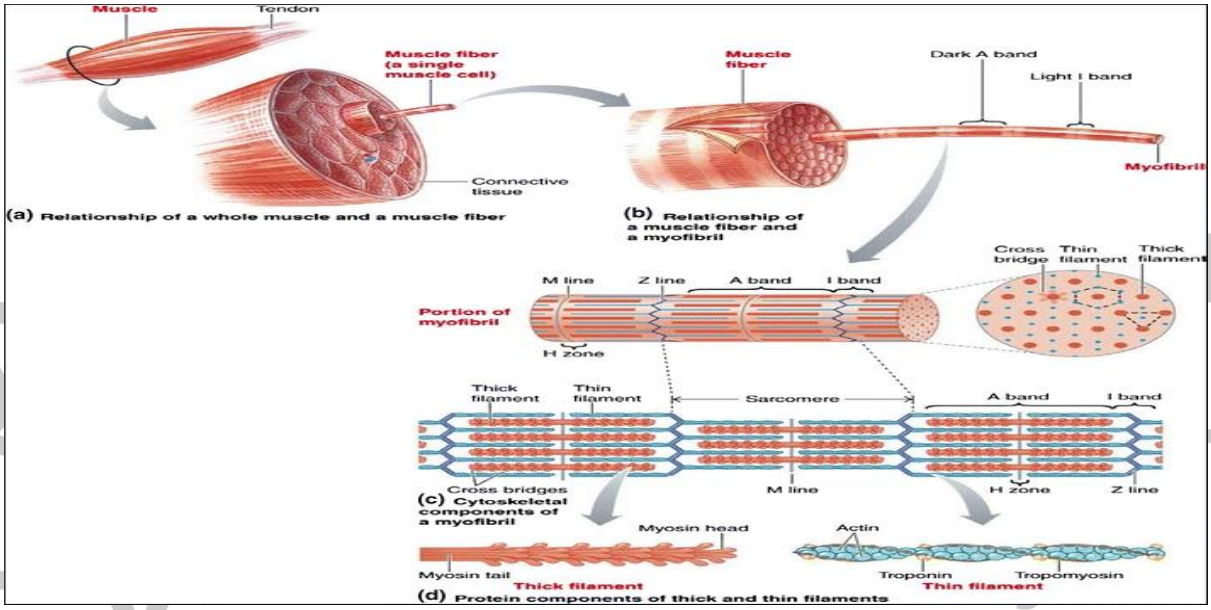

Fig 1. This figure comprehensively illustrates the hierarchical structure of skeletal muscle, from the whole muscle to muscle fibres and myofibrils, along with the detailed arrangement of filaments within sarcomere, which are important for muscles contraction.(Raven *et al.*, 2013; Sherwood and Pysiology, 2010).

Skeletal muscle myogenesis is an extremely synchronized developmental process which is essential for the formation and maintenance of skeletal muscle tissue. The process of myogenesis begins with the specification of mesodermal progenitor cells into myoblasts, which later proliferate, differentiate, and then fuse to form multinucleated myotubes that mature into functional muscle fibres. Pax3, Pax7, MyoD, Myf5, and MRF4 are the myogenic regulatory factors (MRFs) which servers as fundamental for myogenesis and drive the assurance of

progenitor cells to the muscle lineage and compose the transcriptional program required for the differentiation of muscles. (Buckingham and Relaix, 2015; Rudnicki *et al*., 1993; Zammit, 2017) (**Figure 2.**) .Additionally, the interaction between myoblasts, extracellular matrix and various signalling pathways, including those which involves Wnt, Notch, and TGF-β, plays a crucial role in myogenesis regulation and muscle repair (Relaix and Zammit, 2012).(Forcina *et al*., 2019)Satellite cells, play key role in muscle regeneration and hypertrophy, by providing a source of myoblasts for tissue repair and growth.

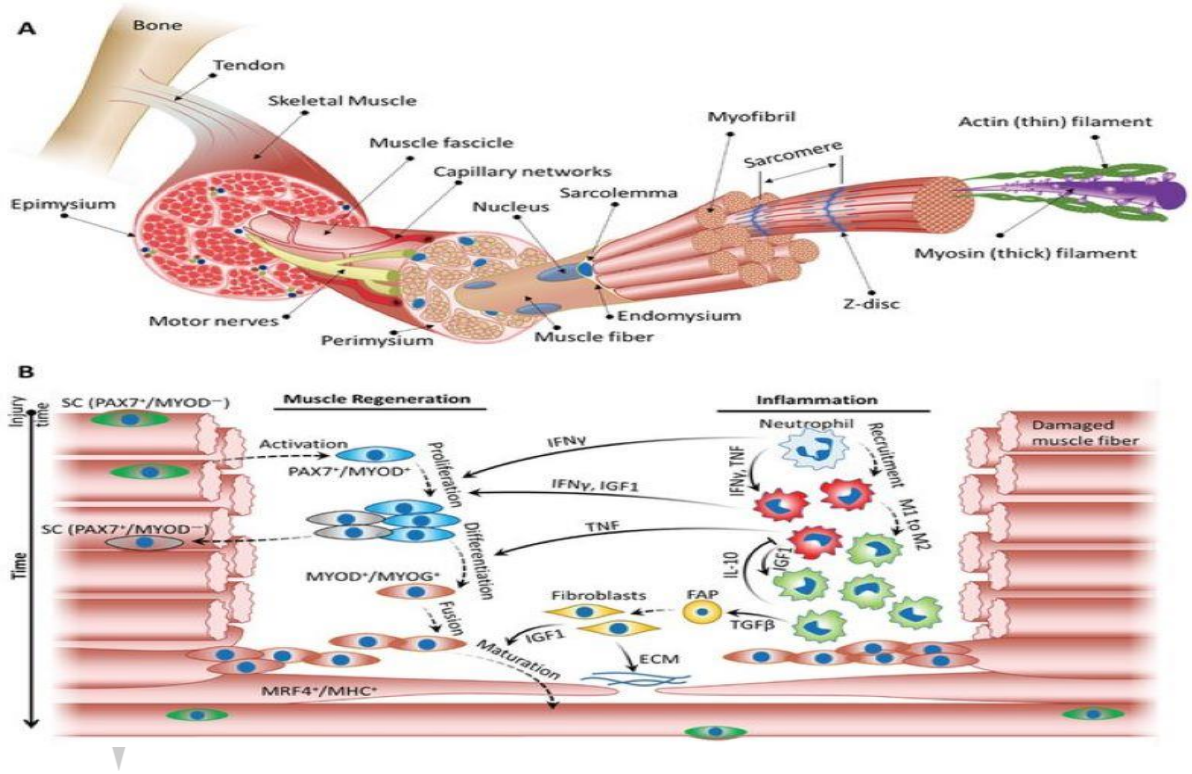

Fig.2. Physiology of skeletal muscle, and myogenesis mechanism following injury (a) Pathophysiology of skeletal muscle structure, containing layers, fibres, and sarcomeres with actin and myosin filaments fundamental for contraction. (b) Timeline of regeneration of muscle and inflammation, illustrating activation of, proliferation, differentiation, and cytokine-mediated fibrosis and post injury repair.(Samandari *et al*., 2022)

1.2. Muscles regeneration and role of satellite cells:

The ability of skeletal muscle to regenerate after injury is extraordinary, and it is mostly due to the existence and activity of satellite cells. The regenerative property of skeletal muscles was

first shown in 1860s (Weber, 1867) and represents as a significant homeostatic process of fully grown skeletal muscle, which have the ability to regenerate and retain, in response to several stimuli, and can repair the injured myofibers (Forcina, Cosentino and Musarò, 2020).Regeneration of injured muscle depends on as satellite cells (SCs), which are muscle stem cells, present between the basal lamina and sarcolemma of myofibers. These cells remain in mitotically quiescent stage until required for growth or repair (Schultz, Gibson and Champion, 1978) . Satellite cells are tissue-specific and play a vital role in the process of regeneration, and are linked with the repairing of muscle fibres. A special category of monopotent stem cells known as satellite cells , are capable of regeneration and to differentiate into myoblasts and myotubes, and play an important role to preserve the structural and functional integrity of skeletal muscle cells. (Weissman, Anderson and Gage, 2001). Satellite cells remain in a quiescent state beneath the basal lamina, exhibiting the expression of paired-homeobox transcription factors, Pax3 (Relaix *et al.*, 2006) and Pax7 (Seale *et al.*, 2000). The process of muscle cell regeneration is shown in (**Figure 3.**).

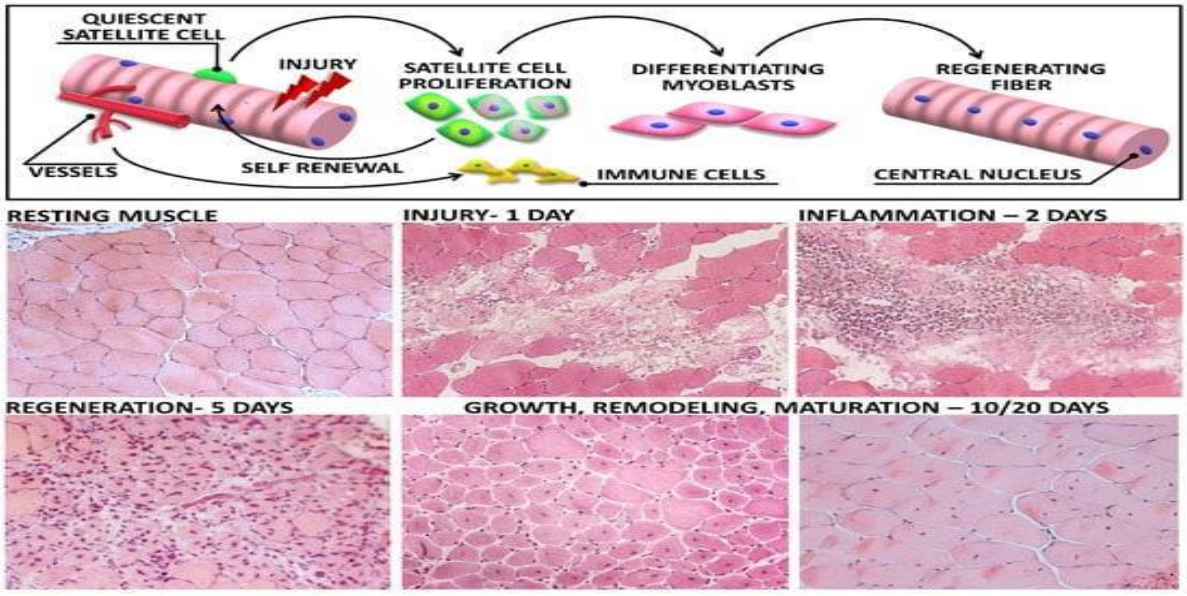

Fig.3. Regeneration of skeletal muscle upon acute injury: The top panel shows a schematic illustration of biological responses initiated in muscle tissue following damage. Lower panel reports images of haematoxylin and eosin muscle sections, each step of muscle degeneration and regeneration was followed by cardiotoxin (CTX) injection. One day after the injection, necrotic myofibers were observed in damaged muscle. Following injury on second day the

lesion was dominated by infiltrated inflammatory cells. Within a week, activated satellite cells underwent proliferation and new fibres were generated., and newly regenerating fibres appears within the first week. The overall structure of tissues restored after ten days of injection and majority of the myofibers exhibit central nuclei. After regeneration, myofibers undergo growth and maturation, highlighted with increased cross-sectional area and relocation of nuclear to the periphery.(Forcina, Cosentino and Musarò, 2020)

The myocytes present within myotome represents the initial population of postmitotic cells that play an important role in skeletal muscle's development during embryogenesis. The elongation of newly formed mononucleated myocytes beside the anterior-posterior axis which is necessary for the covering of the entire somite length is regulated by Wnt11 signalling (Christ et al., 1983) (Denetclaw Jr et al., 1997; Gros et al., 2009). The dermomyotome lips gradually undergo through cellular addition into the myotome, where these cells combine with existing myocytes to create myofibers (Gros et al., 2004; Sieiro-Mosti et al., 2014). The process of stem cells transition from a dormant state to an activated, committed, and differentiated state involves genetic and epigenetic modifications, which allows then to meet new biological roles. This process involves ongoing changes in the expression pattern of protein throughout the body. One the satellite cells (SCs) are activated they can maintain the expression Pax7, Mcad, VCAM1, Caveolin 1, and Integrin 7 expression levels stable. Furthermore, these cells show an increased expression of proliferative and myogenic markers such as desmin, Myf-5, and MyoD (Scharner and Zammit, 2011).

1.3. Impact of nutrition and aging muscles:

Ageing has various consequences which impact human body and affects both physical Capabilities and appearance. Moreover, skeletal muscle mass and strength gradually decline after attaining a peak in early adult years. **(Figure 4.)**

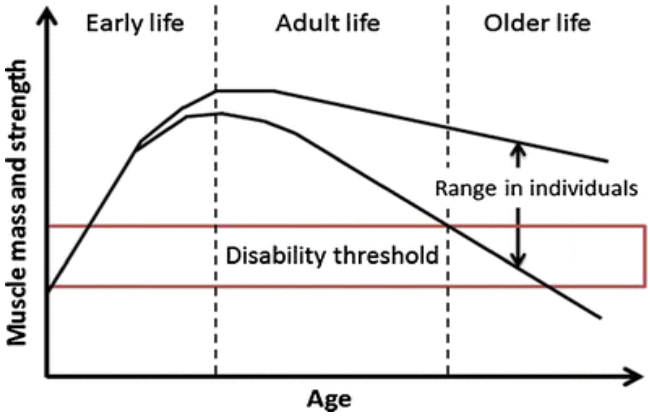

Fig.4. Relation between age to muscle mass and strength. This graph illustrates an increase in mass and strength of muscles during early life, with peak in adult life, and progressive decline in old age. Individuals falling below disability threshold, are more likely to get muscular dystrophies.

Irwin Rosenberg in 1989 introduced the word "sarcopenia" to describe the gradual decline of muscle mass associated with older individuals. (Rosenberg, 1989; Rosenberg, 1997) This disorder is also referred as "age-related muscle wasting." Sarcopenia can be defined using two main approaches: the first way involves measuring of muscle mass alone. (Baumgartner *et al.*, 1998; Pichard *et al.*, 2000), or by other, more comprehensive approach which involves measuring of physical performance, strength and mass of muscles (Cruz-Jentoft *et al.*, 2010). Many studies have shown that sarcopenia prevalence exhibits a wide range with documented rates between 5% and 70%, likely due to many factors such as gender and age and varying diagnostic methods. (Castillo *et al.*, 2003; Janssen, 2006; Rolland *et al.*, 2003). Some evidence shows that sarcopenia prevalence is higher among women than in males. Moreover, poor nutrition intake, inactive lifestyle, and the presence of related health conditions may by the accelerate factors for the progression of sarcopenia (Dutta and Hadley, 1995) .Furthermore, hormonal imbalance, genetic vulnerability, neuromuscular disfunction, and physical trauma, are possibly considered to be the contributing factors which can potentially cause sarcopenia (Thomas, 2010).

1.4. Muscle health and Quercetin supplementation:

Quercetin is a carbohydrate free flavonoid, naturally present in appreciable amounts in fresh produce such as fruits, vegetables, and grains (Harwood *et al.*, 2007). Quercetin has drawn interest due to its possible advantages in supporting muscle health, especially in relation to ageing and being well-known for its strong anti-inflammatory, antioxidant, anti-mutagenic, and anticarcinogenic qualities, it has been researched for its capacity to fight oxidative stress and inflammation, two major causes of muscle deterioration and the onset of sarcopenia in older adults (MacRae and Mefferd, 2006).

Moreover, extensive research has demonstrated that quercetin show notable pharmacological properties that confer protective effects on the cardiovascular system (da Silva, Tsushida and Terao, 1998; Prince and Sathya, 2010). A phenolic organic compound quercetin present in certain food sources, also exhibit potential in regulating the mass of body and lipid profiles. In vivo and in vitro studies conducted on animal models, have provided strong evidence suggesting that quercetin could impede the absorption of dietary energy, regulate the metabolic rate of adipose tissue, and reduce the triglycerides accumulation . A study by conducted by (Rivera *et al.*, 2008) found that chronic administration of quercetin considerably improves several metabolic abnormalities, such as obesity, dyslipidaemia, hypertension, and insulin resistance in obese rats. Furthermore, it has been observed that this intervention tends to reduce body weight and potentially extend the lifespan of rodents.

A study conducted in 2009 by (Davis, Murphy and Carmichael, 2009) brought this effect to light that short term supplementation with quercetin greatly decreased markers of inflammation and muscle injury in healthy volunteers who had completed a rigorous exercise regimen. According to the study, quercetin may be able to lessen the post-exercise oxidative damage that happens immediately in muscle tissues. Moreover, quercetin supplementation has also shown to be beneficial for recovery from muscle damage caused during exercise, supporting its potential use as a supplement for athletes or individuals undergoing intense physical activity (Rojano-Ortega *et al.*, 2023; Ortega *et al.*, 2021). The anti-inflammatory properties of quercetin are also essential for the health of muscles (Bazzucchi *et al.*, 2020).

1.5. Malnutrition and sarcopenia:

Malnutrition causes changes in the composition of body by reducing adipose and muscle tissues. Various studies have shown that advancing age leads to decrease in F.F.M, surrounding muscle, skin, tissue, organ, and bones.(Forbes and Reina, 1970; Baumgartner *et al.*, 1995). The importance of the muscle mass % present in person's body depends on in its relation with, strength, morbidity and physical function. Studies have presented that even minor reductions by as little as 10% in lean tissue of an individual who in general is in good health might have compromised immune function, raise their vulnerability to infections, and possibly contribute to a increased mortality (Broadwin, Goodman-Gruen and Slymen, 2001; Landers *et al.*, 2001) .Subsequently, elderly persons with any preceding history of fat-free mass (FFM) are at high risk for adverse consequence.

A complex geriatric syndrome sarcopenia is caused by multifactorial conditions and is characterised by progressive loss of skeletal muscle mass and strength with a risk of adverse consequences like physical incapacity, poor quality of life and even death. Although the underlying mechanism of sarcopenia is unclear, there are several age-related factors which contribute to the structural and functional aggravation of skeletal muscle leading to sarcopenia. The development of sarcopenia is unquestionably accelerated by lack of physical activity. Research has shown that inactivity causes loss of muscle mass, whereas exercising slows and lowers this process (Roubenoff, 2000). According to European Working Group on Sarcopenia in Older People (EWGSOP), sarcopenia can be defined as a progressive and extensive decrease in muscle mass and function, this decline can be identified through a low muscle strength or through reduced physical performance(Cruz-Jentoft *et al.*, 2010). While the decline of muscle mass and function is a part of natural phenomenon of ageing, sarcopenia is specifically diagnosed when these factors fall below a specific threshold. For accurate diagnosis of sarcopenia, it is essential to consider and evaluate multiple factors like muscle mass, muscle strength and their physical performance (Cooper *et al.*, 2013). Sarcopenia, in general is a significant problem within the realm of public health due to its significant influence on clinical outcomes, economic implications, and public well-being(Bruyère *et al.*, 2019b; Beaudart *et al.*, 2014). As of expanding prevalence of sarcopenia and due to its resulting debilitating effects,

successful implementation of preventive and therapeutic measures for the elderly population is significantly challenging. (**Figure 5.)**

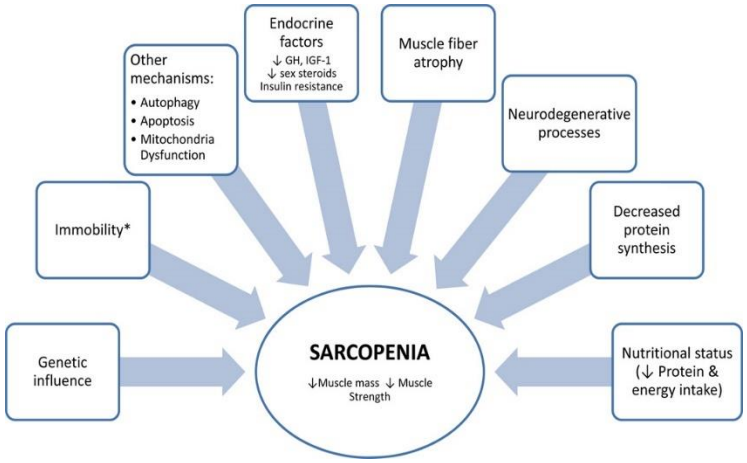

Fig.5. Causes of Sarcopenia (Ali and Garcia, 2014)

1.6. Aim:

Muscle dysfunction, mainly sarcopenia, presents a major challenge for the elderly population, specifically those over 50, as it leads to decline in muscle mass, their strength and function. Despite the prevalence of this condition no effective therapy or medicine has been established which can effectively cure sarcopenia. Advance research indicates that nutrient supplementation in combination with exercise might assist in managing this condition, though it is important to note that these strategies does not provide a convincing cure. Against this backdrop, the objective of this research is to potential of quercetin, to enhance the myogenic differentiation of human skeletal muscle stem cells (SkMCs) using an in-vitro model. By investigating whether quercetin can improve SkMCs proliferation, differentiation, and function, the research seeks to determine its potential as a therapeutic agent in preserving muscle health and extenuating the effects of sarcopenia in mature individuals, contributing to the development of more effective management approaches for this condition.

1.7. Objective:

The objective of this dissertation is to:

- Create an in-vitro human muscular model for experiment, and for this purpose human myogenic stem cells were cultured and allowed to proliferate in growth medium.
- Evaluate the effect of quercetin on the proliferation and differentiation of muscle cells.
- Analyse quercetin's impact on fusion of muscle cells.
- Determine quercetin's cytotoxicity at different concentrations.
- Perform microarray assay for the evaluation of expression of growth factors.

1.8. Purpose and Hypothesis:

The ultimate purpose of this investigative study was to scrutinise the structural composition of skeletal muscles and to examine the possible impact of a nutrient named quercetin, on myogenic differentiation. The triumph of this objective could be enhanced by the development of a distinctive and entirely functioning humanoid model.

The hypothesis for this project as follows:

Quercetin supplementation potentially enhances muscle health by facilitating myogenic differentiation of human muscle stem cells by promoting the formation of myotubes and optimizing the development of nuclei, moreover, leading to modifications in the Myogeography and morphology of skeletal muscles

2. Methodology:

2.1. Culturing Skeletal muscle stem cells:

Human immortalized myoblasts cell line ("C25") was achieved from the Institute of Myology.(Mamchaoui *et al.*, 2011). This cell line was established from biopsy of semi-tendinosis muscles of a 25-year-old male donor. This donation of tissues was anonymously conducted through Myobank, a tissue bank affiliated to EuroBioBank and is authorized by the French Ministry of Research [authorization AC-2013-1868]). Afterwards of attaining 80% of the confluence, cells were then seeded in six-well plates recoated with gelatine (0.5%) at a concentration of 1.5×10^5 cells/mL in growth media. (**Table 1.)**

Table 1. Composition of Growth Media for SkMCs proliferation.

Components of Growth Medium	Concentration
Dulbecco's standard Modified Eagmes medium (DMEM) from Lonza (Basel, Switzerland)	60% (v/v)
Medium 199 with Earle's balanced salt solution(BSS) form Lonza, Nottingham, UK)	20% (v/v)
Heat inactivated Foetal bovine serum (FBS) from Thermo Fisher Scientific (Waltham, MA,USA)	20 % (v/v)
L-glutamine from Lonza (Nottingham, UK)	1 %
Fetuin from FBS from sigm-aldrich (St Louis, MO, USA)	0.5 ng/mL
recombinant human EGF from Thermo Fisher Scientific	5 ng/mL
recombinant human hepatocyte growth factor from Sino Biological Inc. (Beijing, China)	2.5 ng/mL
recombinant human insulin from Sigma-Aldrich	5 µg/mL
dexamethasone from Sigma-Aldrich	0.2 µg/mL
gentamicin from Thermo Fisher Scientific.	1 %

2.2. Cells Proliferation and counting:

C25 immortalised skeletal muscle cells were grown in growth medium (GM) and were then transferred into the flasks, afterwards subjected for incubation with 1 % gelatine, at a temperature of 37°C in an atmosphere having 5% of carbon dioxide (CO^2) for 4 – 5 days. Use of Gelatine is important as it will help in cells growth as well as sticking of the cells. 1 ml of gelatine was then poured into each well of 6 well plate. The experiment was performed until 80% of confluence for cell counting, cell density has been achieved. The attained confluency was then assessed with ImageJ programme. (Schneider, Rasband and Eliceiri, 2012b) (Busschots *et al.,* 2015). Myoblast area (MA) calculations were assessed by combined area occupied by myoblast within the specific field / whole image. (Kim, Qiu and Kuang, 2020).The obtained value is then multiplied by 100.

Once the incubated flasks reach 80 % of the confluence, the growth medium (GM) was gathered from the flasks. After that, the cells experienced two washing rounds with Dulbecco's Phosphate Buffered Saline (DPBS) to remove all the medium, dead cells and the traces of serum. After that, cell dissociation was induced with 1.5 ml of TrypLE™ Express Enzyme. At 37°C the cells were incubated alongside 5% CO^2 for 10 minutes. The dissociated solution then shifted into a conical tube and exposed to homogenise with growth medium (GM) to neutralize the effect of enzyme. The experimental protocol included the use of a 50 µl of 0.4 % Trypan Blue Stain in combination with an equivalent amount of cell suspension and then vortexed and loaded onto the slides for the counting of cells in haemocytometer (**Figure 6.)**. Formulas used to calculate the concentration of live cells per millilitre was as follows:

[The average cells count present in a single large corner square, X by the dilution factor, and then X by 10^4]

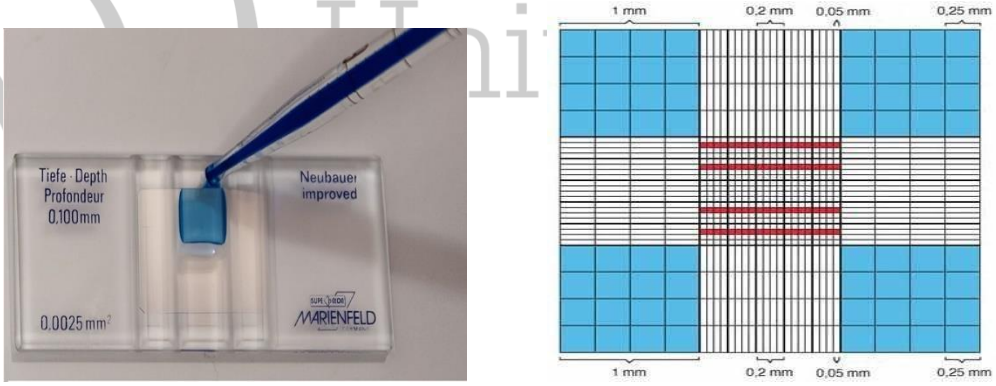

Fig.6. An image representing Haemcytometer along with enlarged counting chamber view used to count the cells after proliferarion.

2.3. Nutrient preparation:

In the experiment, the quercetin was investigated at different concentrations to determine the optimal level for the growth of skeletal muscle stem cells. The amount of quercetin was

calculated by considering its concentration, solute mass and molecular weight. As quercetin has low water solubility , DMSO (Dimethyl sulfoxide) was added to assist its dissolution. The quercetin was dissolved in DMSO, and Eppendorf tubes were labelled corresponding to the concentrations used for testing. For differentiation analysis the concentrations used were 1µM, 5µM, 15µM, and 25µM, whereas those for the cytotoxicity assay were 1µM, 5µM, 15µM, 25µM, 50µM, 75µM, and 100µM.

2.4. Differentiation of SkMCs, Nutrient treatment and cytotoxicity:

In 96-well plates already coated with 1 ml of 1% gelatin solution, specimens were incubated for a period of 24 hours at 37°C with the environment containing 5% CO^2. After completing 24 hours, the cells were washed twice with 1 ml of DPBS, following subsequent removal from the growth medium (GM). Onwards, 2 ml aliquot of differentiation medium was dded to each well, and incubation continued for 72 hours at 37°C and in 5 % CO^2.

Afterward, different concentrations of quercetin, ranging from 1 µM to 100 µM, were introduced into the culture medium. The cytotoxicity assay aimed to evaluate the potential toxicity of the substances used. Skeletal muscle cells were cultured in the 96-well plates and treated with different concentrations of quercetin, then the medium was removed after a set time.

MTT assay/ Cytotoxicity assay steps:

1. On multi-well plate skeletal muscle cells were cultured, and then were treated with the MTT reagent, and metabolized by viable cells, producing purple formazan crystals.

2. Onwards the cells were exposed to different concentrations of quercetin (1 µM to 100 µM) to determine the optimal concentration for bringing cytotoxicity.

3. Cytotoxicity reagent WST-8 (10 µl) was then added to each well, and incubated at a temperature of 37°C for 60 minutes, furthermore DMSO was used to dissolve the formazan crystals.

4. After the treatment period, a microplate reader at 450 nm was used to measure the absorbance of the solution, followed by incubation which continued for 1 hour at 27°C, and absorbance was again measured at 460 nm. (**Table 2**).

Cell viability % = absorbance of treated wells / absorbance of control wells X 100

Table 2. Calculated OD Values for Quercetin treatment at 460nm.

	Control-DMS0	1µM	5µM	10µM	15µM	25µM	50µM	75µM	100µM
Mean	0.9612	0.9656	0.9792	0.9596	0.25044	0.9678	0.25044	0.14916	0.12074
Viability %	100	100.457761	101.8726592	99.83354	101.7478	100.6866	26.05493	15.5181	12.56138
n (SD)	0.039833403	0.05040635	0.053096139	0.047263	0.03871	0.051809	0.030346	0.005828	0.010302
Viability % Control	100	100	100	100	100	100	100	100	100

2.5. Differentiation of SkMCs nutrient treatment and differentiation parameters:

For differentiation of skeletal muscle stem cells, 1ml of 1% gelatine solution was used to coat 6-well plate. Onwards cellular specimens were incubated for 1 day, at a temperature of 37 ºC, with environment containing 5 % of CO^2. The incubation conditions were strictly regulated. The cellular specimens after being removed from growth medium were immediately went through wash, the wash was done twice with 1ml of DPBS, which came immediately after the removal from the growth medium (GM). Onwards in each-well 2ml of differentiation

medium was poured, which then underwent incubation for 72 hours at a temperature of 37°C and with 5% of carbon dioxide (CO^2). Differentiation media components are indicated in Whereas the cells were put through two washes with Dulbecco's Phosphate Buffered Saline (DPBS), the designated medium (DM) would periodically aspire. Onwards for nutritional supplementation various concentrations of quercetin, ranging from 1uM to 50uM, were added into the culture medium. Cellular specimens of 6 well plates after nutrient treatment were then treated by a solution containing1ml of 4% formaldehyde to make sure correct fixation. Onwards the samples were incubated very carefully for 15 minutes at room temperature. The process was then followed by permeabilization which has been done by using 0.5 % Triton x-100 solution. 0.5 micro molar of 0.5 % Triton x-100 was mixed with 10ml of DPBS. Perm/wash buffer was used, followed by washing with DPBS, twice in a row, this caused the membranes to become more permeable. Afterwards cells were incubated for 15-minutes at room temperature. Followed by the removal of the permeabilizing and washing buffer, cellular specimens went through 2 final rinses with DPBS. Afterwards, blocking was done using Bovine serum for which 2ml of 5% BSA was added in well plates and each cell then incubated at room temperature for about hour. This was done for the blocking of every non-specific binding site. After permeabilization cellular samples were stained, using a solution that contained 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and green stain (Myosin Heavy Chain **Alexa Fluor® 488**). 300 µl of the staining solution was poured into each well and then incubated at room temperature for about 30 minutes to 1 hour. The stain was then removed from each well by washing them twice with DPBS. Using Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). images of the stained cells from 6-well plates were captured. For quantitative analysis of the differentiation parameters programme ImageJ was used to which is an image processing software.

2.6. Differentiation metrics:

Fusion index is a key differentiation parameter used to measure the extent to which human skeletal muscle stem cells (SkMCs) have undergone myogenic differentiation, particularly assessing the myoblast fusion into multinucleated myotube (Domenighetti *et al.*, 2018; Zhang *et al.*, 2023). Fusion Index is determined by calculating the % of nuclei present within the myotube relative to the total nuclei in the field. **(Table 3).**

Fusion Index % = No. of nuclei within the multinucleated cell (myotube) / total no. of nuclei in the field x 100

Myotube is an early, intermediate structure formed during the development of skeletal muscle fibers. Myotubes are formed during the process of myogenesis. Myotube is as a syncytial structure that has elongated tubular shape containing minimum of two nuclei, which is one of their defining characteristics.(Jones *et al.*, 2018) (Nishiyama et al., 2004).

The ratio between Short and long axis, has shown relationship with both contractility and differentiation of myotubes (Chamley-Campbell, Campbell and Ross, 1979).

Table 3. Differentiation Parameters calculations formulas:

Differentiation Parameter	Formula
Fusion Index %	nuclei present per myotube/ nuclei present in the field X 100
Myotube Area %	Area of myotubes / l area of the field X 100
Aspect Ratio %	Length of myotube / Width of myotube

2.7. ImageJ analysis:

The programme ImageJ appeared as a powerful platform to analyse scientific photographs and for image processing. (Schneider, Rasband and Eliceiri, 2012a; Rueden *et al.*, 2017). Images taken with a **phase contrast microscope** were subjected to analyse using the ImageJ software. A total of four images were taken for each well on a six-well plate, with various angles along with control well. The software program tools were only used to measure the dimensions i.e. length and width alongside the number of nuclei present in myotubes (**Figure 7**). Before measuring the Area of myotubes, a scale was adjusted for each of the image. Scale was being adjusted at 2 pixels/µm the unit for measurement is set as µm (a universal scale for all the images). ROI manager of ImageJ software gives the measured data like area, angle and mean.

All the parameters were studied and afterwards the results were gathered on Microsoft excel sheet for further computations.

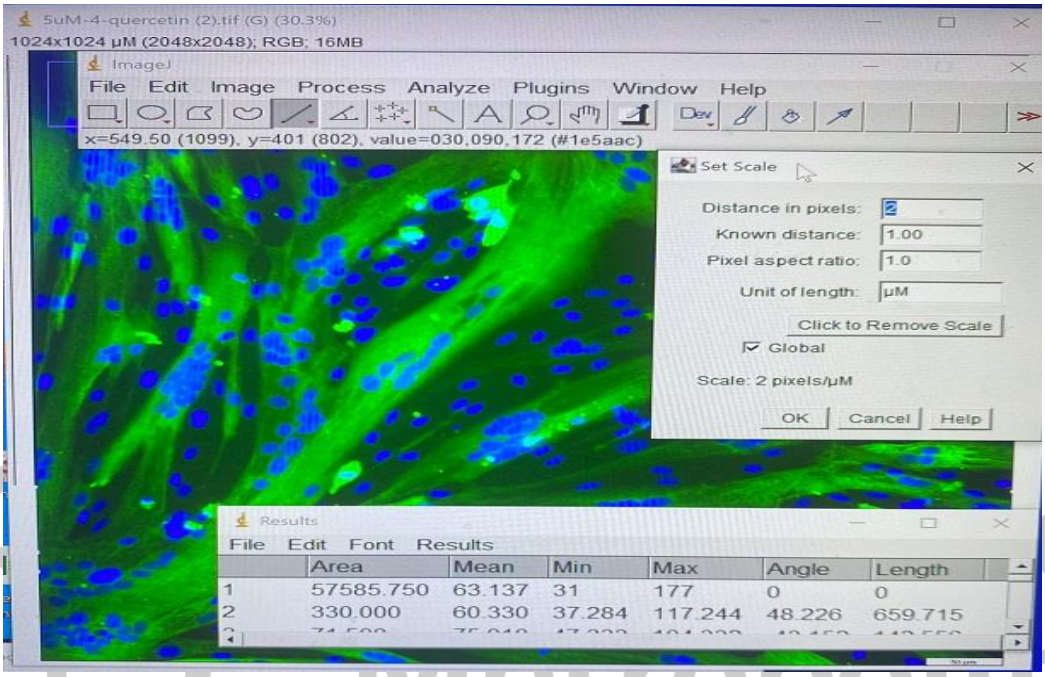

Fig.7. Immunofluorescent image showing formation of myotubes: Leica **TCS SP5 confocal microscope** captured the images showing stained green myotubes marking muscle cells differentiation whereas the nuclei are counterstained in blue colour. The scale was set at 2 pixels/µM. Analysis steps shown are as follow: click on ImageJ "Analyse → Set scale → Analyse → Tools→ ROI Manager" than tap to measure the length and width of myotube with **"/"**. Same steps were followed for all the images.

Microarray Assay:

For ELISA-based protein array using the RayBio® C-Series Human Growth Factor Antibody Array C1 for myoblast stem cells, following the principles defined by Huang et al., start by myoblast cells culturing until they reach 70-80% of confluence. Onwards harvest and lyse the cells for the preparation of a protein lysate by using a lysis buffer that preserves activity of protein. The array membrane, already spotted with 40 antibodies (including 16 key growth factors), is first blocked to avoid any non-specific binding. The membrane then was incubated

with the lysate overnight at a temperature of 4°C to allow the target proteins to bind to their specific antibodies. After the completion of incubation, the membranes were thoroughly washed to remove all the unbounded proteins, then biotin-conjugated detection antibody was applied, which binds to the captured proteins. Following another wash, the membrane was incubated with HRP-conjugated streptavidin. The signals then were detected by using chemiluminescence, which provides the sensitivity needed for multiplexed analysis. The resulting signals were captured with a CCD camera, and the protein levels quantification was done using densitometry software, normalizing against control spots. This procedure, based on of Huang et al., supported techniques has allowed the synchronised quantification of multiple growth factors from myoblast stem cells, with a comprehensive view of protein expression.

2.8. Statistical Analysis:

The data analysis was done using a software called GraphPad Prism version 10.3.1, which was developed by GraphPad Software Inc. located in La Jolla, California, USA. Recent investigations used statistical analytical techniques by adding data into GraphPad prism to undergo comparative analysis of the averages among several groups. One way ANOVA i.e. analysis of variance was used to calculate the total differences between the means. Moreover, , Tukey's multiple comparison test was used to verify the accurate pairwise particularities between the means. Statistically significant values were indicated using asterisks (*). (*) denoting a significant reading of p value ≤ 0.05, (**) denoting $p \leq 0.01$, (***) representing $p \leq 0.001$, and (****) signifying $p \leq 0.0001$ respectively. Results presented were in terms of the mean values obtained from standard deviation. The threshold for statistical significance was set at $p < 0.05$.

2.9. Ethical Consideration:

This research is based on Human skeletal muscles stem cell line "C25" obtained from Myo bank, a tissue bank which is affiliated to Eurobank (authorized by the French ministry of

research (Mosmann)).Assuring that no animal or human sources were used for any cause while conducting this research. And hence consent, confidentiality and anonymity are not applicable.

Furthermore, Ethical approval has been taken from …… All the chemical handling follows COSHH regulations and ETHOS protocols were fully followed and approved.

3. Results:

3.1. Results Outline:

The main aim of this investigative study was to determine the effect of quercetin supplementation. Quercetin if taken in safe quantity, would be highly beneficial for muscle development. In in-vitro environment the human skeletal muscle stem cells were sown in a suitable environment in a stable inoculant. The cells multiplied and differentiated over the course of the experiment. Afterwards treated with quercetin, at variable concentrations of 1µM, 15µM, 25µM, and 50µM. The growth of the cells was measured by comparing it to a control group which only contained that differentiation medium and stem cells. This efficiently served as the benchmark. From the performed experiment, it was discovered that favourable changes were taking place, such as an increase in differentiation parameters, with the increasing concentration of nutrients. There was an evident rise in both the area of the myotubes and the fusion index and aspect ratio.

3.2. Cytotoxicity:

The results from cell viability and cytotoxicity shows that quercetin has a concentration dependent effect on cell viability. The viability at lower concentrations (1uM - 25uM) is relatively high and is like control group. However, at high quercetin concentrations (50uM - 100uM), cell viability has significantly decreased suggesting that quercetin is toxic to cells at high concentrations. The graph does not explicitly depict statistical significance. **(Figure8.)**

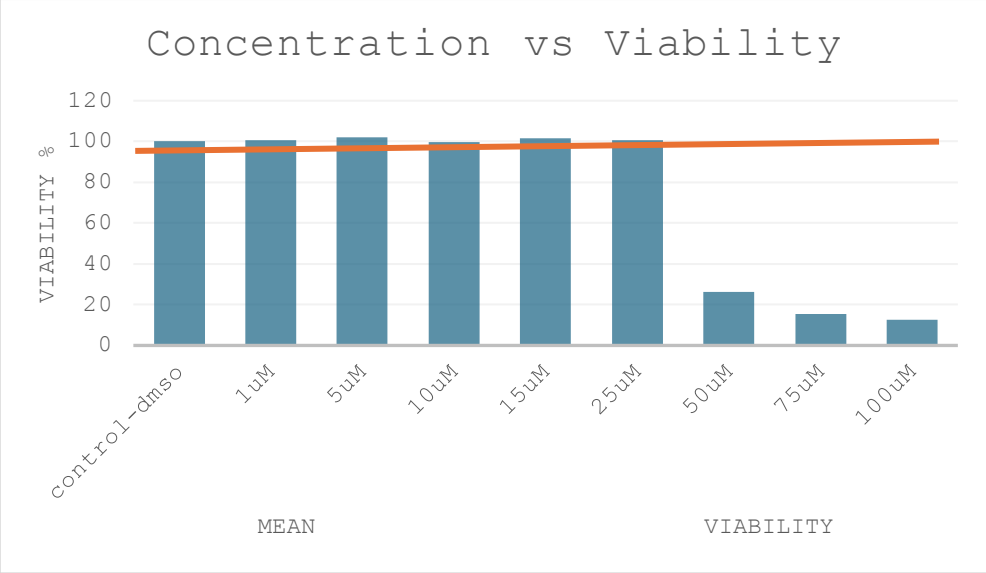

Fig.8. Cytotoxicity assay of quercetin treated cells. At lower concentrations from 1 µM – 25 µM, quercetin exhibits minimum cytotoxicity whereas viability remaining above 90%, while at high quercetin concentrations from 50 µM – 100 µM significant decrease in cell viability can be seen. For each concentration data is presented as mean values.

Differentiation Parameters:

The graph analysis of the effects of quercetin on myogenic differentiation of human skeletal muscle cells (SkMCs) provide a comprehensive insight into dose dependent impact of quercetin on differentiation parameters (fusion index, myotube area and expect ratio). The effect of quercetin on fusion index, myotube area and aspect ratio was assessed across various concentrations (1 µM, 5 µM, 15 µM, and 25 µM) and was compared with a control group (**Figure 9. (a-e)**.

The fusion index data clearly shows that quercetin has significantly increased the fusion of myoblasts into multinucleated myotubes and statistically significant increase in the Fusion Index was observed in all treated groups in comparison to the control group. Explicitly, the Fusion Index drastically increased at 1 µM ($p<0.0001$), 5 µM ($p<0.0001$), 15 µM ($p<0.0001$), and 25 µM ($p<0.0001$) concentrations of Quercetin when is compared to the control group. Moreover, significant differences were observed between different concentrations of Quercetin. Between 1 µM and 5 µM F.I was statistically significant ($p<0.001$). However, no statistically significant difference was observed in the Fusion Index between 5 µM and 15 µM,

as well as between 15 µM and 25 µM, demonstrating that quercetin's effect plateaued after 5 µM. **(Figure 10.a.)**

Correspondingly, a significant increase in Myotube Area was also observed at 1 µM ($p<0.01$), 5 µM ($p<0.0001$), 15 µM ($p<0.0001$), and 25 µM ($p<0.0001$) compared to the control group. The enhancement in Myotube Area between 1 µM and 5 µM concentrations was statistically significant ($p<0.01$), as was the increase between 5 µM and 15 µM ($p<0.0001$). Whereas no statistically significant difference was observed between 15 µM and 25 µM, demonstrating that the effect on Myotube Area reached a plateau at 15 µM concentration. These findings have suggested that Quercetin promotes an increase in Myotube Area in a dose-dependent manner, with its maximum effect at concentrations of 15 µM and above. **(Figure 10.b.)**

Conversely, the Aspect Ratio has significantly decreased in all treated groups when is compared to the control group. Specifically, a reduction in Aspect Ratio was observed at 1 µM ($p<0.001$), 5 µM ($p<0.0001$), 15 µM ($p<0.0001$), and 25 µM ($p<0.01$) concentrations of Quercetin. Conspicuously, the decrease between the control group and 1 µM of quercine concentration was statistically significant ($p<0.001$), and a further significant decrease was also observed at a concentration of 5 µM ($p<0.0001$) when compared to 1 µM. However, there were no significant differences in the Aspect Ratio between the 5 µM, 15 µM, and 25 µM groups, indicating that the effect of Quercetin on Aspect Ratio plateaus after reaching 5 µM. Suggesting that Quercetin decreases the Aspect Ratio in a dose-dependent manner, with its maximum effect at 5 µM, beyond which further significant reductions were not detected. **(Figure 10.c.)**

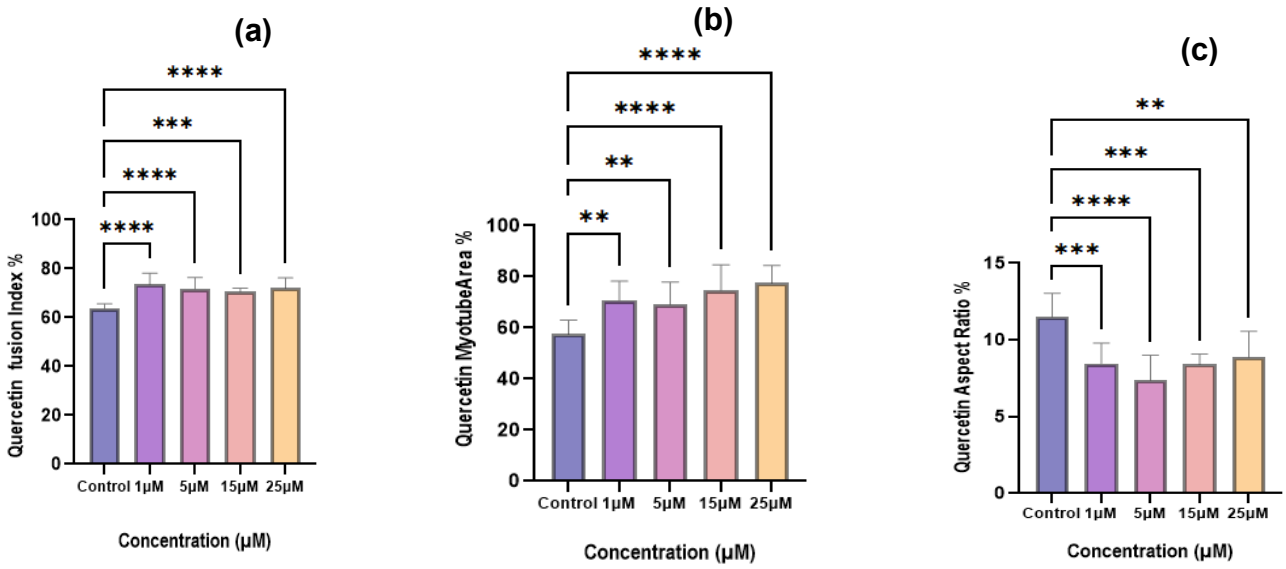

Fig.10. (a), (b), (c).

The graphs illustrating changes occurring across three differentiation parameters i.e., Fusion index, myotube area, and expect ratio as the concentration of quercetin supplementation increased in comparison with control group.

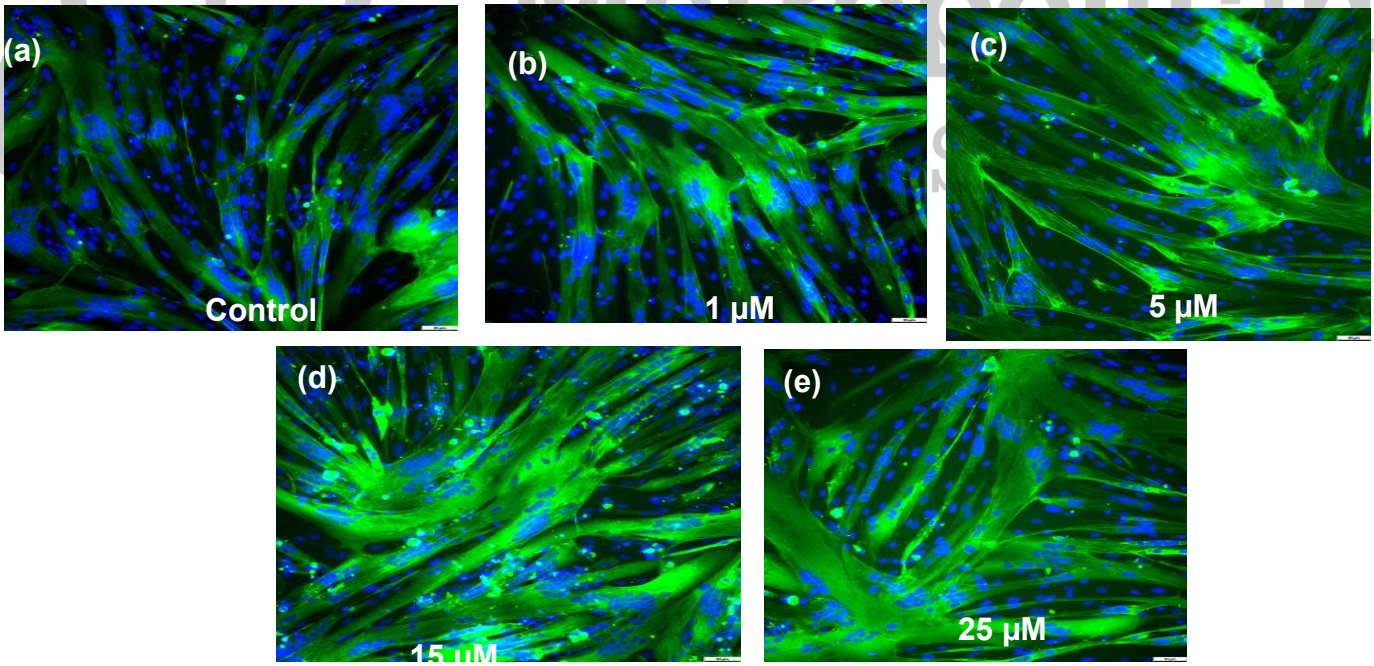

Fig.9. (**a-e**). The obtained immunofluorescent images show myogenetic and Myo morphological changes occurred in myotubes after Quercetin supplementation at various concentration levels. With the increase in quercetin concentration, size of myotubes and number of nuclei in myotubes also increase.

These results together demonstrate that quercetin could enhance myogenic differentiation in SkMCs by increasing cell fusion and myotube formation while reducing the Aspect Ratio. These effects generally plateau beyond 5 µM. Suggesting that a potential optimal concentration range for quercetin promotes muscles cells differentiation.

3.3. Microarray Assay:

To analyse Quercetin's effects on human skeletal muscle cells (SkMCs), a comprehensive protein expression study was conducted using 16 key growth factors involved in myogenic differentiation, cellular signalling, and muscle function **(Table.4.)**. This analysis aimed to understand the molecular mechanisms underlying Quercetin's influence on myogenic differentiation of skeletal muscle stem cells and to identify potential protein markers that respond to Quercetin treatment.

MCSF-R:

MCSF-R (macrophage colony-stimulating factor receptor) has shown no significant difference which are denoted as "ns" (non-significant) on human skeletal muscle cells between control group and any of the quercetin treated groups. The control group demonstrates baseline level for the expression of MCSF-R, whereas the treated groups, despite receiving increasing concentrations of quercetin, have shown no substantial deviation. Suggesting that expression of MCSF-R is not modulated with quercetin even in a dose dependent manner. This indicates that MCSF-R may not be directly involved in myogenic differentiation of skeletal muscle stem cells.

NGF-R

NGF-R (nerve growth factor receptor) has shown progressive increase in its expression level in skeletal muscle stem cells with the rising concentration of Quercetin from 1µM to 25 µM. The expression level is lowest for the control group whereas, highest levels are observed at 15 µM and 25 µM, which shows nearly identical results. NFG-R expression level suggest that

quercetin may enhance neurotropic signalling of SkMCs, which can potentially support muscle cells regeneration and repair. NGF-R is important for neurons i.e to survive and differentiate, and in SkMCs its increased expression level indicates that quercetin can potentially promote neurogenic environment, influencing muscle-nerve interactions for myogenic differentiation.

NT-3

NT-3 did not show any significant dose dependent expression in skeletal muscle stem cells following quercetin treatment; however, a significant variation was observed at a specific concentration. Most of the quercetin treated groups (1µM, 15 µM, and 25µM) showed no significant differences from the control group, whereas, at 5 µM (*P value < 0.05 compared to control group) a statistically significant decrease in the expression of NT-3 was observed, followed by return to standard baseline level for high concentration of quercetin (15 µM, and 25µM). This temporary reduction at 1 µM indicate short-term inhibitory effect on neurotropic support, impacting muscle nerve interaction. However, at high concentration the restoration of NT-3 expression indicates that quercetin does not have prolonged suppression, and the initial NT-3 signal disruption was eventually balanced with continued treatment.

NT-4, PGDF-AA, PIGF, SCF, SCF-R, VEGF-R3, VEGF-D

The graphs for the expression of NT-4, PGDF-AA, PIGF,SCF,SCF-R, VEGF-R3, VEGF-D have shown no statistically significant differences (“ns”) at all concentrations of quercetin compared to the control suggesting that quercetin does not significantly alter the expression of NT-4, PGDF-AA, PIGF,SCF,SCF-R, VEGF-R3, VEGF-D in skeletal muscle stem cells, irrespective of concentration .

OPG:

OPG has shown statistically significant upregulation in response to different quercetin concentrations, particularly at 1 µM and 5 µM 9with *P values < 0.05 and at 25 µM with ****p value < 0. 0001.The expression of OPG at 25 µM is evidently higher than all the other

concentrations, signifying dose dependency of OPG at high levels of quercetin. This sharp rise at 25 μM suggests that quercetin at higher concentrations exhibit stimulatory effect on OPG. Furthermore, the results indicates that high concentrations of quercetin can effectively modulate pathways associated with OPG, possibly via NF-Kb signalling, which is known to be a regulator for OPG expression. **(Figure 11.a.)**

TGFa, TGFb1, TGFb3

The graph for the expression of TGFa shows statistically significant differences at 1 μM and 25 μM (*** $p < 0.0001$ and **** $p < 0.0001$) compared to the control group. Whereas at 5 μM and 15 μM no significant differences have been observed for the expression of TGFa. The graph indicates that TGFa expression is dose- dependent for different concentration of quercetin. Moreover, TGFb1 expression has shown statistically significant increase at 25 μM (****$p< 0.0001$) as compared to control group however, at 1 μM, 5 μM and 15 μM the expression of TGFB1 remained like control group. TGFb3 expression has shown a significant increase at 5 μM (*$P <0.05$) as compared to control group while at 1 μM, 15 μM and 25 μM non-significant ("ns") values are observed. **(Figure 11.b., 11. c.)**

VEGF. VEGF R2

VEGF expression graph illustrates statistically significant upregulation at 1 μM , 15 μM and 25 μM (***$p <0.0001$, **** $p <0.0001$, *** $p <0.0001$) compared to control group. At 1 μM no significant value is observed for the expression of VEGF. The graph indicates dose dependent response of VEGF at high concentrations of quercetin. The graph illustrating VEGF R2 expression shows zero value at 1 μM and 5 μM however at 15 μM and 25 μM some extent of VEGF expression can be observed with lack of statistically significant values suggesting that these changes have not occurred due to any biological response rather than that can be due experimental variability. **(Figure 11.d.)**

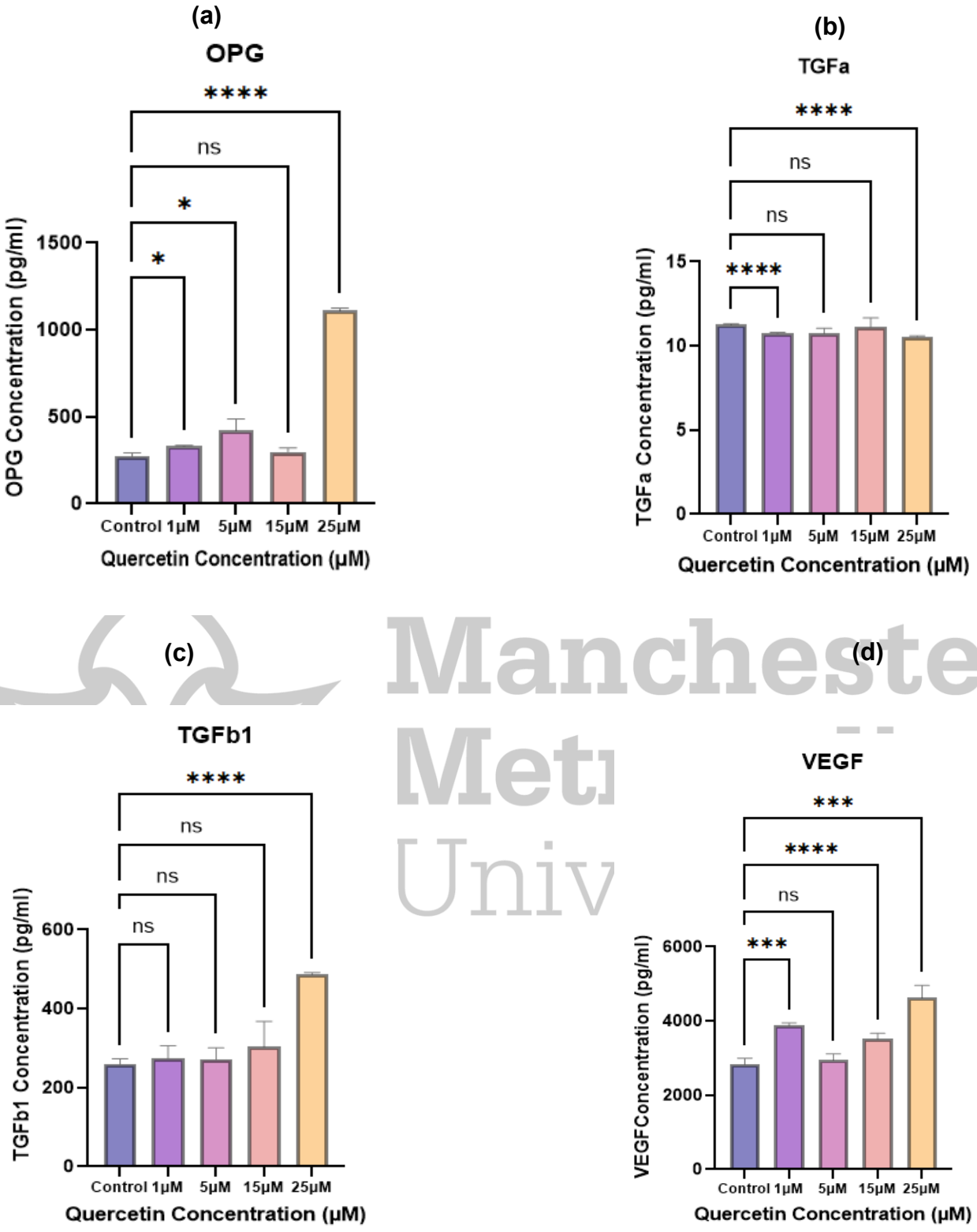

Fig .11. (a – d): Microarray analysis showing the expression of growth factors OPG, TGFa, TGFB1, VEGF involved in skeletal muscle stem cells differentiation across with varying quercetin concentrations (1 µM, 5 µM, 15 µM, and 25 µM). Each graph highlights key growth factor, showing up regulations and down regulation as compared to control groups.

Table (4): Statistic analysis Growth factors expression:

The following table shows the non-significant and statisticaly significant growth factors:

Growth Factor	Statistical analysis
MCSF R	Non significant (ns)
NGF R	Non significant (ns)
NT-3	Non significant (ns)
NT-4	Non significant (ns)
OPG	Statistically significant
PDGF-AA	Non significant
PIGF	Non significant
SCF	Non significant
SCF R	Non significant
TGFa	Statistically significant
TGFb1	Statistically significant
TGFb3	Non significant
VEGF	Statistically significant
VEGF R2	Non significant

4. Discussion:

Quercetin belongs to a broad group of natural polyphenolic flavonoids and is being investigated for its enormous health benefits(Deepika and Maurya, 2022). These benefits have been generally recognized by its antioxidant and anti-inflammatory property, but recent in vitro studies also suggests its role in increased mitochondrial biogenesis of skeletal muscles and brain cells, indicating that short term feeding of quercetin can increase mRNA expression of PGC-1a and SIRT1, and mtDNA and cytochrome c in both skeletal muscles and brain (Davis *et al.*, 2009; Boots, Haenen and Bast, 2008) . Moreover, some studies have investigated that quercetin may improve recovery and performance of muscle by reducing inflammation, improving blood flow, and by providing support in muscle metabolism (Anhê *et al.*, 2012; Wang *et al.*, 2022a).

Furthermore, Various studies conducted on muscle differentiation and regeneration have demonstrated that nutrient supplementation enhances growth whereas, nutrient supplement deficiencies lead to adverse damaging effects on skeletal muscles. Due to these metabolic abnormalities after a particular age people do suffer from muscle illness such as dystrophies or

atrophies.(Motlagh, MacDonald and Tarnopolsky, 2005) Evidence have shown that exercises for 2-3 times a week along with nutritional value diet have certain beneficial effects on muscle growth, regeneration and on muscular strength.

Anthocyanins and epicatechin have shown subsequent role in muscle regeneration through various molecular mechanisms. These are potential nutraceuticals and improve skeletal muscle health by their ability of modulating oxidative stress, enhancing mitochondrial function, and can also promote myogenesis (Khairani *et al.*, 2020; McDonald *et al.*, 2021; Byun *et al.*, 2014; Kim *et al.*, 2017). Studies have also shown that silibinin can enhance muscle regeneration by promoting migration and differentiation of myoblasts (C2C12) through increased ROS production and by down-regulating the activity of oestrogen receptor α (Long *et al.*, 2020). A natural flavonoid, Luteolin, has also shown ability to promote the differentiation of porcine myoblasts by activating the PI3K/Akt/mTOR signalling pathway (Guan *et al.*, 2022). More studies have also shown the ability of daidzein, a soy isoflavone, to down-regulate ubiquitin-specific protease 19 (USP19) expression through estrogen receptor β (ERβ) and then increase skeletal muscle mass in female mice (Ogawa *et al.*, 2017).All of the studies produced brilliant results and immunofluorescent images but still there is a lot more to discover as these researches have some limitations.

Hence, inspiring from these investigative studies, this study aimed to focus on Investigating the effects of quercetin, a naturally occurring flavonoid, on myogenic differentiation of SkMCs. The study was conducted on human skeletal muscle stem cells which have been cultured in -vitro to obtain further reassuring evidences by precisely working on cells obtained from humans. Particularly, with the focus to explore the influence of quercetin on key differentiation parameters i.e. fusion index (F. I), myotube area (M. A), and aspect ratio (A. R). Furthermore, its effect on gene expression via microarray analysis, as well as its cytotoxicity profile. The findings of this study shed light on the potential therapeutic applications of quercetic for the regeneration of muscles and vascularization. This comprehensive research provides a strong foundation in understanding the role of quercetin in muscle cell biology and foundation for future research into its clinical applications.

Fusion index which, measures the no. of nuclei within multinucleated myotubes, has significantly increased following quercetin treatment. This finding aligns with previous study which highlighted the positive effect of quercetin in muscle cell fusion. In human skeletal muscle satellite cells quercetin enhances muscle cell fusion by upregulating gene expression related to muscle differentiation and induces slow myofibers in human skeletal muscles(Nagai *et al.*, 2024). Reported that quercetin enhances myogenesis by activating key pathway particularly AMPK pathway, increasing GLUT4 expression, and inducing calcium signalling in muscle cells (Dhanya *et al.*, 2017).Suggesting that quercetin can facilitate initial muscle regeneration , thus promoting formation of large and complex myotubes.

Similarly, myotube area also showed the growth and increase in size of myotubes, particularly ay high quercetin concentrations supporting the fact that quercetin not only contributes to fusion of myoblasts but also promotes growth and maturation of myotubes. Quercetin can inhibit the activity of myostatin both in silico and in vitro, when used as a media supplement, promoting increase in myotube area and improved muscle differentiation (Ahmad *et al.*, 2024).

The aspect ratio has also increased in response to treatment with quercetin at different concentrations. Myotube elongation is a critical indicator to access muscle maturation, as myotubes need to attain optimal length for the formation of proper muscle fibre. Studies such as (Ostrovidov *et al.*, 2014; Bruyère *et al.*, 2019a)have supported that the elongation of myotube correlates with improved muscle contractility and function, proposing that quercetin may not only promote the early stages of myogenesis but also enhance the process of maturation.

Quercetin's ability to increase myotube size highlights its therapeutic potential, especially during muscle wasting or injury, conditions where it is critical for large and functional myotubes to regenerate. One of the recent studies showed that Quercetin can enhance migration of skeletal muscle and promote myogenic differentiation, advocating its role in improving muscle regeneration and repair (Hour *et al.*, 2022).

These findings together suggest that quercetin plays an important role in promoting myogenic differentiation and its dose dependant effects on fusion index, myotube area and aspect ratio

has further highlighted the need for optimization of quercetin dosage in future therapeutic applications.

Quercetin has complex and concentration-dependent effects on the expression of growth factor in human skeletal muscle stem cells. From 16 screened growth factors some showed quite significant results related to their function in muscle cells via being directly involved in the regulation of critical cellular processes like cell growth, their differentiation, survival and integrity.

Quercetin does not significantly affect the expression of MCSF-R, SCF, or SCF-R at the concentrations tested. These pathways (MCSF-R and SCF/SCF-R) are essential for the survival and proliferation of stem cells, including muscle stem cells. The absence of significant changes suggests that Quercetin does not disrupt the normal activity of these pathways, helping to maintain the stem cell niche and support regular myogenic processes. This is important for preserving the self-renewal and differentiation capabilities of muscle stem cells without triggering unwanted proliferation or differentiation.

The effects of Quercetin on neurotrophic factors, such as NGF-R, NT-3, and NT-4, along with members of the TGF family, were dependent on concentration. Notably, Quercetin significantly upregulated NGF-R expression in a dose-dependent manner, especially at higher concentrations. This suggests that Quercetin may enhance neurotrophic signalling, potentially benefiting muscle-nerve interactions during myogenesis.

In contrast, NT-3 expression showed a temporary decrease at 1 μM, returning to baseline at higher concentrations. This suggests that low levels of Quercetin may briefly disrupt neurotrophic support, but the effect isn’t lasting. Similarly, NT-4 expression remained largely unchanged, indicating that Quercetin's impact on neurotrophic support is minimal and specific to certain factors.

The members of the TGF family (TGFα, TGFβ1, and TGFβ3) exhibited varying responses to Quercetin treatment. TGFα was significantly downregulated at low concentrations, but this effect did not persist at higher doses. In contrast, TGFβ1 showed a notable upregulation at 25 μM, raising concerns that high concentrations of Quercetin could potentially create a fibrotic environment. TGFβ3 was significantly upregulated only at 5 μM, indicating a specific concentration range where Quercetin may affect tissue remodelling processes. These findings underscore the complexity of Quercetin's role in regulating growth factor signalling, suggesting that its effects on myogenic differentiation may involve a delicate balance between promoting and inhibiting certain pathways.

One of the most notable findings of this study is the strong and dose-dependent upregulation of VEGF expression by Quercetin, especially at higher concentrations. VEGF is a critical regulator of angiogenesis, and its increased levels suggest that Quercetin may enhance vascularization in developing muscle tissues. This finding is consistent with existing studies where quercetin has shown ability to upregulate VEGF in endothelial cell, promoting the formation of blood vessels.(Jeon *et al.*, 2007).This pro-angiogenic effect presents a promising therapeutic opportunity for improving muscle regeneration and repair, particularly in ischemic conditions or following injury.

However, the expression of VEGF receptors (VEGF R2 and VEGF R3) and VEGF-D showed variability and was not significantly impacted by Quercetin (Yu *et al.*, 2009). This suggests that while Quercetin effectively boosts VEGF production, its influence on the associated receptors and lymphangiogenic factors is less consistent. The primary angiogenic response in skeletal muscle stem cells (SkMCs) seems to be driven by elevated VEGF levels, with receptor expression playing a more secondary or variable role. This finding highlights the importance of VEGF as a key mediator of Quercetin's pro-angiogenic effects, while also implying that the downstream signalling may be more intricate and context dependent.

Another important finding is the significant upregulation of OPG (Osteoprotegerin) at higher concentrations of Quercetin. OPG is known for its crucial role in bone metabolism by inhibiting osteoclast differentiation in muscles (Indridason, Franzson and Sigurdsson, 2005). Its increased

expression in skeletal muscle stem cells (SkMCs) suggests that Quercetin may enhance communication between muscle and bone, potentially creating an environment that supports both muscle regeneration and bone health (Kanter *et al.*, 2007). This dual effect could be especially beneficial for aging populations or in conditions where the integrity of both muscle and bone is compromised.

The results of this study have significant implications for the potential therapeutic use of Quercetin in muscle regeneration and angiogenesis. The dose-dependent effects observed, particularly the upregulation of VEGF and OPG, suggest that Quercetin could be effectively utilized to enhance both vascular and musculoskeletal health. However, these findings also emphasize the necessity for careful dose optimization to mitigate potential adverse effects, such as the pro-fibrotic response indicated by the increased levels of TGFβ1 at higher concentrations (Ismaeel *et al.*, 2019).

Further research is required to clarify the specific signalling pathways involved and to assess the functional outcomes of the observed changes in growth factor expression. This deeper understanding of how Quercetin modulates myogenic differentiation and angiogenesis could guide the development of targeted therapies for muscle and vascular regeneration factors and the TGF family are more nuanced and require further investigation. These results contribute to our knowledge of quercetin's potential as a therapeutic agent for improving muscle regeneration and vascularization, suggesting its applicability in the treatment of various musculoskeletal and vascular conditions.

In the study of skeletal muscle cells, conducting a cytotoxicity test aims to investigate the possible negative effects that different drugs or substances may have on cell viability, health, and functionality of these muscles (Adan, Kiraz and Baran, 2016). These assays play a crucial role in assessing the toxicity of medications, chemicals, or other compounds that could encounter with skeletal muscle tissue in animal. MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide is a most commonly used colorimetric assay(Tolosa, Donato and Gómez-Lechón, 2015). This methods was used to access the cytotoxic effects of of nutrients or drugs as well as to cell viability (Mosmann, 1983; Berridge, Herst and Tan, 2005; Adan, Kiraz and Baran, 2016).

Cytotoxicity analysis of this study revealed that quercetin exhibits low cytotoxicity across different concentrations of quercetin. At low concentration (1 μM - 25 μM) which were effective to promote myogenic differentiation and angiogenesis, quercetin showed no significant cytotoxic effect on skeletal muscle stem cells demonstrating favourable safety profile of quercetin in different cell types (Ma *et al.*, 2024). However, high doses (50 μM - 100 μM) showed cytotoxic effects and risk for potential fibrosis. A study performed on glioblastoma cell lines further supported this by highlighting quercetin ability to induce apoptosis and stress response especially at high concentrations. emphasizing on the need for dose optimisation in therapeutic applications (Kusaczuk *et al.*, 2022).

4.1. Future of the Research:

The findings of this research open promising opportunities for future studies on the effect of quercetin on myogenic differentiation and on muscles regeneration. One of the key areas to explore is the transition from in-vitro to in-vivo models. Investigating quercetin's impact on animal models or clinical trials could provide more comprehensive understanding of its therapeutic potential in treating muscles related disorders like muscular dystrophies or sarcopenia.

Further research should also dive into the molecular mechanisms that underly the effect of quercetin on SkMCs, specifically its role in gene expression modulations, oxidative stress, and inflammatory responses. Insight into these pathways could lead to the development of targeted therapies that harness quercetin's benefits by minimizing any of the possible adverse effects.

4.2. Strengths of the Research:

This study has explored an under-researched area of quercetin's effects on the myogenic differentiation of human skeletal muscle stem cells (SkMCs). It provides valuable insights into the growing interest in natural compounds for therapeutic purposes, particularly in muscles regeneration and in the treatment of muscle-related disorders. By systematically analysing the impact of quercetin across several key parameters such as fusion index, myotube area, aspect ratio, protein expression through microarray analysis, and cytotoxicity, this research offers a detailed understanding on how quercetin a nutrient supplement can affect various aspects of muscle cell differentiation and health.

In this research the application of ImageJ software for image analysis is providing significant strength, as it presents a systematic and quantifiable approach for measuring differentiation parameters like myotube area, aspect ratio and fusion index. Despite of having certain limitations this software adds a layer of accuracy to data analysis. By indicating positive effects of quercetin on SkMCs differentiation, as well as its ability to modulate oxidative stress and inflammation. This study lays a foundation for future research on natural compounds, as potential therapeutic agents for muscle-related conditions.

One of the key strengths is using in-vitro model for the study in combination with microarray to provide comprehensive understanding of quercetin treatment on muscle cell differentiation. Furthermore, the research has identified specific pathways and effects of quercetin that warrant further investigation, providing a clear path for future studies. The findings contribute to the broader field of muscle regeneration research and open potential avenues for clinical applications.

The study has not only presented its findings but has also critically assessed its limitations, such as the challenges associated with using ImageJ software and the need for in vivo studies. This transparency enhances the credibility of the research and provides valuable guidance for future work in this field.

4.3. Limitations of the research:

While this study has provided valuable insight into the effects of quercetin on myogenic differentiation, still there are several limitations. Firstly, the research was conducted in vitro, and the results may not fully replicate when performed in in-vivo environment, due to complex in vivo environment of human muscle tissues, where factors like blood supply, human immune responses, and extracellular matrix interactions play a significant role .Furthermore, the study has focused on limited range of quercetin concentrations, whereas further exploration across broad spectrum of doses could offer a more comprehensive understanding of its effect and cytotoxicity.

One more limitation identified in the present research pertains to be the reliance on ImageJ software for obtaining differentiation parameters data. The programme may introduce variability depending upon the experience of user as well as the quality of image acquisition. Furthermore, due to programs lack automatic functionality this software is proved to be challenging. In this research, the ImageJ program is used as a tool for the quantification and computing of the differentiation parameters. The individual had to focus on each image and had to manually perform the analysis to count the number of nuclei present within each of the myotube to determine the fusion index. This task can be both time-consuming as well as prone to errors due to the clustered arrangement of nuclei, making precise analysis difficult. Likewise, measuring metrics such as aspect ratio and myotube area a scale within the program is used to determine the length and width of myotubes, adding to the time burden.

Moreover, the measurements obtained using ImageJ can vary slightly when the same image is analysed on different systems or even is re-analysed on the same system, mainly for field area, myotube area, and myotube length and width. To minimize these differences, it's crucial that the selected image or set of images be evaluated in single continuous session. However, in future software advancements in terms of automation could significantly reduce the time and effort required while minimizing errors and inconsistencies.

Lastly, the study has not assessed long-term effects or potential side effects of quercetin treatment, which are critical for evaluating its therapeutic potential. Future research should address these limitations by integrating in-vivo studies, increasing dosage ranges, and conducting longitudinal analyses to fully explicate quercetin's impact on muscle health and their regeneration.

5. Conclusion:

This research demonstrates that a naturally occurring flavonoid named quercetin, can significantly influence the myogenic differentiation of human skeletal muscle stem cells (SkMCs) which have been cultured in-vitro, and can also primarily modulate key regulatory factors involved in myogenesis, as demonstrated by microarray analysis. In this study several key factors are analysed such as fusion index, myotube area, and aspect ratio, gene expression profiles using microarray analysis and cytotoxicity.

Quercetin was found to increase fusion index, by indicating high percentage of SkMCs forming multinucleated myotubes, which is crucial for effective regeneration of muscles. More-over, an increase in myotube area has also been observed by treating the cells with quercetin. Additionally, the results showed that the cytotoxic test performed on the quercetin influenced myotube differentiation, which can possibly affect regeneration of muscles. The study has also emphasized on the importance of dosage, depicting that despite quercetin being a promising therapeutic agent for muscle regeneration, careful consideration of its dosage is critical to avoid its adverse effects.

In conclusion, we can say that quercetin as a modulator of muscles differentiation and regeneration of human SkMCs which have been cultured in vitro has shown significant potential and hence can be explored as a therapeutic agent especially in treating muscle-related disorders. This study has provided a foundation for future research on clinical applications of quercetin in muscle health and regeneration, yet further research is necessary to fully determine quercetin’s safety and efficacy in clinical settings and to fully elucidate its molecular mechanism

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