University of Piemonte Orientale

School of Medicine
Department of Health Science

Master's Degree in Medical Biotechnologies

Vaccinium Macrocarpon Counteracts Advanced Glycation End Product-Induced Atrophy in Skeletal Muscle Cells

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Summary

Advanced Glycation End Products (AGEs) are a group of compounds formed through the non-enzymatic Maillard reaction between reducing sugars and proteins, lipids, or nucleic acids. They can be exogenous, derived from high-heat cooking methods prevalent in Western diets, or endogenous, physiologically formed in the body. Despite their significant implications in chronic diseases such as atherosclerosis, cancer, and aging, the impact of AGEs on skeletal muscle health remains underexplored.

Preliminary results from Prof. Riuzzi's laboratory (University of Perugia) indicate that AGEs induce atrophy in C2C12-derived myotubes in a dose-dependent manner. Since it is well established that AGEs trigger oxidative stress, we hypothesized that AGEs negatively impact muscle homeostasis by impairing mitochondrial function.

Our findings indicate that AGEs induce mitochondrial fission, exacerbate mitophagy, and increase ROS production, likely through the activation of distinct pathways. Furthermore, we investigated the putative protective effect of Vaccinium Macrocarpon (VM), known as cranberry. Our results suggest that VM effectively counteracts the detrimental effects of AGEs, offering a potential natural therapeutical approach for muscle pathologies associated with AGE accumulation.

Introduction

Advanced Glycation End Products (AGEs)

Advanced glycation end products (AGEs) are a group of heterogeneous chemical compounds, primarily formed through the Maillard reaction, that have been associated with adverse health effects, such as chronic diseases, atherosclerosis, and inflammation (Fishman et al. 2018). They are naturally produced when a reducing sugar reacts with proteins, lipids, or nucleic acids in a non-enzymatic way. This spontaneous and slow process, under physiological conditions, takes several days or weeks to complete because it does not involve an enzyme catalyst (Mengstie et al. 2022), and it is followed by the structural rearrangements of different molecules (e.g., protein and sugar) that can lead to the formation of these irreversible end-products (Twarda-Clapa et al. 2022).

AGEs can be either exogenous or synthesized endogenously within the body (Van Dongen et al., 2022). Exogenous AGEs primarily derive from the consumption of highly processed foods, which are common in modern diets, especially in the so-called "Western diet" (in Table 1 is reported the amount of AGEs in common food). This diet is characterized also by preparations like grilling, roasting, broiling, searing, and frying. These thermal processes, especially when applied to products with high levels of sugar and proteins, promote glycation leading to the rapid formation of AGEs (Twarda-Clapa et al. 2022). In our society, the consumption of large quantities of processed foods is increasing, leading to a continuous increase in the production and accumulation of AGEs in our bodies, which pose potential health risks.

Products	AGE [1] (kU/100 g)	Products	AGE (kU/100 g)
Meats		Carbohydrates	
Beef, raw	707	Bread, pita	53
Beef, steak, pan fried with olive oil	10,058	Bread, Greek, hard, toasted	607
Chicken, breast, skinless, raw	769	Biscuit (McDonald's)	1470
Chicken, boiled with lemon	957	Cookie, biscotti, vanilla almond	3220
Chicken, breast, breaded, deep-fried, 20 min	9722	Donut, chocolate iced, crème filled	1803
Chicken, skin, back or thigh, roasted, barbequed	18,520	Apple, baked	43
Chicken, skin, thigh, roasted	11,149	Apple, Macintosh	13
Turkey, ground, raw	4957	Fig, dried	2663
Turkey, ground, grilled, crust	6351	Carrots, canned	10
Bacon, fried, 5 min, no added oil	91,577	Tomato	23
Bacon, microwaved, 2 slices, 3 min	9023	Vegetables, grilled (broccoli, carrots, celery)	226
Lamb, leg, raw	826	Honey	7
Lamb, leg, broiled, 450°F, 1 min	2431	Fats	
Fish		Almonds, roasted	6650
Salmon, fillet, microwaved	912	Butter, whipped	26,480
Salmon, fillet, broiled	3347	Margarine, tub	17,520
Trout, raw	783	Walnuts, roasted	7887
Trout, baked, 25 min	2138	Eggs	
Liquids		Egg white, large, 12 min	63
Milk, whole (4% fat)	5	Egg yolk, large, 12 min	1680
Juice, apple	2	Egg, omelet, pan, low, butter, 13 min	507

Table 1. The dietary AGE content in food (Twarda-Clapa et al. 2022).

When ingested, exogenous AGEs are absorbed by the gastrointestinal tract. Some enter the circulatory system and accumulate in tissues, while others are excreted in urine or feces as non-absorbable AGEs (Mengstie et al. 2022). The absorption level of these compounds in the gut is determined by several factors, such as their molecular weight and hydrophobicity. Indeed, small hydrophobic AGEs can cross the basolateral membrane easier than large hydrophilic ones (Twarda-Clapa et al. 2022).

On the other hand, endogenous AGEs are typically formed in all tissues and body fluids under physiological conditions through glycation reactions. The accumulation of endogenous AGEs within the body occurs during the aging process, in which the repair and degradation systems are no longer functional, leading to the accumulation of modified biomolecules (Ott et al. 2014).

Numerous studies show that excessive accumulation of AGEs promotes oxidative stress and inflammation by activating several pro-inflammatory transcription factors (Fishman et al. 2018), through the binding of their receptor RAGE (Receptor for advanced glycation end-products). This process plays a critical role in the development and progression of many diseases, including cancers (Wang et al. 2015). Moreover, AGEs can accumulate in musculoskeletal tissues in old age and are thought to play a crucial role in the development of sarcopenia (Dao et al. 2020). Furthermore, recent studies demonstrated that the increase in ROS induced by AGEs is a contributing factor to subsequent changes in mitochondrial structure and dysfunction (Wang et al. 2015).

Over the past decades, natural products have been extensively tested *in vitro* and *in vivo*, demonstrating significant medical properties (Salvadori et al. 2024). Indeed, natural active metabolites have emerged as promising agents to reduce and mitigate the harmful activity of AGEs in various diseases, reducing oxidative stress and inflammation (Salvadori et al. 2024). Interestingly, *Vaccinium macrocarpon* (VM), commonly known as cranberry, has been shown effective in mitigating AGE formation (data not shown) among a pool of thirty different natural compound.

Role of mitochondria in skeletal muscle health

Mitochondria play a crucial role in a wide range of cellular processes, including homeostasis, cell death, inflammation, and senescence. They are also essential in the maintenance of skeletal muscle homeostasis. Indeed, efficient muscle bioenergetics hinge on mitochondria, and mitochondrial dysfunction is recognized as a major hallmark and cause of muscle atrophy (Harper, Gopalan, e Goh 2021).

Mitochondria are dynamic organelles capable of altering their morphology through fusion and fission events. Fusion leads to mitochondrial elongation, while fission fragments the mitochondria.

Mitochondrial fusion is regulated by Mfn1 and Mfn2 on the outer mitochondrial membrane and by Opa1 on the inner mitochondrial membrane. On the other side, fission is mainly mediated by the cytoplasmic protein Dynamin-related protein 1 (Drp1) that is recruited to the outer mitochondrial membrane, where it binds to its adaptor proteins, in order to activate the fission machinery (Leduc-Gaudet et al. 2021).

In various pathological conditions associated skeletal muscle wasting, mitochondrial quality control and the regulation of mitochondrial fusion and fission proteins are often dysregulated (Leduc-Gaudet et al. 2021).

Equally indispensable for the muscle health is also the removal of dysfunctional mitochondria. Skeletal muscle and other cell types have a mechanism to do so: a selective form of autophagy, termed mitophagy. Failure of this process can negatively regulate metabolism and muscle mass ((Harper, Gopalan, e Goh 2021). The balance between mitochondrial fission, fusion, and mitophagy plays a pivotal role in regulating skeletal muscle health, and any disruption of this equilibrium can lead to muscle atrophy in different physiopathological conditions, including sarcopenia ((Harper, Gopalan, e Goh 2021).

Aims of the project

As previously pointed out, the accumulation of AGEs can lead to various pathological condition, including muscle atrophy. Consistently, preliminary results from the laboratory of prof. Francesca Riuzzi (University of Perugia) indicate that AGEs induce a dose-dependent myotube area reduction in C2C12, a widely used model to study skeletal muscle homeostasis *in vitro*. However, the underlying mechanisms by which AGEs cause atrophy have not yet been investigated.

During my thesis internship, I focused on deciphering the effects of AGEs on skeletal muscle homeostasis, with specific attention to mitochondrial function. To this aim, we utilized AGE-BSA, produced by the glycation of bovine serum albumin (BSA), representative of endogenous AGEs, to study the impact of internally produced AGEs. Additionally, we used methylglyoxal (MG), an exogenous AGE, to investigate the effects of dietary AGEs. These compounds were chosen to comprehensively examine the roles of both exogenous and endogenous AGEs in skeletal muscle wasting.

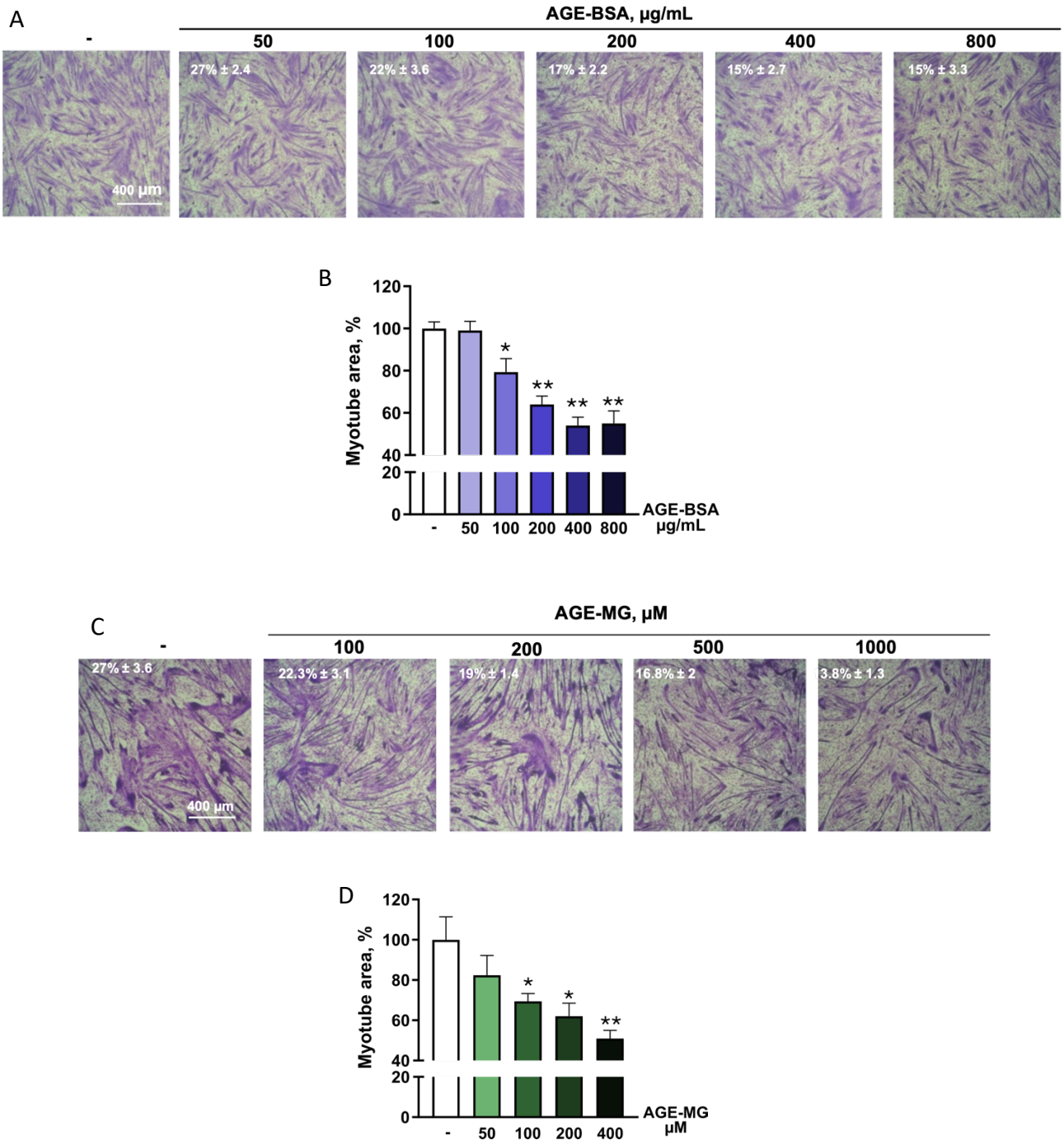


Figure 1. Both exogenous and endogenous AGEs reduced C2C12 myotube area. (A,C) C2C12 myotubes were treated with different doses of AGE-BSA (50-800 µg/mL) or AGE-MG (100-1000 µM). May-Grünwald/Giemsa staining was performed after 48h and myotube areas were measured by Image J software. (B,D) Representative images with the percentages of myotube areas.

Natural compounds have the potential to reduce the harmful effects of AGEs, decreasing oxidative stress and inflammation (Salvadori et al. 2024). Thus, this project also aims to investigate the potential therapeutic effects of VM on AGE-induced muscle atrophy.

Materials and methods

Reagents

To obtain AGE-BSA 1g of BSA and 1,8g of D-glucose were dissolved in 20 mL of PBS and incubated at 50°C for 4 days. MG solution was purchased from Merck Life Sciences (Milan, Italy). Standardized dried extract of VM was provided by Laboratory Biokyma S.r.l (Anghiari, Italy). The same batch of product was used in the various experiments. Compounds were solubilized in water at a concentration of 10 mg/mL, and then opportunely diluted to be tested at the final concentrations reported in the several bioassays. *CellROX R Deep Red Reagent* was purchased from Thermo Fisher Scientific. Recombinant rat agrin was purchased from Biotechne. All other reagents were from Merck Life Sciences.

Cell cultures and treatments

C2C12 myoblasts were grown at low density in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL antimycotic. To induce differentiation, cells were allowed to become confluent, and the medium was switched to differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum (GE Healthcare Bio-Sciences), penicillin, streptomycin, and antimycotic as described above. Myotubes were treated after at least 4 days of differentiation in serum-free medium with AGE-BSA (50-800 µg/mL) or MG (100-1000 µM) in the presence or absence of 100 µg/mL VM. Myotube diameters were measured after 48 h treatment, while all the other analysis were performed after 24 h treatment.

Immunofluorescence and myotube analysis

In order to measure myotube diameters, MyHC was detected through immunofluorescence. Briefly, myotubes were fixed with 4% paraformaldehyde (PFA), permeabilized using 0.1% Triton X-100 in PBS, blocked with blocking buffer containing 1% glycine (SERVA) and 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, and incubated in a humid chamber overnight at 4 °C with mouse anti-MyHC-II primary antibody (eBiosciences) in PBS 3% BSA. The next day, coverslips were incubated with anti-mouse Alexa Fluor 488-conjugated antibody (Thermo Fisher Scientific) in a light-tight humid chamber. Nuclei were counterstained with DAPI. Samples were mounted with fluorescent mounting medium and viewed by an epifluorescence microscope (Leica DMRB, Milan, Italy) equipped with a digital camera. Average diameters of at least 100 myotubes from 10 randomly chosen fields for each condition were determined. The width of each myotube was measured at 3 different points along the longitudinal axis of the cell using Image J software.

Cellular fractionation

At the end of the indicated treatments, cells were trypsinized, resuspended in the fractionation buffer (20 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4), supplemented with protease inhibitor cocktail, and incubated for 15 min on ice. Cells were then lysed with a 29-gauge needle and incubated again on ice for 20 min. Afterwards, lysates were centrifuged at 720 × *g* for 5 min at 4°C to remove the nuclei. The supernatant was centrifuged once more at 10,000 × *g* for 5 min at 4°C. The pellet, which contains the mitochondrial fraction, was resuspended in TBS 0.1% SDS and sonicated briefly to obtain mitochondrial lysate, while the supernatant was collected and used as cytoplasmic fraction. For each experiment, the fractionation efficacy was evaluated through western blotting, assessing the presence of cytosolic- or mitochondrial-specific markers in the corresponding fractions. Both the mitochondrial and cytosolic fractions were then analyzed by western blotting.

Western Blotting

After cellular fractionation, proteins were separated by SDS- PAGE and transferred to polyvinylidene difluoride filters (PVDF) (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Membranes were saturated with 4% BSA, incubated with the primary antibodies overnight, washed with Tris buffered saline (TBS)-0.1% Tween, incubated with the appropriate secondary antibody (Bio-Rad, Hercules, California, USA), visualized with Western Lightning Chemiluminescence Reagent Plus (Thermo Fisher Scientific), acquired with ChemiDoc Touch (Bio-Rad), and analysed with ImageLab (Bio-Rad).

Cellular respiration

Cellular respiration was measured using an Oroboros oxygraph-2K high-resolution respirometer (Oroboros Instruments) and the "substrate, uncoupler, inhibitor, titration" (SUIT) protocol SUIT-003_O2_ce_D012 recommended by the manufacturer of the Oroboros instrument as previously described (Raiteri et al. 2021). At the end of the treatments, C2C12 myotubes were trypsinized, centrifuged at 300 × *g* for 5 min, resuspended in mitochondrial respiration medium MiR05 (0.5 mM EGTA, 3.0 mM $MgCl2·6H2O$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM $KH2PO4$, 20 mM HEPES, 110 mM sucrose, 1 g/L bovine serum albumin, pH 7.1) and transferred to the chambers of the Oroboros oxygraph. Control and treated samples were assessed simultaneously. After initial stabilization of O_2 flux, 5 mM pyruvate was used to sustain TCA-linked respiration in MiR05 medium. 5 nM oligomycin (Omy), an ATP synthetase inhibitor, was added, and the oxygen consumption was quantified to determine the oligomycin-sensitive and -insensitive respiration.

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a protonophore and uncoupler of oxidative phosphorylation, was then added at 0.5 µM increments to achieve maximum respiration to quantify the maximum respiratory capacity. This was followed by the inoculation of 500 nM rotenone (Rot) to inhibit complex I of the ETS, and, finally, 2.5 µM antimycin A (Ama), an inhibitor of complex III, was added to determine the non-mitochondrial respiration (ROX). Oxygen consumption rates were calculated using the accompanying software DatLab7 (Oroboros). Rates of O2 consumption (flux) were normalized to total protein content. Briefly, at the end of the experimental procedure, the cellular suspension from the two chambers was centrifuged at 1,000 × *g* for 5 min. The cellular pellet was lysed in 200 µL of lysis buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% NP40, 1 mM DTT) and then centrifuged at 15,000 × *g* for 15 min at 4°C. The concentration of the protein in the supernatant was measured with Bradford Reagent.

Acetylcholine receptor clustering assay

To examine the formation of Acetylcholine receptor (AChR), C2C12 myotubes were co-treated with 5nM agrin for 16h and AGEs for 24h. After 24 hours myotubes were fixed with 4% PFA at room temperature for 20-30 min and AChR clusters were labeled in myotubes using Alexa Fluor488-conjugated with α-Bungarotoxin (α-BTX) (code) and DAPI (1:100). Images of AChR clusters were captured by using a 20x objective and a fluorescence microscope. The area of AChR clusters were quantified from random fields using imageJ software.

Reactive Oxygen Species (ROS) production

To measure cellular ROS production, C2C12 myotubes were stained with *CellROX R Deep Red Reagent* for 30 min at 37°C and washed with PBS. Images were taken using the EVOS™ XL Core Imaging System, and the mean fluorescence signal intensity was measured using ImageJ. For every experiment assessing cellular oxidative stress, at least three myotubes in each field, five different fields for each replicate, three technical replicates for each treatment were measured.

RNA extraction and analysis

Total RNA from muscles or myotubes was extracted by Nucleozol (Macherey Nagel). The RNA was retro-transcribed with High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) and real-time PCR was performed with the StepOnePlus Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific), using the following TaqMan probes (Thermo Fisher Scientific): Mm00499518_ m1 (*Mfn2*), Mm00515325_g1 (*Atf4)*, Mm01197698_m1 (*Gusb*).

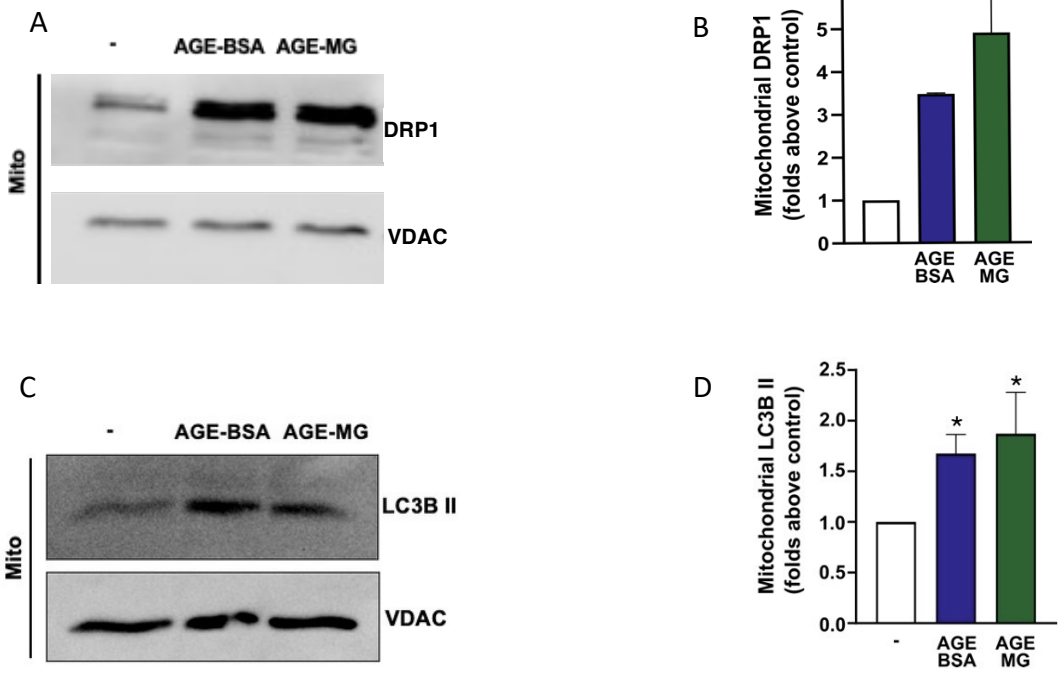
Statistical analysis

Data are presented as the mean ± SEM. Outliers in the measurements were identified by mean of the interquartile range (IQR), as either below Q1 − 1.5 IQR or above Q3 + 1.5 IQR and excluded from the analysis. The variation among groups was evaluated using Student's t test unless otherwise stated. Statistical significance was assumed for $P < 0.05$. All statistical analyses were performed with GraphPad Prism 8.

Results

AGEs impact on mitochondrial dynamic and degradation

The maintenance of mitochondrial function and integrity is critical for skeletal muscle homeostasis. Fission and fusion are dynamic events essential for segregation of damaged mitochondria and their subsequent degradation through mitophagy (Harper, Gopalan, e Goh 2021). To evaluate the impact of AGEs on skeletal muscle mitochondria, C2C12 myotubes were treated with AGE-BSA and AGE-MG for 24 h. A substantial increase in the expression of DRP1, a fundamental protein required for mitochondrial fission, was observed in the mitochondrial fraction of both AGE-BSA and AGE-MG-treated myotubes (Fig. 2A, B). Since mitochondrial fission can promote mitophagy, we assessed the expression levels of the autophagic marker LC3B II in the mitochondrial fraction. We found that both AGEs exacerbated mitophagy, as evidenced by the accumulation of LC3B II at the mitochondrial level (Fig. 2C, D). To understand if these dysregulations in mitochondrial dynamics translated into an impairment of mitochondria functionality, the oxygen consumption rate (OCR) of C2C12 myotubes was measured using an Oroboros oxygraph-2K high-resolution respirometer. Notably, a reduction in mitochondrial respiration was observed exclusively after AGE-BSA treatment. In particular, AGE-BSA reduced maximal respiration capacity (*i.e.,* ET state; fig. 2E) and reserve respiratory capacity, a critical component of mitochondrial oxidation that can be utilized during states of increased ATP demand (Fig. 2E-G). These findings suggest that AGEs disrupt mitochondrial dynamics and degradation in skeletal muscle cells, with AGE-BSA having a pronounced effect also on mitochondrial respiration.



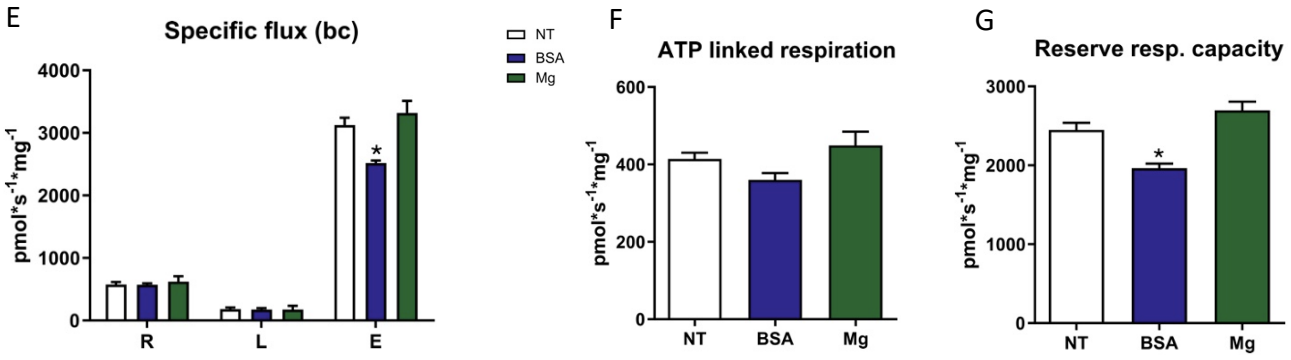


Figure 2. Both AGEs increase mitochondrial fission and mitophagy but only AGE-BSA affects mitochondrial respiration. C2C12 myotubes were treated with 200 μg/mL AGE-BSA or 200 μM AGE-MG for 24 h. (A) The recruitment of the fission marker DRP1 at the mitochondria was analyzed by western blotting of the mitochondrial fraction (Mito) upon AGEs treatment. (C) To assess mitophagy mitochondrial LC3II was analyzed by western blotting of the mitochondrial fraction. (B-D) Relative intensity was normalized on VDAC. (E-G) Analysis of oxygen flux through Oroboros 2K high-resolution respirometer. (E) Mitochondrial respiration in the routine state [R], leakage [LEAK] state after addition of oligomycin, and after the addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation to induce maximal respiratory capacity [E]). All data are expressed as specific flux, i.e., oxygen consumption normalized to the sample protein content. (F) Oxygen consumption linked to ATP production, i.e., oligomycin-sensitive respiration obtained by the subtraction of LEAK from Routine. (G) Reserve respiratory capacity obtained by the subtraction of Routine from ET. Values are means±SEM. Statistical analysis was conducted using t-test *$p<0.05$, significantly different from untreated control.

Vacciunium Macrocarpon counteracts AGE-induced atrophy

Previous experiments carried out in Prof. Riuzzi's Lab investigated the potential of VM to counteract the detrimental effect of AGEs on skeletal muscle. To this aim, C2C12-derived myotubes were treated with 200 μg/mL AGE-BSA and 200 μM AGE-MG, in the presence or absence of 100 μg/ml VM for 48 h. In this model, atrophy could be evaluated by measuring variation in myotube diameters. The morphological analysis confirmed the atrophic activity of both AGEs, with AGE-BSA reducing myotube diameter by 27% and AGE-MG by 35%. Remarkably, co-treatment with the phytoextract completely abolished this effect, resulting in negligible differences in myotube diameters compared to untreated cells (Fig. 3).

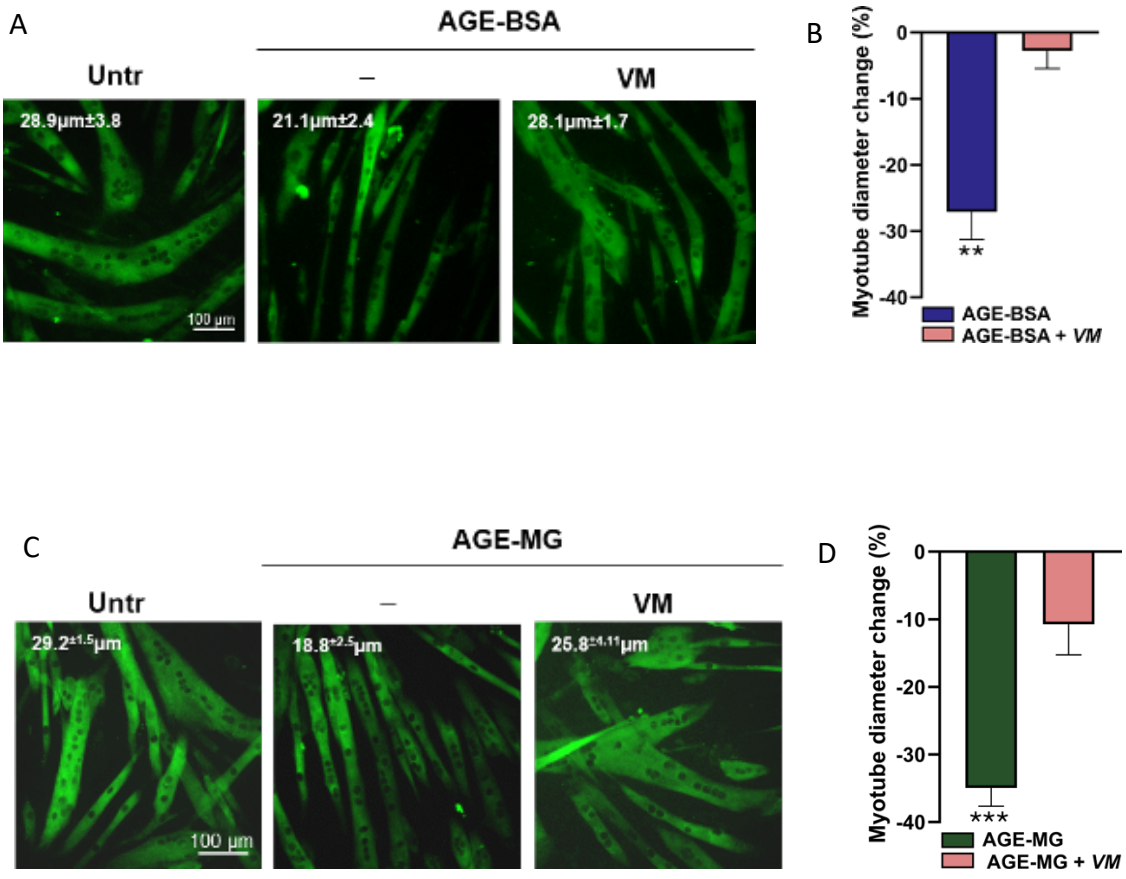


Figure 3. AGE-induced C2C12 atrophy is abolished by VM treatment. C2C12 myotubes were treated with 200 µg/mL AGE-BSA, or 200 µM AGE-MG for 48h in the absence or presence of VM (100 µg/ml). (A,C) Immunofluorescence analysis for MyHC-II was performed after 48 h, and myotube diameters were measured by Image J software. (B, D) The values (µm) and the percentages of myotube diameters vs untreated control are reported. Reported are the means±SEM. Statistical analysis was conducted using t-test *p<0.05, **p<0.01, significantly different from untreated control.

The reduction of mitochondrial respiration induced by AGE-BSA is abolished by VM

Since mitochondrial respiration was affected exclusively by AGE-BSA treatment (fig. 2E-G), we investigated whether VM could contrast this impairment. Myotubes were treated with 200 µg/mL AGE-BSA in the presence or absence of 100 µg/ml VM for 24 h, and mitochondrial respiration was assessed using the Oroboros oxygraph-2K high-resolution respirometer.

The analysis revealed that VM treatment effectively prevented the reduction in mitochondrial respiration induced by AGE-BSA. Specifically, in myotubes treated with both AGE-BSA and VM, the OCR was restored to levels comparable to untreated controls, in all phases of the mitochondrial

respiration, indicating a protective effect of VM against AGE-BSA-induced mitochondrial dysfunction.

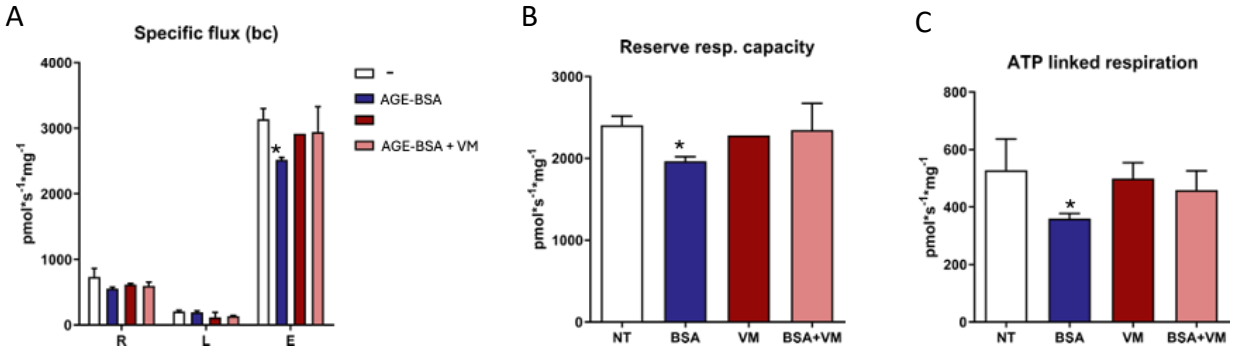


Figure 4. OCR reduction caused by AGE-BSA is reverted by VM. (A-C) Analysis of oxygen flux through Oroboros 2K high-resolution respirometer in C2C12-derived myotubes treated for 24 h with AGE-BSA in the presence or absence of VM. (A) Mitochondrial respiration in the routine state [R], leakage [LEAK] state after addition of oligomycin, and after the addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation to induce maximal respiratory capacity [E]). All data are expressed as specific flux, i.e., oxygen consumption normalized to the sample protein content. (B) Oxygen consumption linked to ATP production, i.e., oligomycin-sensitive respiration obtained by the subtraction of LEAK from Routine. (C) Reserve respiratory capacity obtained by the subtraction of Routine from ET. Values are means±SEM. Statistical analysis was conducted using t-test *$p<0.05$, significantly different from untreated control.

VM abolished AGE-induced ROS production

ROS play a crucial role in skeletal muscle physiology, influencing muscle function. However, an imbalance between ROS production and antioxidant defenses leads to oxidative stress, which contributes to mitochondrial dysfunction and muscle atrophy (Liguori et al. 2018). Excessive ROS production, commonly seen in aging and age-related diseases, induces mitochondrial damage, ultimately compromising muscle health. Furthermore, it is well-established that AGEs can enhance ROS production via RAGE activation. Given these notions, we decided to investigate whether AGEs directly increase ROS levels in skeletal muscle cells and whether VM could provide protection against this phenomenon.

To assess the impact of AGE-BSA and AGE-MG treatments on oxidative stress in C2C12-derived myotubes, we measured ROS levels using the CellROX Deep Red staining assay. The analysis revealed a significant increase in ROS levels following treatments with both AGEs, as evidenced by the enhanced fluorescence intensity. Notably, co-treatment with VM effectively restored ROS levels to those observed in untreated controls (Fig. 5A-D).

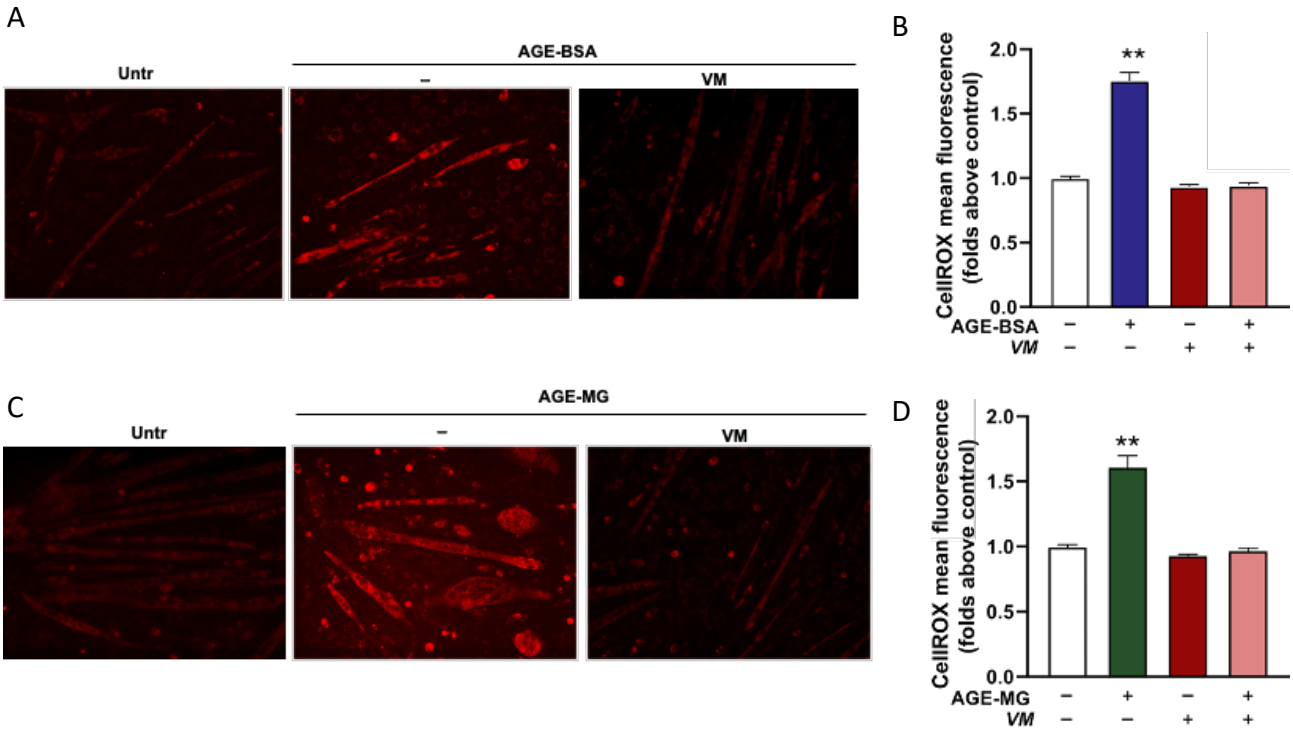


Figure 5. AGE-induced ROS production is reverted by VM. (A,C) After treatment with 200 µg/mL AGE-BSA, or 200 µM AGE-MG for 48h in the absence or presence of VM (100 µg/ml) Cell ROS production was evaluated by CellROX Deep Red reagent and quantified as the fluorescence mean intensity of each myotube. (B,D) Reported are the means±SEM. Statistical analysis was conducted using t-test *p<0.05, **p<0.01, significantly different from untreated control.

AGEs induce acetylcholine receptors dismantling

The neuromuscular junction (NMJ) is a specialized synapse that connects the motor neuron to the muscle fiber, allowing muscle contraction and movement. *In vivo*, dysfunction in NMJ functionality can lead to skeletal muscle atrophy (Rudolf et al., 2016). Furthermore, the degenerative change of NMJ promotes the process of muscle atrophy in the elderly (REF?).

In vitro, NMJ formation can be studied in C2C12 myotubes by inducing the clustering of acetylcholine receptors (AChRs) with agrin treatment (Marangi et al., 2001). To assess whether AGEs affected the formation of AChR clusters, we induced the formation of AChR clusters in the presence or absence of AGEs and quantified the positivity for fluorescent α-Bungarotoxin (α-BTX), which binds to AChRs.

Through fluorescence staining of AChRs, we observed that the area of acetylcholine receptor clusters in AGE-treated myotubes was significantly reduced compared to non-treated controls. Remarkably, co-treatment with VM effectively reversed this condition, restoring AChR clustering to levels

comparable to untreated myotubes. These data suggest that AGE-induced NMJ dismantling could be another mechanism through which AGEs induce skeletal muscle atrophy, in particular a denervation-like atrophy.

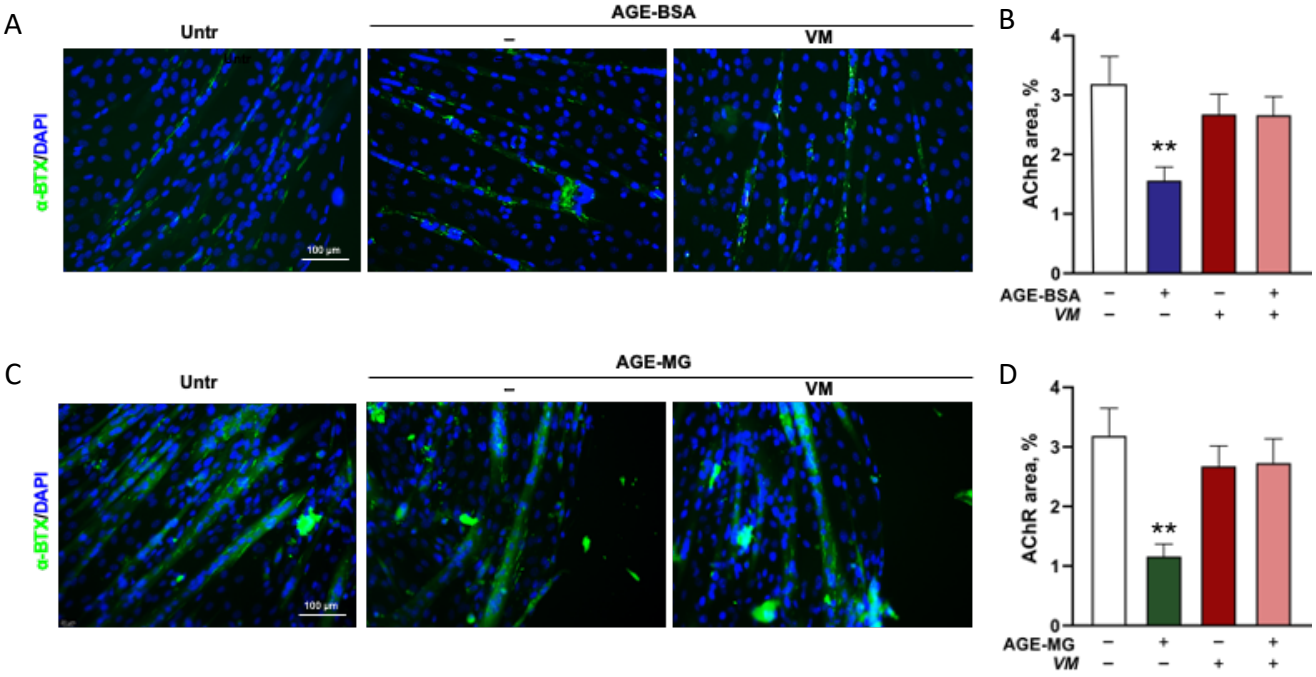


Figure 6. VM prevents AGE-induce acetylcholine receptors dismantling. (A,C) Representative images of C2C12 myotubes incubated with 5 nM agrin for 16 h in the presence or absence of 200 μg/mL AGE-BSA or 200 μM AGE-MG and stained with AlexaFluor-conjugated α-bungarotoxin. (B,D) Percentage of AChR clusters was quantified 24 h after the treatment with AGEs Reported are the means±SEM. Statistical analysis was conducted using t-test **p<0.01.

Different AGEs activate different pathways upon short treatments

To evaluate the early impact of AGEs on different cellular stress pathways, we treated C2C12-derived myotubes with AGE-BSA and AGE-MG for one hour. The expression levels of key genes involved in mitochondrial dynamics and endoplasmic reticulum (ER) stress were assessed to understand the immediate cellular responses contributing to skeletal muscle damage. AGE-BSA treatment resulted in a significant downregulation of Mitofusin 2 (Mfn2), a crucial marker of mitochondrial fusion. The reduction in Mfn2 expression indicates an early impairment in mitochondrial dynamics, which is essential for maintaining mitochondrial integrity and function in skeletal muscle cells. In contrast, AGE-MG treatment significantly induced an increase in the expression of activating transcription factor 4 (ATF4), a pivotal regulator of ER stress, suggesting an early activation of ER stress pathways, leading to potential cellular dysfunction and damage that can eventually result in muscle atrophy. These findings underscore that, despite the similar ultimate effects of AGEs in inducing mitophagy, ROS production, and skeletal muscle atrophy, AGE-BSA and AGE-MG activate distinct early cellular pathways.

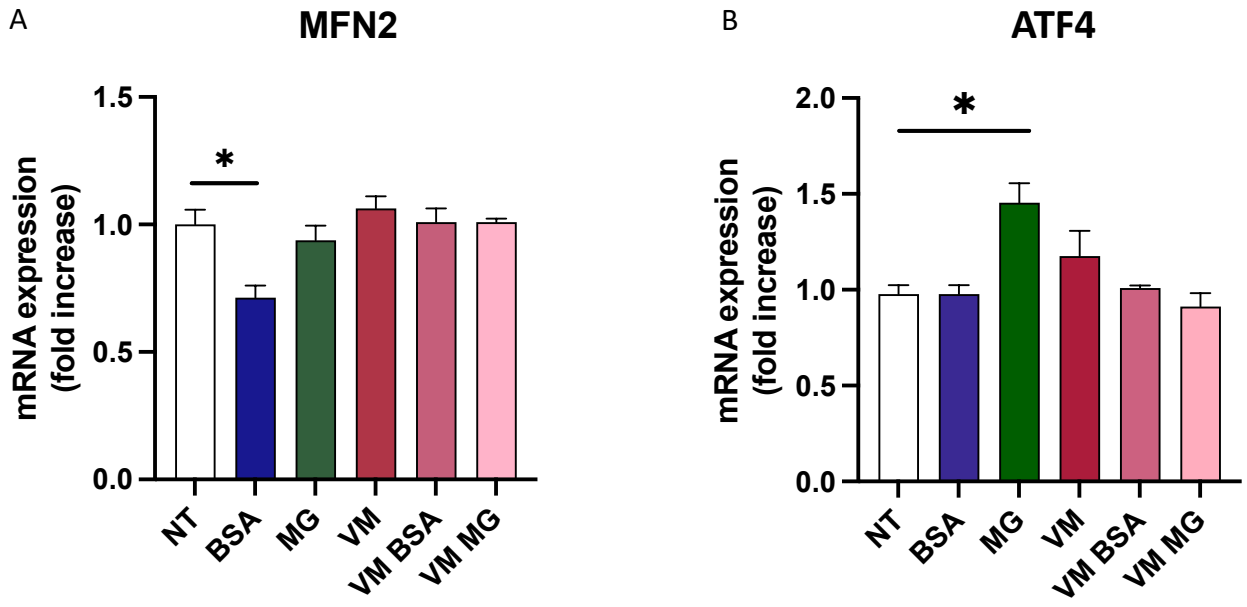


Figure 7. C2C12 myotubes were treated with 200 µg/mL AGE-BSA and AGE-MG for 1h in the absence or presence of VM (100 µg/ml). (A,B) Real-time PCR analysis was performed to evaluate Atf4 and Mfn2 levels after 1h. Gene expression was normalized to Gapdh.

Discussion

Today, in developed countries, we live longer but not always healthier. However, this extended lifespan usually coincides with an increased risk of developing chronic diseases, including musculoskeletal and metabolic disorders, that represents a novel growing socio-economic burden to face. As a matter of fact, it is not surprising that nowadays aging is seen as a social problem with a global impact whose solution seems to be still out of range.

Among the different age-related diseases, the slow decline in muscle mass and function, called sarcopenia, represent one of the most dramatic and significant aging-associated condition. However, since to date the causes of sarcopenia have not been fully understood, therapeutic options to treat this condition are still missing.

During the last decades it has been shown that lifestyle (diet, physical exercise, alcohol consumption, cigarette smoking…) has a crucial impact on the aging process and in (un)healthy aging. Physical exercise is considered the best therapeutic option to counteract the aging process due to its numerous benefits, including improving muscle strength, enhancing cardiovascular health, and reducing inflammation (Lu et al. 2021). Nevertheless, this intervention is not always feasible, especially in patients where sarcopenia co-occurs with other debilitating pathologies. Thus, the need of molecules with a broad activity that could possibly counteract multiple diseases at once, is becoming increasingly urgent.

AGEs, a heterogeneous group of non-enzymatic compounds that can be endogenously formed during physiological aging or introduced with the diet, have been shown to promote oxidative stress and inflammation (Fishman et al. 2018). Since it is well established that these processes concur to induce muscle atrophy, during my thesis internship I delved into exploring the role of AGEs in skeletal muscle wasting, with a particular emphasis on mitochondria.

Here, we demonstrated that AGEs induce skeletal muscle atrophy in C2C12-derived myotubes, likely through causing mitochondrial fission and subsequent increase of ROS (Fig. 5 A-D) Indeed, both dietary (*i.e.,* AGE-MG) and endogenous (*i.e.,* AGE-BSA) AGEs increase the recruitment of DRP1 at the mitochondrial level (Fig. 2 A,B), activating the fission machinery. In this scenario, the augmented mitochondrial fission increases the production of ROS, that directly cause myotube atrophy (Liguori et al. 2018). Moreover, these events may concur for the establishment of a reinforcement loop of mitochondrial damage, leading to the hyperactivation of mitophagy and, ultimately, exacerbating the atrophy. This is supported by the increased localization of the autophagic marker LC3II B in the mitochondria (Figures 2 C,D). However, further investigations are necessary to determine whether the augmented mitophagy is preceded by a the dissipation of the mitochondrial membrane potential, as this event typically anticipate the recruitment of the machinery required to initiate the autophagic degradation of damaged mitochondria (Leduc-Gaudet et al. 2021).

Nowadays there is a growing interest in employing natural plant compounds with antioxidant, anti-inflammatory, anti-glycation, and anti-neurodegenerative properties to slow down the onset of age-related diseases. Indeed, they can be an interesting solution compared to normal drugs since they are easier to introduce in lifestyle, more accessible, and seem to avoid side effects until excessive introduction (Babich et al. 2022). Along this line, it has been recently demonstrated as *Equisetum arvense*, a perennial fern, have a pro-myogenic effect on myoblast and an anti-atrophic ability in myotubes and delay the onset of sarcopenia in mice, preventing the loss of both muscle mass and functionality (Salvadori et al. 2024).

Remarkably, preliminary results conducted in the laboratory of Prof. Riuzzi (University of Perugia), demonstrated that VM not only reduces AGE formation at 24 h, but also preserves myotube size in the presence of both exogenous and endogenous AGEs. Based on these promising findings, we conducted further investigations to explore the putative mechanisms through which VM is able to counteract the detrimental effects of AGEs on skeletal muscle cells. Here, we demonstrated that VM can abolish the AGE-induced ROS production (Figures 5 A-D), and this is sufficient to protect C2C12 myotubes from atrophy, suggesting the augmented oxidative stress as the principal mechanism through which AGEs induce skeletal muscle wasting. This protective effect of VM can be mediated by the high content of polyphenols (flavonoids, phenolic acids, anthocyanins, tannins), ascorbic acid, and triterpene compounds, all known for their antioxidant properties (Babich et al. 2022).

Even though the final effect of both exogenous and dietary AGEs is heightened mitochondrial fission and oxidative stress, the early initial pathways they activated appear to be distinct. Indeed, our result suggest that AGE-BSA induces a reduction in mitochondrial fusion (*i.e.,* decrease expression of Mfn2 gene), while AGE-MG triggers ER stress (*i.e.,* increase of Atf4 gene) after only one-hour treatment (Figures 7 A,B). These intriguing findings led us to speculate that endogenous AGEs may have a more direct impact on mitochondria, as also indicated by the reduction of mitochondrial respiration, which, in contrast, was not observed following exogenous AGE treatment (Fig. 2E-G).

The relationship between the accumulation of endogenous AGEs, characteristic of the aging process, and the reduction of Mfn2, is consistent with the progressive decline of Mfn2 observed during aging. Along this line, studies in mice have shown that skeletal muscle Mfn2 ablation leads to a gene signature associated with aging, inducing mitochondrial dysfunction and sarcopenia (Sebastián et al. 2016).

On the other hand, the effects of exogenous AGE-MG on mitochondria may be more indirect, initially activating ER stress. The increased ER stress induced by AGE-MG may contribute, in the long run, to mitochondrial dysfunction and oxidative stress, leading to muscle atrophy. Coherently, during

skeletal muscle aging, ATF4 promotes the repression of transcripts involved in mitochondrial function (Miller et al. 2023).
Importantly, our study shows that VM revers the deregulation of both Mfn2 and Atf4 genes, indicating again its ability to maintain skeletal muscle health in the context of AGE-induced atrophy (Fig. 7A,B).

Even though the majority of research to date has focused on muscle abnormalities as the primary cause of sarcopenia, a growing body of evidence suggests that the NMJs may be a critical mediator of muscle wasting (Rudolf et al. 2016). Our data show that AGEs affect the formation of NMJs *in vitro*, destabilizing agrin-induced AChR clusters (Fig. 6A-D). Therefore, we can speculate that AGE-induced deterioration of NMJs could represent another mechanism mediating muscle wasting. Interestingly, our findings also indicate that VM prevents the AChR dismantling, induced by AGEs, suggesting a broader protective role beyond mitochondrial and ER stress pathways. This ability of VM to preserve NMJ integrity underscores its potential as a multifaceted therapeutic agent against AGE-induced skeletal muscle damage.

In conclusion, VM, as a bioactive compound, demonstrates efficacy in neutralizing the adverse impacts of AGE-induced skeletal muscle damage, highlighting its potential as a therapeutic agent for preventing or mitigating muscle atrophy associated with AGE accumulation. Further investigations are warranted to explore its potential applications in clinical settings for conditions characterized by increased AGE levels, such as aging.

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