UNIVERSIDAD FINIS TERRAE

Faculty of Medicine and Health-PhD in Exercise Science

Universidad
Finis Terrae

DOCTORAL THESIS

Thesis Title:	Urolithin A modulates myogenic differentiation, mitochondrial functional adaptation, and mTOR signaling during C2C12 differentiation
Spanish Title:	La urolitina A modula la diferenciación miogénica, la adaptación funcional mitocondrial y la señalización de mTOR durante la diferenciación de C2C12
PhD Student:	Ronald Alejandro Vargas Foitzick
Primary Supervisor:	Dra. Elisa Balboa (Universidad Finis Terrae)
Co-Supervisor:	Dra. Paola Haeger (Universidad Católica del Norte)
Thesis Committee:	Dr. Carlos Henríquez, Committee Member Dr. Rodrigo Mancilla, Committee Member Dra. Paola Llanos, External Examiner
Submission Date:	[16 / 07 / 2026]
Draft Version:	Draft V2

Declaration of Authorship

I hereby declare that this doctoral thesis is my own original work and has not been submitted, in whole or in part, for any other academic degree or qualification. All sources of information used in this thesis have been properly acknowledged and cited. I further declare that the research presented in this thesis was conducted under the supervision of the people named on the cover page.

Declaration on the Use of Artificial Intelligence (AI)

Artificial intelligence (AI) tools were used in the preparation of this doctoral thesis: Yes _x_ No ___

If yes, the AI tools used were Perplexity

Their use was limited exclusively to the following activities:

Assistance in improving academic writing style (grammar and coherence), and translation from Spanish to English. AI tools were not used to design experiments, acquire or process data, perform statistical analyses, interpret experimental results, or generate scientific conclusions.

Under no circumstances were AI tools used to generate, interpret, or modify the research findings, conduct the scientific analysis, develop the conclusions, or replace the doctoral candidate's critical judgment or intellectual authorship. The doctoral candidate assumes full responsibility for the originality, accuracy, academic integrity, and quality of the content of this dissertation and declares that all AI-assisted content has been carefully reviewed and validated. Furthermore, the use of AI tools complied with the principles of honesty, transparency, academic integrity, respect for intellectual property, confidentiality of information, and all applicable ethical and institutional regulations.



Ronald Alejandro Vargas Foitzick

Date: [13/07/2026]

Ethical Statement

This study was reviewed and approved by the Scientific Ethics Committee of the Finis Terrae University (CEC-UFT) under protocol ID 25-054. The committee determined that the methodology was appropriate for the stated objectives and that the investigator had the necessary experience to conduct the study. All procedures were carried out in accordance with institutional, national, and international ethical standards, including the principles of good clinical practice (ICH-GCP) and applicable Chilean regulations.

Acknowledgements

I would like to express my sincere gratitude to my supervisors, Dr. Elisa Balboa Castillo and Dr. Paola Haeger Soto, for their guidance, support, and constant encouragement throughout this thesis project. I am also grateful to the members of my research environment and all collaborators who contributed to the development of this work.

I am deeply grateful to my wife, María Fernanda Aguilar Gomez, for her love, patience, and unwavering support during every stage of this doctoral journey. Her encouragement has been fundamental to completing this thesis. I also want to thank my family for their constant support, understanding, and motivation, which have sustained me throughout this process.

Finally, I would like to thank my friends for their companionship, encouragement, and for being an important source of balance and motivation during this period.

Abstract

Background: Skeletal muscle differentiation is a highly energy-demanding process that requires coordinated regulation of anabolic signaling, protein synthesis, and mitochondrial adaptation. In the final stages of this process (late-stage myogenesis), newly formed myotubes undergo structural maturation and oxidative remodeling to support increased contractile and metabolic demands. Compounds that enhance mitochondrial function may therefore play a key role in optimizing this process. Urolithin A, a gut microbiota-derived metabolite of ellagitannins, has been shown to improve mitochondrial function and muscle performance in other experimental and clinical contexts, suggesting that it may influence the coupling between anabolic signaling and mitochondrial adaptation. However, its specific role during late-stage differentiation remains insufficiently defined.

Aim: To evaluate the effects of urolithin A on myogenic differentiation through the quantification of mitochondrial functional adaptation, mTOR signaling, and protein synthesis during late-stage C2C12 differentiation.

Methods: C2C12 myoblasts were differentiated and treated with urolithin A (2 μM) from day 3 to day 6. Cell viability was assessed by MTT assay. Myogenic markers such as MyoD, myogenin, MRF4 and myosin heavy chain (MHC) were analyzed by western blot. mTOR-related proteins (mTOR, AKT, 4E-BP1 and p70S6K), mitochondrial related-biogenesis markers (PGC-1α and TOM20), mitochondrial dynamics markers (MFN2 and OPA1), and OXPHOS complexes (I-V) were quantified by western blot. Protein synthesis was measured by SUnSET puromycin incorporation, fusion index was determined by H&E staining, and mitochondrial respiratory capacity was evaluated by high-resolution respirometry. Mitochondrial ultrastructure was examined by transmission electron microscopy, and statistical analyses were performed using non-parametric tests and two-way ANOVA, as appropriate.

Results: Non-cytotoxic Urolithin A (2 μM) increased myogenic markers levels, including MyoD and MHC, suggesting enhanced differentiation.

Although fusion index was not altered, urolithin A increased total mTOR, phosphorylated mTOR, and total p70S6K levels, while reducing phosphorylation ratios, suggesting remodeling of the mTOR pathway with feedback regulation. Consistent with these changes, urolithin A increased protein synthesis rate in the SUnSET assay. In parallel, urolithin A increased AMPKα phosphorylation, PGC-1α expression, OXPHOS Complex I and II abundance, and maximal respiratory capacity. Mitophagy/autophagy markers and mitochondrial ultrastructure were largely unchanged under these conditions.

Conclusion: Urolithin A modulates late-stage C2C12 differentiation and is associated with alterations in myogenic markers, mTOR signaling, protein synthesis, and mitochondrial respiratory capacity, without clear changes in mitophagy/autophagy markers or mitochondrial morphology. These results suggest that Urolithin A may support myotube maturation by jointly influencing anabolic and mitochondrial adaptations.

Keywords: urolithin A; C2C12; mitochondrial biogenesis; mTOR; myogenesis; oxidative phosphorylation.

Resumen

Antecedentes: La diferenciación del músculo esquelético es un proceso altamente demandante de energía que requiere la regulación coordinada de la señalización anabólica, la síntesis proteica y la adaptación mitocondrial. En las etapas finales de este proceso (miogénesis tardía), los miotubos recién formados experimentan una maduración estructural y una remodelación oxidativa para sostener el aumento de las demandas contráctiles y metabólicas. Por ello, los compuestos que potencian la función mitocondrial podrían desempeñar un papel clave en la optimización de este proceso. La urolitina A, un metabolito de los elagitaninos derivado de la microbiota intestinal, ha demostrado mejorar la función mitocondrial y el rendimiento muscular en otros contextos experimentales y clínicos, lo que sugiere que podría influir en el acoplamiento entre la señalización anabólica y la adaptación mitocondrial. Sin embargo, su papel específico durante la diferenciación tardía permanece insuficientemente definido.

Objetivo: Evaluar los efectos de la urolitina A sobre la diferenciación miogénica mediante la cuantificación de la adaptación funcional mitocondrial, la señalización de mTOR y la síntesis proteica durante la diferenciación tardía de células C2C12.

Métodos: Mioblastos C2C12 fueron diferenciados y tratados con urolitina A (2 μM) desde el día 3 hasta el día 6. La viabilidad celular se evaluó mediante ensayo MTT. Marcadores miogénicos como MyoD, miogenina, MRF4 y cadena pesada de miosina (MHC) se analizaron por western blot. Proteínas relacionadas con mTOR (mTOR, AKT, 4E-BP1 y p70S6K), marcadores de biogénesis mitocondrial (PGC-1α y TOM20), marcadores de dinámica mitocondrial (MFN2 y OPA1) y complejos OXPHOS (I-V) se cuantificaron por western blot. La síntesis proteica se midió mediante incorporación de puromicina (ensayo SUnSET), el índice de fusión se determinó por tinción de hematoxilina-eosina (H&E) y la capacidad respiratoria mitocondrial se evaluó por respirometría de alta resolución. La

ultraestructura mitocondrial se examinó por microscopía electrónica de transmisión, y los análisis estadísticos se realizaron mediante pruebas no paramétricas y ANOVA de dos vías, según correspondiera.

Resultados: La urolitina A (2 μM), no citotóxica, aumentó los niveles de marcadores miogénicos, incluidos MyoD y MHC, lo que sugiere una mayor diferenciación. Aunque el índice de fusión no se modificó, la urolitina A incrementó los niveles totales de mTOR, de mTOR fosforilado y de p70S6K total, a la vez que redujo las razones de fosforilación, lo que sugiere una remodelación de la vía de mTOR con regulación por retroalimentación. De forma consistente con estos cambios, la urolitina A aumentó la tasa de síntesis proteica en el ensayo SUnSET. En paralelo, la urolitina A incrementó la fosforilación de AMPKα, la expresión de PGC-1α, la abundancia de los complejos I y II de la OXPHOS y la capacidad respiratoria máxima. Los marcadores de mitofagia/autofagia y la ultraestructura mitocondrial se mantuvieron prácticamente sin cambios en estas condiciones.

Conclusión: La urolitina A modula la diferenciación tardía de las células C2C12 y se asocia con alteraciones en los marcadores miogénicos, la señalización de mTOR, la síntesis proteica y la capacidad respiratoria mitocondrial, sin cambios claros en los marcadores de mitofagia/autofagia ni en la morfología mitocondrial. Estos resultados sugieren que la urolitina A podría favorecer la maduración de los miotubos al influir de manera conjunta en las adaptaciones anabólicas y mitocondriales.

Palabras clave: urolitina A; C2C12; biogénesis mitocondrial; mTOR; miogénesis; fosforilación oxidativa.

Table of Contents

Chapter 1. Introduction.

1.1 General Context.

1.2 Theoretical Background.

1.3 State of the Art and Research Gap.

1.4 Justification and Scientific Relevance.

Chapter 2. Hypothesis and Objectives.

2.1 General Hypothesis.

2.2 General Objective.

2.3 Specific Objectives.

Chapter 3. Results.

3.1 Urolithin A enhances mitochondrial biogenesis-related markers and maximal respiratory capacity during C2C12 differentiation.

3.2 Urolithin A modulates mTOR signaling components and enhances baseline protein synthesis during late-stage C2C12 myogenesis.

Chapter 4. Conclusions.

4.1Study Limitations and Future Directions.

4.2 General Conclusion.

4.3 Specific Conclusions.

4.4 Scientific Contribution.

Chapter 5. Bibliography.

List of Abbreviations

AUC	Area under the curve
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
BNIP3L/NIX	BCL2 interacting protein 3 like
C2C12	Murine myoblast cell line
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
CV	Complex V
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
H&E	Hematoxylin and eosin
IQR	Interquartile range
LC3	Microtubule-associated protein 1 light chain 3
MFN2	Mitofusin 2
MHC	Myosin heavy chain
MyoD	Myogenic differentiation 1
OCR	Oxygen consumption rate
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
p70S6K	Ribosomal protein S6 kinase beta-1
p62	Sequestosome 1
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SUnSET	Surface sensing of translation
TBST	Tris-buffered saline with Tween-20

TEM	Transmission electron microscopy
TOM20	Translocase of outer mitochondrial membrane 20
UA	Urolithin A
ULK1	Unc-51-like autophagy activating kinase 1

CHAPTER 1 — INTRODUCTION

1.1 General Context.

Skeletal muscle is a highly plastic and metabolically active tissue that is essential for locomotion, posture, and systemic metabolic regulation. Beyond generating force, it contributes to whole body energy homeostasis, glucose disposal, and metabolic flexibility in response to physiological and pathological demands (1-3). These functions rely on the capacity of muscle stem cells (satellite cells) and other myogenic precursor cells to become activated, proliferate as myoblasts, differentiate, and fuse into multinucleated myotubes that ultimately mature into functional muscle fibers (4, 5).

The multistep developmental program encompassing precursor activation, proliferation, differentiation, fusion, and fiber maturation is referred to as myogenesis (1, 3). Within this program, myogenic differentiation denotes the phase during which proliferating myoblasts exit the cell cycle, initiate the myogenic transcriptional program, and fuse to form multinucleated myotubes. During myogenic differentiation, myoblasts activate a coordinated transcriptional cascade driven by myogenic regulatory factors such as MyoD and myogenin, accompanied by chromatin remodeling and the action of non-coding RNAs (e.g., microRNAs and long non-coding RNAs) that regulate gene expression (3, 6). Finally, late-stage differentiation designates, specifically in this thesis, the terminal 72-hour window (days 3-6 of the standard C2C12 protocol) in which newly fused myotubes undergo structural and bioenergetic maturation. When myogenic differentiation is compromised, skeletal muscle-wasting conditions such as sarcopenia, cachexia, and muscular dystrophies can emerge, with substantial clinical and societal consequences (1, 3).

During differentiation, myoblasts synthesize contractile proteins, reorganize their cytoskeleton, and fuse to form elongated, multinucleated myotubes (5,

7). To sustain this structural transition, mitochondria undergo marked changes in content, morphology, and function, reflecting the increased ATP demand and specific redox requirements of maturing myotubes (8, 9).

Mitochondria play a pivotal role in skeletal muscle by regulating oxidative ATP production, reactive oxygen species (ROS) homeostasis, and apoptotic signaling. During myogenic differentiation, mitochondrial biogenesis, dynamics (fission and fusion), and quality control mechanisms such as mitophagy are tightly coordinated with the activation of myogenic gene programs and metabolic reprogramming (8-11). Together, these complementary processes maintain mitochondrial homeostasis by regulating mitochondrial content, morphology, and quality, thereby ensuring the metabolic adaptations required for myotube maturation. Proteins including dynamin-related protein 1 (Drp1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) govern mitochondrial network remodeling, while regulators such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial transcription factor A (TFAM) drive mitochondrial biogenesis and respiratory capacity (12-15). Likewise, mitophagy selectively removes dysfunctional mitochondria, thereby preserving the integrity and functionality of the mitochondrial network during differentiation. Failure to appropriately adjust mitochondrial content and quality during differentiation is increasingly recognized as a contributor to impaired muscle function and aging-related decline (8, 15).

Among the signaling pathways that orchestrate myogenic differentiation, the mechanistic target of rapamycin (mTOR) pathway occupies a central position at the interface between growth and metabolism. mTOR integrates signals from nutrients, growth factors, and mechanical stimuli to regulate mRNA translation, protein synthesis, and autophagy (16-18). It exists in two functionally distinct complexes, mTORC1 and mTORC2, which differentially control muscle growth, fiber type specification, and metabolic adaptation. In skeletal muscle cells, mTORC1 activation promotes protein synthesis through downstream effectors such as p70 ribosomal S6 kinase (p70S6K)

and 4E-binding protein 1 (4E-BP1), thereby supporting myoblast differentiation, myotube growth, and hypertrophic responses (19-21). mTORC2, in turn, contributes to cytoskeletal organization and insulin/AKT signaling, influencing both structural and metabolic aspects of muscle cells (17). Importantly, mTOR signaling is closely linked to mitochondrial function. mTORC1 activity has been implicated in the regulation of mitochondrial biogenesis and dynamics, whereas suppression or dysregulation of mTOR can alter autophagy and mitophagy, thereby impacting mitochondrial quality control (18, 22). During myogenic differentiation, the temporal alignment between mTOR driven anabolic signaling and mitochondrial remodeling, including biogenesis, fission/fusion balance, and selective clearance of damaged mitochondria by mitophagy, is critical to ensure that newly formed myotubes achieve both structural maturity and bioenergetic competence (8). Disruption of this coordination can result in insufficient protein synthesis, compromised oxidative capacity, and increased susceptibility to stress, features that are frequently observed in aging muscle and chronic disease (8, 23). These observations suggest that enhancing muscle cell differentiation may represent a potential therapeutic strategy for the treatment of these conditions.

In this context, bioactive compounds capable of modulating mitochondrial function and muscle signaling have attracted considerable interest for promoting muscle health. Urolithin A (UA), a gut microbiota derived metabolite of dietary ellagitannins found in fruits such as pomegranate, has emerged as a promising candidate for targeting mitochondrial health and muscle function (24-26). Following the consumption of ellagitannin rich foods, UA is generated by specific microbial populations, absorbed, and reaches circulating concentrations that can influence peripheral tissues, including skeletal muscle (24, 27, 28). Experimental studies across multiple models indicate that UA enhances mitophagy, improves mitochondrial function, and exerts anti-inflammatory effects with reported benefits on muscle performance and aging-related phenotypes (29-31).

In vivo, UA has been shown to induce mitophagy and improve muscle function across several models. In Caenorhabditis elegans, dietary exposure to UA prevented the accumulation of dysfunctional mitochondria with age, maintained mitochondrial respiratory capacity, and extended lifespan, while preserving locomotor activity (32). In the same study, oral UA at 25-50 $mg \cdot kg^{-1} \cdot day^{-1}$ administered for several weeks in aged rodents improved exercise capacity in two different mouse models of age-related muscle decline as well as in young rats, indicating that UA induced mitophagy can translate into enhanced muscle performance *in vivo* (32). More recently, UA has been evaluated in preclinical models of Duchenne muscular dystrophy. In the classic mdx mouse model, 3-week-old male C57BL/10ScSn-Dmdmdx/J mice received chow supplemented with UA at 50 $mg \cdot kg^{-1} \cdot day^{-1}$ for 10 weeks, starting in early life. Under these conditions, UA rescued mitophagy defects in dystrophic muscle, increased skeletal muscle respiratory capacity, and improved muscle strength and exercise performance on uphill and downhill treadmill tests compared with untreated mdx mice, indicating a functional recovery of muscle performance. In line with these findings, complementary experiments in primary human myoblasts derived from pediatric DMD patients showed that treatment with 25 µM UA for 2-24 hours rescued mitophagy-related defects and improved mitochondrial function in dystrophic muscle cells, suggesting that UA can directly act on human myogenic cells in addition to whole-organism models. (33)

Recent human trials further characterize the dose range and functional impact of UA supplementation. In a first in human randomized, placebo-controlled study in healthy older adults, single ascending oral doses of 250, 500, 1000, and 2000 mg UA, followed by 28 days of daily supplementation at 250, 500, or 1000 mg, were safe and well tolerated, with UA detectable in plasma and skeletal muscle biopsies. Pharmacokinetic analyses from this and related work indicate that oral doses in the 250-1000 mg range produce low micromolar plasma concentrations of UA conjugates (approximately 1-3 µM), consistent with sustained systemic exposure and tissue uptake. UA

administration up-regulated mitochondrial pathway gene expression in skeletal muscle and lowered plasma medium-chain acylcarnitines in a dose-dependent manner, supporting improved mitochondrial health without adverse events (29). In middle-aged adults, a 4-month randomized, placebo-controlled trial using daily oral UA at 500 or 1000 mg showed approximately 10-12% increases in hamstring and knee-flexor muscle strength, clinically meaningful improvements in aerobic endurance (10% increase in VO_2peak) and 6-minute walk distance (increased 33 m at 1000 mg), as well as reductions in plasma acylcarnitines and C-reactive protein and increased expression of proteins linked to mitophagy and mitochondrial metabolism in skeletal muscle biopsies (26). Complementary trials in older adults have reported that 500-1000 mg UA daily for several months improves muscle endurance and mitochondrial health, further supporting the functional relevance of UA-induced mitochondrial remodeling in human skeletal muscle (28).

At the cellular level, *in vitro* studies using differentiated muscle cells provide mechanistic insight into how UA modulates mitochondrial homeostasis. In C2C12 myotubes, UA at 50 μM for 24 h on day 6 of differentiation attenuated lipopolysaccharide (LPS, 1 $\mu g \cdot ml^{-1}$, 3 h), induced activation of the TLR4/MAPK inflammatory pathway, prevented LPS-associated reductions in mitochondrial area and perimeter, reduced phosphorylation of the fission marker DRP1, and increased the LC3II/LC3I ratio, PINK1 protein expression, and AMPKα phosphorylation, consistent with enhanced mitophagy initiation and partial normalization of mitochondrial morphology under inflammatory stress (34). Studies in primary human myotubes further indicate that UA at physiologically relevant concentrations (0.002-50 μM, 24 h) can enhance basal and insulin-stimulated glucose uptake, up-regulate GLUT4 expression, and reduce myostatin expression, suggesting that UA may improve anabolic and metabolic sensitivity in human skeletal muscle cells (35). These findings complement preclinical and clinical evidence on

UA-induced mitochondrial remodeling and muscle function and support its potential as a nutraceutical to target muscle metabolic health.

Taken together, these *in vivo* and *in vitro* findings suggest that UA can modulate mitochondrial quality control and muscle performance in both whole organism and muscle cell contexts. This evidence provides a mechanistic rationale to investigate whether UA might also influence late-stage differentiation and mitochondrial functional adaptation during this stage of the process, a developmental window characterized by high energetic demand and intensive coordination between anabolic signaling and mitochondrial remodeling.

Despite this growing body of evidence, the precise molecular mechanisms by which Urolithin A influences myogenic differentiation, particularly mTOR signaling, protein synthesis, and mitochondrial adaptation during the transition from proliferating myoblasts to late-stage differentiated myotubes remain incompletely defined, in the context of C2C12 model. Most mechanistic insights have been derived either from mature muscle *in vivo,* from animal interventions, or from acute exposures in already differentiated myotubes, leaving a knowledge gap regarding how UA interacts with anabolic and mitochondrial programs during the dynamic process of myotube formation. The murine C2C12 myoblast cell line provides a well established *in vitro* model to study myogenic differentiation, as it recapitulates the progression from proliferating myoblasts to multinucleated myotubes under controlled conditions; this system allows temporal analysis of myogenic markers, protein synthesis, and mitochondrial function in response to defined UA exposure, making it particularly suitable for mechanistic studies of skeletal muscle remodeling (3, 5, 36, 37).

Within this framework, the present thesis is situated at the intersection of skeletal muscle differentiation, mTOR-dependent anabolic signaling (mTOR-p70S6K-4E-BP1 axis) that controls protein synthesis and growth in skeletal muscle cells, and mitochondrial functional adaptation. By

examining how Urolithin A modulates these processes during late-stage differentiation in C2C12 cells, it aims to characterize the effects of a mitochondrial-targeted nutraceutical on growth-related signaling pathways and bioenergetic remodeling, thereby providing a conceptual basis for future translational strategies in muscle aging and disease. This raises the central research question of whether Urolithin A modulates late-stage myogenic differentiation through coordinated effects on mTOR signaling and mitochondrial functional adaptation.

1.2 Theoretical Background

1.2.1 Skeletal Muscle Differentiation and Mitochondrial Remodeling.

Skeletal muscle differentiation is a multistep process in which myogenic precursor cells undergo proliferation commitment, and terminal differentiation, culminating in the formation of multinucleated myotubes and the subsequent development of mature muscle fibers (1-3). This program is conducted by a sequence of regulatory events, including the activation of myogenic transcription factors such as MyoD, myogenin, MRF4, and Myf5, which control lineage specification and fusion (3, 7). Structural remodeling during this differentiation process involves cytoskeletal reorganization, synthesis of contractile proteins, and cell fusion of myoblasts into multinucleated myotubes, all of which are essential for establishing the mechanical and functional properties of muscle tissue (6).

Simultaneously, myogenic differentiation demands substantial metabolic adaptation. Maturing myotubes require increased ATP production to fuel protein synthesis, membrane expansion, and contractile activity. This is achieved through robust mitochondrial biogenesis, governed by transcriptional coactivators such as PGC-1α and TFAM, which upregulate the expression of genes involved in oxidative phosphorylation (15, 23, 37). Mitochondrial morphology shifts from a fragmented network toward a more interconnected reticular network, driven by proteins such as MFN1, MFN2,

and OPA1, which promote mitochondrial fusion, together with DRP1, which regulates fission, thereby optimizing energy distribution and enhancing resistance to metabolic stress (8, 37).

The alignment of myogenic and metabolic programs ensures functional competence of muscle fibers. Together, these coordinated structural and metabolic adaptations depend on tightly regulated anabolic signaling and bioenergetic remodeling. Given this reliance on protein synthesis and mitochondrial function, the mechanistic target of rapamycin (mTOR) pathway emerges as a central hub linking myogenic differentiation with translational control and mitochondrial adaptation (4).

1.2.2 mTOR Signaling and Protein Synthesis in Myogenesis.

The mechanistic target of rapamycin (mTOR) is a master regulator of cell growth, metabolism, and protein synthesis, acting through two functionally distinct complexes, mTORC1 and mTORC2, which differentially control muscle growth, fiber-type specification, and metabolic adaptation (16, 18, 19). In C2C12 cells specifically, pharmacological inhibition (rapamycin, or the more selective inhibitor KU0063794) and genetic manipulation of raptor confirm that mTORC1 activity is required for myogenic differentiation, acting through downstream effectors such as p70 ribosomal S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1) to support myoblast differentiation, myotube growth, and hypertrophic responses (38, 39). mTORC2, by contrast, is required specifically for late-stage differentiation, rapamycin-induced disruption of mTORC2 assembly and shRNA-mediated depletion of rictor block MHC expression and myotube formation, while expression of phosphomimetic AKT-S473D rescues differentiation, indicating that mTORC2 acts largely through AKT-Ser473 phosphorylation to support cytoskeletal remodeling and survival signaling, complementing translational and anabolic role of mTORC1 (17, 19, 21, 39, 40).

Beyond its role in differentiation per se, mTOR signaling is closely coupled to mitochondrial adaptation, although the two roles are mechanistically distinct and should not be conflated. In skeletal muscle cells, mTORC1

supports mitochondrial oxidative function through a YY1-PGC-1α transcriptional program; inhibition of mTORC1 with rapamycin reduces expression of oxidative and mitochondrial genes and lowers respiratory capacity (41). In parallel, mTORC1 negatively regulates autophagy, and its dysregulation can impair mitochondrial quality control, including mitophagy, thereby affecting organelle homeostasis. mTORC1 controls mitochondrial activity and biogenesis by promoting the translation of nucleus-encoded mitochondria-related mRNAs through inhibition of 4E-BP-dependent repression, thereby linking mTORC1 directly to mitochondrial remodeling at the level of protein synthesis (42). Accordingly, mTOR-dependent regulation of muscle differentiation and of mitochondrial remodeling should be understood as interconnected but mechanistically distinct processes, a distinction relevant to interpreting how a compound such as UA might act on one, the other, or both.

1.2.3 Mitochondrial Biogenesis, Dynamics, and Respiratory Capacity During Differentiation.

Mitochondrial biogenesis refers to the process by which cells increase mitochondrial mass and copy number through the coordinated action of transcriptional regulators such as PGC-1α, NRF1/2, and TFAM (Review by Scarpulla (10)). During myogenic differentiation, mitochondrial biogenesis increases mitochondrial content to meet energetic and biosynthetic demands. Mitochondrial dynamics, encompassing fusion, fission, biogenesis, and mitophagy, drive organelle remodeling and quality control. Key regulators including Mfn2, OPA1, and Drp1 shape the mitochondrial network and enable selective removal of damaged mitochondria via mitophagy (43-45).

During C2C12 differentiation, autophagic flux increases transiently at the onset of the myogenic program; its functional necessity was demonstrated by pharmacological inhibition (3-methyladenine) and shRNA-mediated Atg7 knockdown, both of which reduced myogenin and MHC expression, delayed

myoblast fusion, and impaired myotube formation, indicating that autophagy is required for differentiation(46).

Respiratory capacity is enhanced during differentiation, reflected in increased expression of electron transport chain components and greater oxidative phosphorylation rates (12, 47). These adaptations are critical for ATP generation and redox balance in maturing myotubes. Autophagy and mitophagy maintain mitochondrial quality; disruption of these processes impairs muscle differentiation and contributes to disease (8, 14, 30).

This conceptual block establishes the centrality of mitochondrial remodeling in muscle development and highlights the potential for targeted modulation, as explored in this thesis.

Taken together, these findings highlight that successful myogenic differentiation depends on the coordinated regulation of anabolic signaling, mitochondrial remodeling, and organelle quality control. When these processes are compromised, skeletal muscle function is impaired, contributing to the development and progression of muscle-related pathologies. In this context, there is growing interest in bioactive compounds capable of supporting or restoring these regulatory pathways under conditions of metabolic stress or dysfunction.

1.2.4 Urolithin A: Origin, Metabolism, and Biological Effects on Mitochondria and Skeletal Muscle.

Urolithin A (UA) is a postbiotic metabolite produced by the gut microbiota through the sequential biotransformation of dietary ellagitannins (ETs) and ellagic acid (EA) (27, 48). These precursors are found in abundance in foods such as pomegranates, walnuts, and various berries (24, 25, 49). After ingestion, ellagitannins are hydrolyzed in the gut to release ellagic acid, which resident colonic bacteria convert into urolithins through a stepwise process: cleavage of one lactone ring of ellagic acid yields the pentahydroxylated urolithin M5, which is progressively dehydroxylated by specific species (notably Gordonibacter and Ellagibacter spp.) into

intermediates such as urolithins M6, M7, and C and, ultimately, urolithin A, with urolithin B arising through a parallel branch (50). Because urolithin A is the convergent product of this main dehydroxylation route and is generated by most urolithin producing individuals, it is typically the most abundant urolithin recovered in plasma and tissues, including skeletal muscle (24, 26, 32, 49, 51).

As summarized in a comprehensive review by García-Villalba et al. (2016), structurally, UA is a small, lipophilic dibenzo-α-pyrone (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one); no specific membrane receptor has been described, and the prevailing view is that it enters cells by passive diffusion to act on intracellular targets. Once absorbed, UA forms glucuronide and sulfate conjugates (50).

UA is best known for stimulating mitophagy through the PINK1/Parkin pathway in C. elegans and rodent muscle, where the selective removal of damaged mitochondria is coordinated with mitochondrial biogenesis, thereby improving respiratory capacity and ATP production (29, 32). In UA also displays anti-inflammatory activity across models: in LPS-stimulated macrophages it inhibits NF-κB activation and lowers NF-κB–dependent pro-inflammatory cytokines such as TNF-α and IL-6 (31), and in C2C12 myotubes it attenuates LPS-induced TLR4/MAPK inflammatory signaling while preserving mitochondrial morphology (34); in human supplementation trials, it reduces systemic inflammatory markers such as C-reactive protein (26, 52). Together, these mitochondrial and anti-inflammatory actions have been linked to improved muscle performance and endurance in models of aging and muscular dystrophy (32, 33). Early clinical trials have confirmed that UA supplementation is safe and bioavailable, and direct supplementation bypasses the inter-individual variability in endogenous UA production, which depends on gut microbiome composition and occurs in only about 40% of individuals. In older adults, UA supplementation improves biomarkers of mitochondrial health, reduces plasma acylcarnitines, and

enhances muscle endurance, effects attributable to UA and/or its circulating metabolites (51).

Given that mitochondrial biogenesis and quality control are essential for the energetic and structural remodeling required during myogenic differentiation, these findings raise the possibility that UA may act not only by supporting general muscle function but also by modulating specific mitochondrial changes. However, the effects of UA during late-stage myogenic differentiation, and particularly its potential interaction with mTOR signaling and dynamic mitochondrial adaptation, remain poorly characterized; to date, there is no direct evidence that UA modulates the mTOR pathway during myogenesis, which defines the specific knowledge gap addressed in this thesis. While related metabolites like urolithin B have been shown to enhance protein synthesis and muscle mass in C2C12 models, the specific role of UA during active myogenesis, particularly regarding the activation and differentiation of muscle stem cells (MuSCs), is a more recent area of exploration (53). In this context, evidence suggesting that UA may improve regenerative capacity by modulating key myogenic genes such as Pax7 and MyoD (33) can be tested directly during myotube formation.

1.2.5 The C2C12 Cell Line as a Model for Myogenesis and Mitochondrial Adaptation.

The C2C12 murine myoblast cell line is a widely used model for studying skeletal muscle differentiation and mitochondrial remodeling(5, 8). Under defined culture conditions, C2C12 cells proliferate, exit the cell cycle, and differentiate into multinucleated myotubes, replicating key aspects of skeletal muscle myogenesis (3). The model enables temporal analysis of differentiation markers, protein synthesis, and mitochondrial function, providing a controlled environment for mechanistic studies (8, 37). C2C12 cells are responsive to pharmacological and genetic interventions, making them suitable for investigating the effects of bioactive compounds

such as UA on myogenic and mitochondrial pathways (33, 51). This system allows for systematic dissection of mTOR signaling, protein synthesis, and mitochondrial adaptation during myogenic differentiation.

The use of C2C12 cells thus offers a versatile platform for examining the coordination of anabolic and mitochondrial programs during myogenic differentiation, and for evaluating the mechanistic impact of nutraceuticals like UA, supporting translational insights for muscle health and disease.

1.3 State of the Art and Identification of the Research Gap

1.3.1 Urolithin A, Mitophagy, and Mitochondrial Quality Control in Skeletal Muscle and C2C12 Cells.

UA has emerged as a potent modulator of mitochondrial quality control, primarily through the activation of mitophagy (32, 33, 48, 54). Several studies demonstrate that UA enhances mitophagy in mature skeletal muscle, improves mitochondrial function, and leads to better muscle performance in aging animals and humans (32, 33). These effects are generally attributed to the activation of the PINK1/Parkin pathway, resulting in increased clearance of dysfunctional mitochondria and preservation of a healthy mitochondrial network (29, 33).

Despite the growing evidence for UA's beneficial effects, most studies target mature muscle tissue or fully differentiated myotubes. For example, Ryu et al. (2016) and Andreux et al. (2019) focused on UA supplementation in rodents and humans with established muscle function, while Singh et al. (2022) examined UA-induced mitophagy in fully differentiated C2C12 myotubes. Similarly, related research has reported improved mitochondrial health and muscle function in aged models but does not extensively investigate the effects during active myogenic differentiation (30, 31, 55). *In vitro* studies using C2C12 cells often apply UA only after myotube formation, thus assessing mitochondrial quality control in a post-differentiation context

(32). Consequently, there is a marked lack of data on UA impact in myoblasts or during the dynamic transition from myoblasts to myotubes, especially during late-stage differentiation (days 3-6), a critical window for myotube maturation and energetic remodeling.

1.3.2 Urolithin A Effects on Mitochondrial Biogenesis and Respiratory Function: Cell, Animal, and Human Studies.

Beyond mitophagy, UA has also been shown to stimulate mitochondrial biogenesis and enhance respiratory function in a variety of models (26-29, 32, 48, 51, 55, 56). UA increases the expression of transcriptional regulators such as PGC-1α and TFAM, leading to upregulation of OXPHOS complexes and improved ATP production (33, 57). In animal studies, UA supplementation was associated with increased oxidative capacity and endurance, as well as improved muscle performance in aging mice (32). Early clinical trials suggest that UA can modulate biomarkers of mitochondrial health and exercise performance in humans, though outcomes may depend on dose and duration (27, 28, 48, 51, 58).

Notably, most *in vitro* studies employ UA at relatively high concentrations, typically 5-10 μM, and short-term exposures lasting 12-24 hours (29, 32). Most experimental designs assess UA effects either in mature muscle or in fully differentiated C2C12 myotubes, seldom examining cells during active differentiation. By contrast, the late stages of C2C12 differentiation (days 3-6) are characterized by increased mitochondrial biogenesis, respiratory adaptation, and myotube maturation (8). There is a paucity of data addressing how lower, physiologically relevant UA doses (e.g., 2 μM) administered over longer periods (72 hours) affect mitochondrial function and biogenesis during the transition from myoblast to myotube.

1.3.3 mTOR Signaling, Protein Synthesis, and Crosstalk with Mitochondrial Adaptation During Myogenesis.

The mechanistic target of rapamycin (mTOR) pathway is a central regulator of muscle growth and protein synthesis, intricately involved in myogenic differentiation and muscle adaptation (16, 21, 40). mTOR integrates signals from nutrients, growth factors, and mechanical stimuli, promoting translation of components essential for muscle growth and maturation (19, 59). Importantly, mTOR also coordinates with mitochondrial biogenesis and quality control programs, ensuring that anabolic and energetic demands are met during myotube formation (60).

Bioactive compounds such as UA can influence mTOR signaling, although the direction of this effect appears to be context dependent. In non-muscle tissues, UA has most often been reported to suppress the mTORC1 axis: in cardiac and neuronal models, UA activates AMPK and downregulates PI3K/AKT/mTOR signaling, thereby promoting ULK1 dependent autophagy and PINK1/Parkin mitophagy (56, 61, 62). This is consistent with the canonical role of UA as an autophagy and mitophagy inducer, since mTORC1 inhibition is a classical trigger of autophagic flux. However, this inhibitory relationship has been characterized mainly under stress or disease conditions and in post-mitotic or mature myotubes. Whether UA exerts the same effect during active myogenic differentiation, a stage in which mTORC1 activity is required to drive the anabolic and translational program that supports myotube maturation, remains unknown. Indeed, very few reports explicitly examine UA impact on mTOR-driven protein synthesis or signaling during myogenic differentiation in C2C12 cells. It is unclear whether, during late-stage differentiation (days 3-6), UA modulates mTOR.

1.3.4 Limitations of Existing UA Studies: Dose, Exposure, and Developmental Stage.

A closer examination of existing UA literature reveals several methodological limitations. Most studies employ relatively high UA concentrations (5-10 μM), short-term exposures (12-24 h), and focus on

mature muscle or fully differentiated C2C12 myotubes (29, 32). UA is often applied after myotube formation, neglecting the dynamic processes during active differentiation. This approach overlooks the possibility that UA effects may be dose, and stage-specific, with distinct outcomes during myotube formation versus in established fibers.

In direct contrast, the present thesis examines UA at a lower, physiologically relevant concentration applied during active myotube maturation rather than after differentiation is complete, thereby addressing a critical gap left by the prevailing literature. This strategy enables the evaluation of UA effects on myogenic differentiation, mTOR signaling, protein synthesis, and mitochondrial functional adaptation within a developmentally relevant window.

1.3.5 Identification of the Knowledge Gap.

Despite substantial progress in elucidating UA effects on mitophagy, mitochondrial biogenesis, and muscle function, it remains unclear how Urolithin A modulates the interplay between mTOR signaling, protein synthesis, and mitochondrial functional adaptation during late-stage C2C12 differentiation (days 3-6). This gap is particularly relevant because this window represents a phase of intense myotube maturation, where anabolic and mitochondrial programs must be tightly coordinated to ensure structural integrity and bioenergetic competence. Current evidence is insufficient to determine whether UA can optimize this coordination, enhance muscle cell differentiation, or improve mitochondrial function under these specific conditions.

Taken together, these gaps lead to the central research question of this thesis: does Urolithin A modulate late-stage myogenic differentiation through coordinated effects on mTOR signaling and mitochondrial functional adaptation during C2C12 differentiation?

By systematically evaluating UA impact under developmentally targeted, physiologically relevant exposure parameters, the present thesis aims to

generate new mechanistic insight into the regulation of muscle differentiation and mitochondrial adaptation. This knowledge will inform the potential translational value of UA as a nutraceutical intervention for muscle health, aging, and disease.

1.4 Justification and Scientific Relevance.

Skeletal muscle formation and regeneration depend on the correct progression of myogenic differentiation, in which proliferating myoblasts exit the cell cycle and fuse into multinucleated myotubes that subsequently mature. The terminal phase of this program (days 3-6 in the C2C12 model) is especially demanding; newly formed myotubes must simultaneously assemble contractile structures, sustain high rates of protein synthesis, and undergo extensive mitochondrial biogenesis and network remodeling to support the shift from glycolytic to oxidative metabolism (8, 63). Because anabolic and metabolic programs are most tightly coupled during this window, it represents a biologically meaningful, yet understudied, target for nutritional or pharmacological modulation. Most available evidence on UA, however, derives from mature muscle or fully differentiated myotubes, leaving unclear whether UA can influence muscle cell fate precisely when these programs are being established.

UA is an attractive candidate to investigate this window. It is a naturally occurring, orally bioavailable metabolite with a well documented capacity to enhance mitochondrial quality control and muscle function, and it is already being tested in humans (29, 32, 51). Despite this therapeutic promise, its mechanism of action in skeletal muscle has not been characterized in detail, and its potential effect on the mTOR pathway, a central hub that integrates nutrient and growth signals to control protein synthesis and coordinates with mitochondrial adaptation, remains largely unexplored during differentiation (See review (18)). Clarifying whether UA engages this pathway is therefore relevant not only to understand how UA works, but also to anticipate

possible context-dependent or off-target effects, given the broad regulatory role of mTOR across cell types.

From a translational standpoint, this question is especially relevant to exercise and health sciences. Skeletal muscle mass and function depend on the capacity of satellite cells to activate, proliferate, and complete myogenic differentiation, a regenerative capacity that becomes progressively impaired with aging and in muscle wasting conditions (64). In sarcopenia, the age-related loss of muscle mass and strength is accompanied by blunted satellite-cell function, impaired myogenic differentiation, mitochondrial dysfunction, and anabolic resistance, all of which limit the ability of muscle to remodel and recover (44, 65). Comparable impairments slow recovery after muscle injury or disuse, where the efficient differentiation and fusion of myoblasts into new myotubes largely determines how quickly functional capacity is restored. In this scenario, a nutraceutical able to support or accelerate late-stage myogenic differentiation, while simultaneously enhancing mitochondrial adaptation, could be a practical strategy to preserve muscle quality and shorten recovery. Determining whether UA also enhances the differentiation program itself, the aim of this thesis would therefore provide a mechanistic rationale for its use in sarcopenia, aging, and exercise or injury-related muscle recovery.

Methodologically, the exposure paradigm was chosen to reflect a physiologically plausible scenario rather than an acute pharmacological challenge. Instead of the short, high dose pulses used in most *in vitro* studies, UA was applied at a low, physiologically relevant concentration, within the low micromolar range of circulating UA and its conjugates reported in human supplementation trials (29, 51) and maintained as a sustained exposure across the late differentiation window, mimicking chronic dietary or supplemental intake. These considerations, however, should be interpreted with caution, because C2C12 myotubes are an *in vitro* model that does not reproduce the full complexity of human skeletal muscle, and the present findings are best regarded as a first mechanistic step whose

extrapolation to human muscle and to clinical or exercise settings will require validation in more physiological and *in vivo* models. Within these boundaries, the integrated analysis of myogenic, translational, and mitochondrial readouts makes this study both feasible and necessary to advance the mechanistic understanding of UA in muscle biology.

CHAPTER 2 — HYPOTHESIS AND OBJECTIVES

2.1 General Hypothesis.

It is hypothesized that exposure to 2 μM Urolithin A during late-stage C2C12 differentiation (days 3-6, 72 hours), coinciding with the onset of mitochondrial biogenesis, promotes myogenic differentiation by modulating mTOR-dependent protein synthesis pathways and enhancing mitochondrial functional adaptation, as reflected by changes in mitochondrial biogenesis-related markers and respiratory capacity.

2.2 General Objective.

To evaluate the effects of urolithin A on myogenic differentiation through the quantification of mitochondrial functional adaptation, mTOR signaling, and protein synthesis during late-stage C2C12 differentiation.

2.3 Specific Objectives.

1. To evaluate the effects of Urolithin A on myogenic differentiation markers and myotube morphology during late-stage C2C12 differentiation.
2. To determine the effects of Urolithin A on autophagy-related markers and mitochondrial ultrastructure, biogenesis and dynamics markers, OXPHOS complex expression, and respiratory capacity, during late-stage C2C12 differentiation.

3. To characterize the effects of Urolithin A on the mTOR signaling activation state and global protein synthesis (SUnSET) during late-stage C2C12 differentiation.

CHAPTER 3 — RESULTS

The results are presented in the order in which the studies were developed and disseminated: the first manuscript, already accepted for publication, addresses mitochondrial functional adaptation, and the second, currently in preparation, addresses mTOR signaling and protein synthesis.

ARTICLE 1 — PUBLISHED MANUSCRIPT

This chapter is based on the following original research article, accepted for publication in Frontiers in Cell and Developmental Biology (Section: Cellular Biochemistry):

Vargas-Foitzick R, Iribarren D, Valero-Breton M, Balboa E. Urolithin A enhances mitochondrial biogenesis-related markers and maximal respiratory capacity during C2C12 differentiation. Front Cell Dev Biol. 2026;10:1854844. doi:10.3389/fcell.2026.1854844.

The content of the following chapter corresponds to the version of the manuscript accepted on 31 May 2026 (Article ID 1854844), with minor formatting adaptations to comply with the Universidad Finis Terrae doctoral thesis requirements. Any subsequent copyediting or layout changes introduced by the journal after acceptance are not reflected here.

Urolithin A enhances mitochondrial biogenesis-related markers and maximal respiratory capacity during C2C12 differentiation

Ronald Vargas-Foitzick [1,2], Diego Irribarra [3], Mayalen Valero-Breton [4,5], Elisa Balboa Castillo [3]

[1] Exercise Science Laboratory, School of Kinesiology, Faculty of Medicine, Universidad Finis Terrae, Santiago, Chile.

[2] Escuela de Ciencias de la Salud, Departamento de Kinesiología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago 1270709, Chile.

[3] Center for Biomedical Research, School of Medicine, Faculty of Medicine, Universidad Finis Terrae, Santiago, Chile.

[4] Exercise and Rehabilitation Sciences Institute, Faculty of Rehabilitation Sciences, School of Physical Therapy, Universidad Andres Bello. Santiago, Chile.

[5] Institute of Health and Sport Sciences, Faculty of Health Science, Universidad Francisco de Vitoria. Madrid, Spain.

Correspondence:
Corresponding Author
ebalboa@uft.cl

Keywords: Urolithin A; C2C12 myotubes; OXPHOS; maximal respiration; myogenic differentiation.

Abstract

Skeletal muscle differentiation in the C2C12 myoblast model requires extensive mitochondrial remodeling to meet rising bioenergetic demands through coordinated changes in biogenesis, dynamics, and respiratory adaptation. Urolithin A (UA), a gut microbiota-derived metabolite of ellagitannins, improves mitochondrial health; however, its role in late-stage myogenic differentiation remains unclear. Here, we investigated whether 72-h UA treatment (2 μM, days 3–6) modulates mitochondrial signaling, respiratory capacity, myogenic morphology, and ultrastructure in differentiated C2C12 myotubes. UA was non-cytotoxic and increased AMPKα phosphorylation and PGC-1α expression, while TOM20, MFN2, and OPA1 remained unchanged. Mitophagy/autophagy related-markers (p-ULK1, p62, BNIP3L/NIX, LC3-II/I) were unaltered, indicating no detectable

changes in steady-state markers under the conditions tested. Notably, UA selectively increased OXPHOS Complex I and Complex II abundance and enhanced maximal uncoupled respiration. In addition, UA increased myotube diameter and myogenic marker abundance, supporting an effect on late myogenic differentiation. However, no overt ultrastructural changes were observed by transmission electron microscopy. These findings suggest that UA promotes mitochondrial functional adaptation during myogenic differentiation, with accompanying changes in myogenic phenotype, without clear evidence of altered steady-state mitophagy/autophagy markers or mitochondrial morphology.

Introduction

Skeletal muscle differentiation is an energy-demanding process requiring extensive remodeling of cellular structure, metabolism, and organelle function during which myoblasts differentiate and fuse to form multinucleated myotubes. This transition is accompanied by a marked increase in mitochondrial content and oxidative capacity, supporting a metabolic shift from glycolysis toward oxidative phosphorylation (OXPHOS) as the primary source of ATP (8, 63, 66), depending on coordinated mitochondrial quality control, including mitophagy for damaged organelle clearance, biogenesis, and network reorganization. Given the high energetic demand and mitochondrial dependency of skeletal muscle differentiation, strategies aimed at enhancing mitochondrial function and metabolic efficiency may represent a promising approach to improve myogenic progression and muscle regeneration. This concept is particularly relevant in physiological and pathological contexts characterized by increased muscle remodeling demands, such as recovery from muscle injury, aging-associated muscle decline, or high-performance athletic adaptation, where accelerated or enhanced differentiation could contribute to improved functional recovery and muscle performance(67). However, despite the recognized importance of mitochondrial remodeling during

myogenesis, the way in which bioactive compounds modulate this process during late-stage differentiation remains insufficiently defined.

The C2C12 myoblast model is widely used to study mammalian myogenesis due to its robust differentiation and conserved mitochondrial dynamics (8, 63). During C2C12 differentiation, mitophagy predominates during early stage (days 1–3), facilitating clearance of dysfunctional mitochondria, followed by increased biogenesis and network maturation at intermediate/late stages (days 3–6) (8, 63, 66). This temporal shift makes the late differentiation window particularly relevant for investigating how compounds influence the balance between organelle turnover and metabolic maturation. In this context, BNIP3L/NIX and PINK1-Parkin pathways mediate mitophagy to selectively remove damaged mitochondria, while mitofusin-2 (MFN2) and OPA1 regulate fusion and cristae organization (44, 68). Disruption of these processes impairs myogenic progression and myotube function (44, 68, 69), underscoring the essential role of mitochondrial remodeling in functional myotube formation. This temporal window is therefore especially useful for determining whether candidate compounds act on mitochondrial turnover, metabolic adaptation, or both.

Urolithins, gut microbiota-derived metabolites of ellagitannins from pomegranates and walnuts, are converted into several metabolites, including urolithin A, B, C, and D (70). Urolithin A (UA) is the predominant circulating metabolite detected in human plasma following consumption of ellagitannin-rich foods (24), with reported concentrations ranging from 0.024 to 35 μM depending on microbiota composition and polyphenol intake(26). UA is associated with improved mitochondrial function and cellular homeostasis (29, 32, 71). Preclinical studies in Caenorhabditis elegans demonstrated that UA induces mitophagy, preserves mitochondrial function, and improves mobility and lifespan (32). These effects translate to rodent models, where UA supplementation enhances exercise capacity in both age-related muscle decline and young animals (32). In randomized,

placebo-controlled clinical trials, UA administration in middle-aged and older adults is safe and associated with improvements in muscle strength, endurance, and physical performance (26).

Despite growing evidence supporting UA's effects on mitochondrial health in mature muscle and systemic models, a critical translational gap remains: it is unknown whether UA can modulate mitochondrial adaptation during the active phase of myogenic differentiation. Current translational models relying on fully differentiated or mature muscle systems do not capture this dynamic remodeling phase, which limits their applicability to regenerative contexts. The C2C12 late-stage differentiation model (days 3–6) provides a tractable *in vitro* system to isolate and characterize UA's effects during this specific biological window, establishing a mechanistic foundation prior to more complex *in vivo* investigation

Therefore, this study investigated whether 2 μM UA, selected as a non-cytotoxic concentration within the physiologically relevant plasma range reported after oral UA supplementation in humans, modulates mitochondrial signaling, respiratory capacity, myogenic morphology, and ultrastructure during late-stage C2C12 differentiation (days 3–6).

Materials and Methods

Cell Culture

The murine C2C12 cell line (ATCC) was used. C2C12 myoblasts were seeded at a density of 1×10^5 cells per well in 6-well plates. Cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (GIBCO), 2% penicillin/streptomycin (GIBCO), 1% non-essential amino acids (NEEA; GIBCO), and 0.1% amphotericin B (GIBCO) in a humidified atmosphere with 5% CO_2 at 37 °C. Upon reaching ~70% confluence, the proliferation medium was replaced with a differentiation medium containing 2% horse serum (GIBCO), 2% penicillin/streptomycin,

1% NEEA, and 0.1% amphotericin B. The differentiation medium was refreshed every day.

On day 3 of differentiation, cells were treated with Urolithin A (2 μM; BOC Sciences, USA), prepared in DMSO (final concentration 0.1% v/v). DMSO was used as vehicle control during differentiation. UA treatment was initiated on day 3 and maintained for 72 h, with medium replacement every 24 h.

Cell viability

Cell viability was determined using the MTT assay (Invitrogen) according to the manufacturer's instructions. C2C12 myotubes were treated on day 3 of differentiation with increasing concentrations of Urolithin A (0, 0.2, 2, 5, 10, and 20 μM) for 72 h.

At the end of the treatment period, MTT solution (1.2 mM) was added to the cells. After 4 h of incubation at 37 °C, the resulting formazan crystals were dissolved in 100 μL of 0.1% SDS-HCl solution and incubated for 18 h in a humidified chamber. Absorbance was measured at 570 nm. Cell viability was expressed as a percentage relative to the control condition, which was set at 100%.

Histological assessment

After differentiation, cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature. Fixed cells were then stained with hematoxylin for 3–5 min, washed twice with running water, and counterstained with eosin for 1 min. After eosin staining, samples were washed three times with distilled water, air-dried, and imaged for subsequent analysis.

Hematoxylin and eosin (H&E)-stained preparations were used to assess myotube morphology and quantify differentiation-related structural features. The myogenic index was calculated as the percentage of nuclei contained within myotubes relative to the total number of nuclei, using the formula: (number of nuclei in myotube/total number of nuclei) x 100. Myotube

diameter was quantified using ImageJ software. For each biological replicate, 10 myotubes per field were analyzed across seven randomly selected fields, and the average diameter of each myotube was obtained from measurements taken at five different points along the cell length.(72). Quantitative analysis of H&E-stained images was performed by an evaluator blinded to the experimental conditions to reduce observer bias

Protein extraction

At the end of the differentiation period, myotubes were rinsed once with PBS and harvested in ice-cold lysis buffer containing 20 mM Tris (pH 7.0), 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT (dithiothreitol), and a protease inhibitor cocktail containing 1 mM EDTA (Roche Applied Science, Belgium).

Homogenates were centrifuged at 12,500 rpm for 10 min at 4 °C, and the resulting supernatants were collected and stored at −80 °C. Protein concentration was determined using the DC Protein Assay Kit (Bio-Rad).

Western Blot Analyses

Cell lysates (18 µg of protein) were mixed with Laemmli sample buffer and heated for 5 min at 95 °C prior to loading onto 12% SDS-PAGE gels. Proteins were separated by electrophoresis at 120 V for 1.5 h and subsequently transferred onto PVDF membranes at 80 V for 1.5 h for Western blot analysis.

Membranes were blocked for 60 min in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat milk. Membranes were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-phospho-AMPKα (Thr172) (1:1000, 2603S, Cell Signaling Technology), mouse anti-PGC-1α (1:500, SC-517380; Santa Cruz, Dallas, TX, USA), rabbit anti-TOM20 (1:1000, 42406S, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-ULK1 (1:1000, 14202S, Cell Signaling Technology),

rabbit anti-BNIP3L (1:1000, 12396S, Cell Signaling Technology), LC3 (1:1000, 4108, Cell Signaling Technology, Danvers, MA, USA), p62 (1:2000, H00008878-M01, Abnova), mouse anti-Total OXPHOS cocktail (1:1000, ab110413, Abcam), mouse anti-MFN2 (1:1000, ab56889, Abcam), mouse anti-optic atrophy protein 1 (OPA1) (612606, BD Biosciences), and mouse anti-MHC (1:500, SC-47724; Santa Cruz, Dallas, TX, USA), mouse anti-MHC (1:500, SC-376157; Santa Cruz, Dallas, TX, USA), mouse anti-MyoD (1:500, SC-377460; Santa Cruz, Dallas, TX, USA), mouse anti-myogenin (1:500, SC-47724; Santa Cruz, Dallas, TX, USA), mouse anti-MRF4 (1:500, SC-47724; Santa Cruz, Dallas, TX, USA)..

Membranes were then incubated with the appropriate secondary antibodies: goat anti-mouse IgG-HRP (1:10,000; 31430, Invitrogen, USA) or goat anti-rabbit IgG-HRP (1:10,000; 7074P2, Cell Signaling Technology, Danvers, MA, USA). After three additional washes, protein bands were detected using an enhanced chemiluminescence (ECL) kit (Bio-Rad). Membranes were imaged using a ChemiDoc MP™ system (Bio-Rad), and band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Oxygen Consumption

Oxygen consumption rates (OCR) were measured using an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) in intact C2C12 myotubes. On day 6 of differentiation (72 h post-Urolithin A treatment), myotubes from each condition were trypsinized, and resuspended in DMEM (Life Technologies). Respiration was recorded sequentially under the following conditions: basal respiration (routine state); oligomycin (2 µM) to inhibit ATP synthase (leak respiration); FCCP (0.5 µM, titrated) to stimulate maximal uncoupled respiration (electron transport system capacity); and rotenone (0.5 µM) plus antimycin A (2.5 µM) to inhibit complexes I and III (residual non-mitochondrial respiration).

Data were acquired using DatLab 7 software and analyzed to determine basal respiration, oligomycin-sensitive respiration (ATP-linked), maximal

uncoupled respiration (FCCP), and spare respiratory capacity. OCR values were normalized to cell number and expressed as pmol/(s·million cells). Experiments were performed in duplicate chambers per biological replicate (n = 5 per condition).

Transmission electron microscopy

Myotubes were treated in the absence or presence of Urolithin A (UA). After 72 h of treatment, myotubes were dissociated using trypsin (Thermo Fisher Scientific), centrifuged, and the resulting pellet was fixed in 2% glutaraldehyde. The fixed pellet was sectioned into fiber bundles and analyzed by transmission electron microscopy (TEM; Talos F200C G2, Microscopy Facility, Pontificia Universidad Católica de Chile)(73)

For each independent experiment, mitochondria from five myotubes were analyzed. Mitochondrial density was calculated as the percentage of mitochondrial area relative to total cellular area, and mitochondrial size was expressed in nm². The width-to-length ratio was calculated to assess mitochondrial circularity. All quantifications were performed in a blind manner by an expert at 13,500× magnification.

Statistical analysis

Statistical significance was assessed using non-parametric tests for two-group comparisons (Mann–Whitney U test) and for three or more groups (Kruskal–Wallis test followed by Dunn's multiple comparisons test). Mitochondrial respiration data were analyzed using two-way ANOVA (condition × substrate), followed by Sidak's multiple comparisons test.

Data are presented as mean ± standard deviation (SD) for viability assays and as median with interquartile range (IQR) for protein expression, respirometry, and morphological analyses. The number of biological replicates (n) is indicated in each figure legend. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

1. C2C12 differentiation and effect of urolithin A on cell viability

To validate C2C12 myogenic differentiation and establish a non-cytotoxic UA concentration during days 3–6, cell viability was first assessed by MTT assay relative to CTRL (0.1% DMSO) (Figure 1a). UA at 0.2 and 2 μM did not affect viability, whereas concentrations ≥5 μM reduced viability by approximately 15% after 72 h. Accordingly, 2 μM UA was selected for subsequent experiments, and representative images were acquired to assess myotube morphology (Figure 1b)

To further characterize the biological effect of the selected UA concentration, we evaluated myogenic markers and myotube morphology during C2C12 differentiation (Figure 1c). UA increased MyoD and myosin heavy chain (MHC) abundance (Figure 1f) and was associated with a significant increase in myotube diameter (Figure 1e), whereas MRF4 and Myog were not significantly modified and the myogenic index showed no clear change (Figure 1e). Overall, these findings suggest that UA may selectively influence late myogenic features rather than broadly enhancing the differentiation program. We next investigated whether these changes were accompanied by alterations in mitochondrial remodeling and respiratory function in differentiated C2C12 cells.

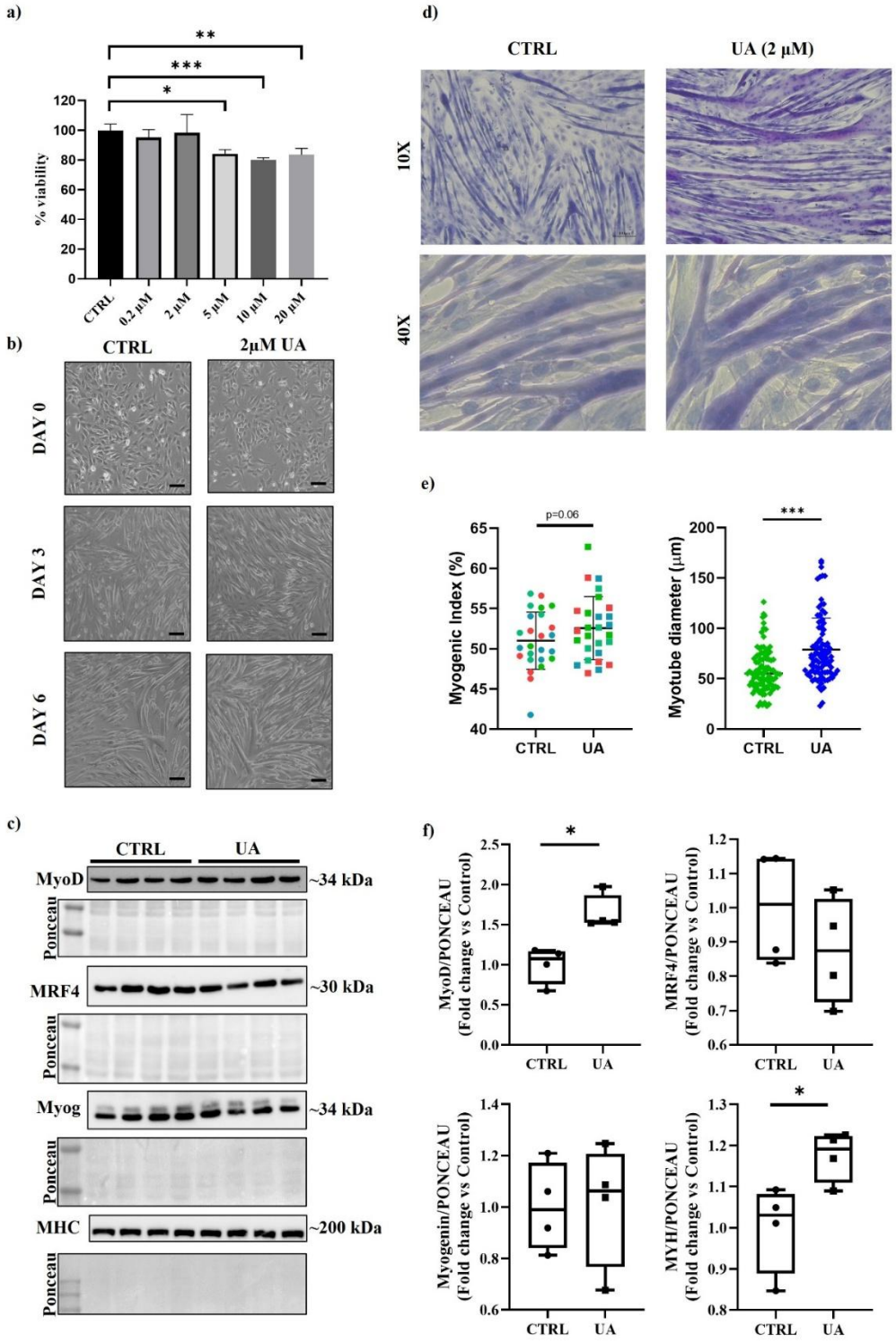


Figure 1. C2C12 differentiation and effects of Urolithin A on cell viability. (a) MTT viability assay. C2C12 cells were exposed to increasing concentrations of Urolithin A (UA; 0.2, 2, 5, 10, and 20 µM) for 72 h starting on day 3 of differentiation. Cell viability is expressed as a percentage

relative to CTRL (b) Phase-contrast microscopy (10×, inverted) showing myoblast-to-myotube progression at day 0 (myoblasts), day 3 (myocytes), and day 6 (myotubes) (CTRL vs. 2 μM Urolithin A [UA] treatment during days 3–6). Scale bar: 100 μm. c) Representative immunoblots of myogenic markers MyoD, MRF4, Myog, and MHC in CTRL and UA-treated cells. (d) Representative H&E-stained images of CTRL and UA (2 μM)-treated myotubes at 10× and 40× magnification. (e) Quantification of myogenic index and myotube diameter in CTRL and UA-treated cells. (f) Quantification of MyoD, MRF4, Myog, and MHC abundance normalized to Ponceau, shown as fold change relative to CTRL. Data are presented as mean ± SD (n = 6; Kruskal–Wallis test). ***p < 0.001, **p < 0.01, *p < 0.05 vs. CTRL.

2. Urolithin A does not alter mitophagy or autophagy-related proteins in C2C12 myotubes after 72 h of treatment (days 3–6 of differentiation).

To determine whether Urolithin A (UA) modulates mitophagy pathways during C2C12 differentiation, cells were treated with 2 μM UA for 72 h, and protein levels of key regulators of autophagy initiation and mitochondrial quality control were subsequently quantified by Western blot (Figure 2). Protein levels were normalized to total protein loading using Ponceau staining. To assess the autophagy machinery, phosphorylated ULK1 (p-ULK1), a key regulator of autophagy initiation, was analyzed. p-ULK1 levels did not differ between CTRL and UA-treated cells (1.00 ± 0.233 vs. 1.23 ± 0.06 AU; p = 0.1) (Figure 2b), indicating that UA did not significantly affect ULK1 activation during days 3–6 of differentiation. Consistent with this observation, protein levels of p62, a selective autophagy receptor and cargo adaptor that binds ubiquitinated substrates for lysosomal degradation, were not significantly altered by UA treatment (1.00 ± 0.10 vs. 1.03 ± 0.37 AU; p = 0.24) (Figure 2c), suggesting no major changes in autophagic steady state under these conditions. Similarly, the mitophagy-related protein

BNIP3L/NIX showed comparable expression between groups (1.00 ± 0.17 vs. 0.95 ± 0.07 AU; p = 0.34) (Figure 2d), indicating that receptor-mediated mitophagy pathways were not activated by UA at this stage of differentiation. Finally, the LC3-II/LC3-I ratio, an indicator of autophagosome formation, was not significantly altered (1.00 ± 0.23 vs. 0.94 ± 0.08 AU; p = 0.24) (Figure 2e), further supporting the absence of major changes in autophagic steady state. Together, these results indicate that 3 days of UA exposure during late-stage C2C12 differentiation does not significantly modify the levels of key proteins involved in mitophagy or autophagy initiation, suggesting that UA-induced mitochondrial adaptations occur without detectable changes in steady-state markers of mitophagy/autophagy under the conditions tested.

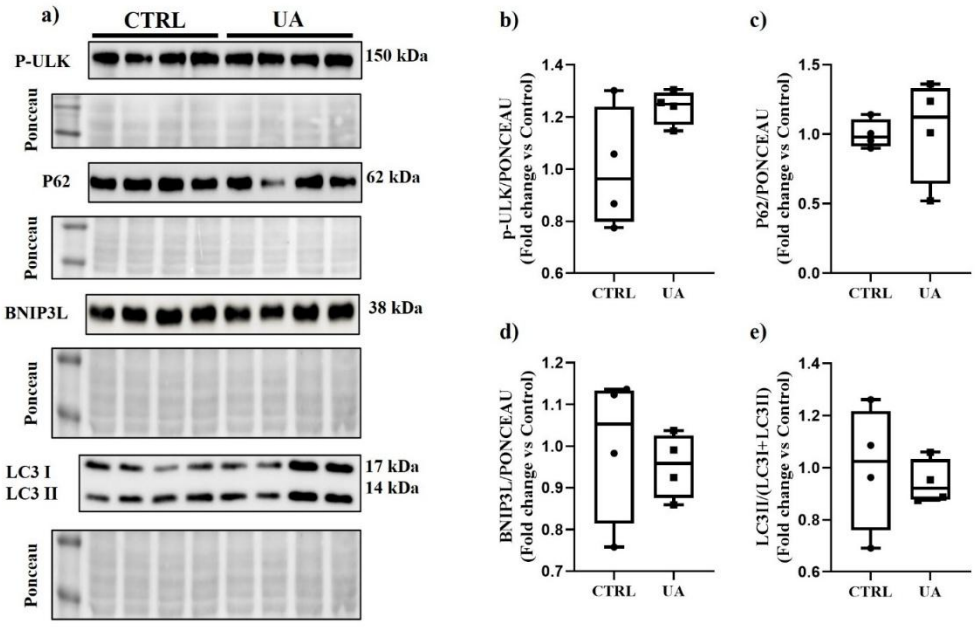


Figure 2. Urolithin A does not alter mitophagy/autophagy-related proteins in C2C12 myotubes. C2C12 myotubes were treated with 2 μM Urolithin A (UA) for 72 h starting on day 3 of differentiation (days 3–6). (a) Representative immunoblots and Ponceau staining. Quantification of Western blot band intensities are expressed as fold change relative to CTRL for p-ULK1 (b), p62 (c), BNIP3L/NIX (d), and the LC3-II/LC3-I ratio (e). Data

are presented as median with interquartile range (Mann–Whitney test; *p < 0.05 vs. CTRL; n = 4).

3. Urolithin A upregulates mitochondrial biogenesis-related markers and fusion protein expression in differentiated C2C12 myotubes.

To assess whether Urolithin A (UA) modulates mitochondrial content and dynamics, protein levels of key regulators of mitochondrial biogenesis and membrane remodeling were analyzed by Western blot (Figure 3). Protein levels were normalized to total protein loading using Ponceau staining.

As a positive control of the reported effects of UA (32), phosphorylated AMPKα (p-AMPKα) levels were significantly increased in UA-treated cells compared with CTRL (1.00 ± 0.21 vs. 1.39 ± 0.17 AUC; p = 0.02) (Figure 3b). Consistent with AMPKα activation, PGC-1α protein levels were also higher in UA-treated cells than in CTRL (1.00 ± 0.14 vs. 1.25 ± 0.07 AUC; p = 0.01) (Fig. 3c). In contrast, TOM20 levels were unchanged in the UA group (1.00 ± 0.27 vs. 1.05 ± 0.21 AU; p = 0.5) (Figure 3d). When normalized to TOM20, OPA1 protein expression remained comparable between groups (1.00 ± 0.30 vs. 0.99 ± 0.26 AU; p = 0.5), indicating that OPA1 was not affected by UA at this stage of differentiation (Figure 3e). Similarly, MFN2 levels normalized to TOM20 were not significantly different between groups (1.00 ± 0.26 vs. 1.08 ± 0.30 AU; p = 0.24) (Figure 3f).

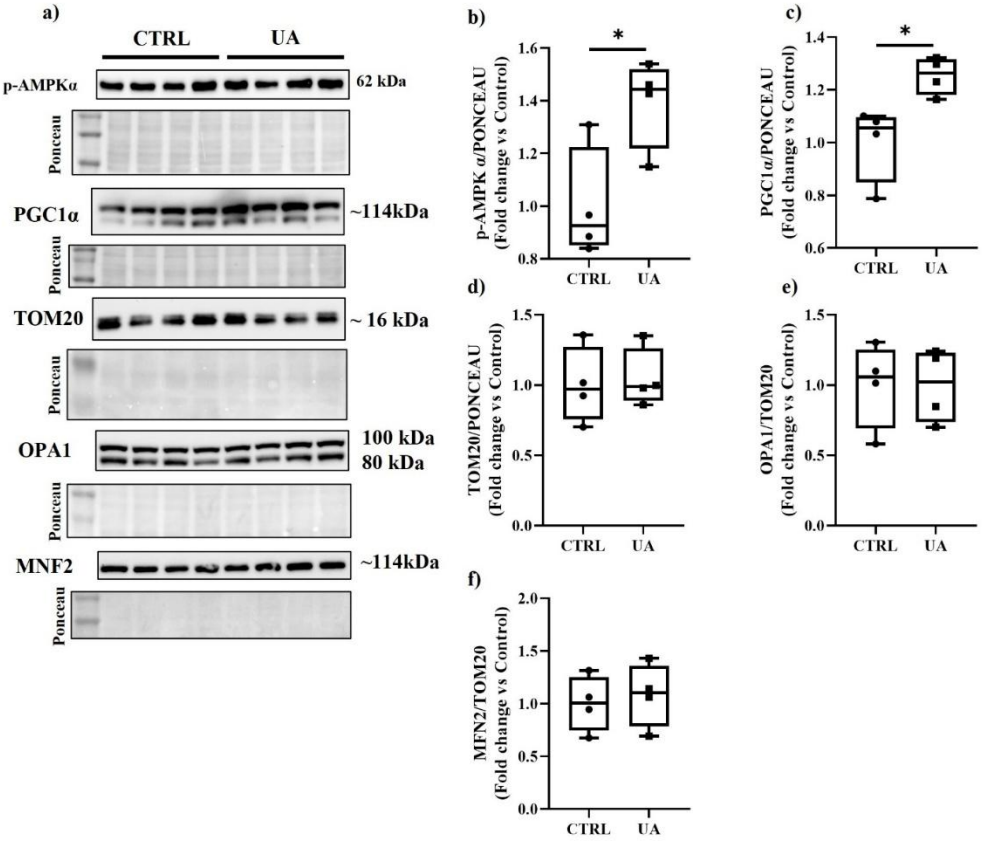


Figure 3. Urolithin A enhances mitochondrial biogenesis-related markers and fusion in differentiated C2C12 myotubes. C2C12 myotubes were treated with 2 μM Urolithin A (UA) for 72 h starting on day 3 of differentiation (days 3–6). (a) Representative immunoblots and Ponceau staining. Quantification of Western blot band intensities is expressed as fold change relative to CTRL for p-AMPKα (b), PGC-1α (c), TOM20 (d), OPA1 (e), and MFN2 (f). Data are presented as median with interquartile range (Mann–Whitney test; *p < 0.05 vs. CTRL; n = 4).

4. Urolithin A upregulates OXPHOS Complex II and maximal respiratory capacity in differentiated C2C12 myotubes

To determine whether Urolithin A (UA) modulates mitochondrial respiratory capacity in late-stage C2C12 myotubes, high-resolution respirometry was performed in intact mature myotubes (Figure 4a). In parallel, the protein abundance of OXPHOS complexes was assessed by Western blot (Figure 4b). Respirometry data were analyzed using two-way ANOVA, with

condition (CTRL vs. UA) and substrate as factors. A significant main effect of condition was observed (p = 0.0033), indicating that UA treatment increased mitochondrial respiration compared to CTRL. A significant main effect of substrate was also detected (p=0.0001), confirming appropriate responses to the sequential substrate–inhibitor protocol. No significant interaction between condition and substrate was found (p=0.0745), suggesting that UA enhanced mitochondrial respiratory capacity in a global manner rather than selectively affecting specific respiratory states. Sidak's multiple comparisons test indicated that UA treatment did not significantly alter basal respiration, oligomycin-sensitive respiration, or non-mitochondrial respiration compared to CTRL. In contrast, UA significantly increased maximal respiration (FCCP) (158.6 ± 53.13 vs. 203.7 ± 79.07 pmol/(s·million cells); p = 0.003) (Figure 4a), indicating enhanced maximal mitochondrial respiratory capacity.

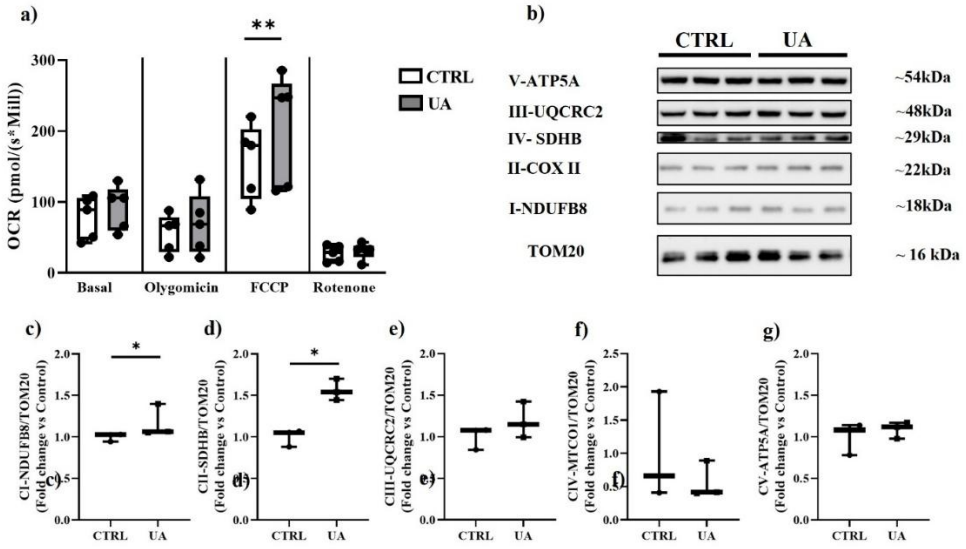


Figure 4. Urolithin A enhances mitochondrial respiratory function in differentiated C2C12 myotubes treated with 2 µM UA (days 3–6). (a) Mitochondrial oxygen consumption rates (OCR) measured by high-resolution respirometry (Oroboros O2k) under basal conditions, followed by sequential addition of oligomycin, FCCP, and rotenone/antimycin A, normalized to cell number and expressed as

pmol/(s*Mill). (b) Representative immunoblots of OXPHOS complexes (I–V), with corresponding Ponceau S staining used as loading control. Quantitative analysis of Western blot band intensities expressed as fold change relative to CTRL for Complexes I (c), II (d), III (e), IV (f), and V (g). Data are presented as median and interquartile range. Statistical significance was determined using two-way ANOVA followed by Sidak's multiple comparisons test for respirometry evaluation (n=5) and Mann–Whitney test for WB analysis (n=4); *p < 0.05 vs CTRL.

In line with the OCR results, UA treatment increased the abundance of selected OXPHOS complexes relative to CTRL. Specifically, UA significantly increased the protein levels of complex I (1.00 ± 0.04 vs. 1.17 ± 0.19 AU; p = 0.05) (Figure 4c) and Complex II (1.00 ± 0.10 vs. 1.56 ± 0.12 AU; p = 0.05) (Figure 4d). However, no significant changes were observed in Complex III (1.00 ± 0.13 vs. 1.18 ± 0.21 AU; p = 0.2) (Figure 4e), Complex IV (1.00 ± 0.81 vs. 0.56 ± 0.27 AU; p = 0.35) (Figure 4f), or Complex V (1.00 ± 0.19 vs. 1.08 ± 0.09 AU; p = 0.35) (Figure 4). Together, these results suggest that UA enhances mitochondrial respiratory capacity during myogenic differentiation.

5. Urolithin A does not alter mitochondrial morphology in differentiated C2C12 myotubes

To examine whether Urolithin A (UA) alters mitochondrial morphology during the late stage of myogenic differentiation, mitochondrial area, perimeter, circularity, and aspect ratio (AR) were assessed by transmission electron microscopy (TEM) and quantified from five myotubes per group (Figure 5). Although UA induced changes in mitochondrial protein expression and function, TEM analysis revealed no significant differences in mitochondrial morphology between CTRL and UA-treated cells. Specifically, mitochondrial area (1.20 ± 0.46 vs. 1.27 ± 0.39 μm²; p = 0.163; Figure 5b), perimeter (4.22 ± 1.03 vs. 4.07 ± 0.91 μm; p = 0.213; Figure 5c), circularity (0.85 ± 0.06 vs. 0.85 ± 0.05; p = 0.33; Figure 5d), and aspect ratio

(1.68 ± 0.34 vs. 1.69 ± 0.33; p = 0.41; Figure 5e) were comparable between groups.

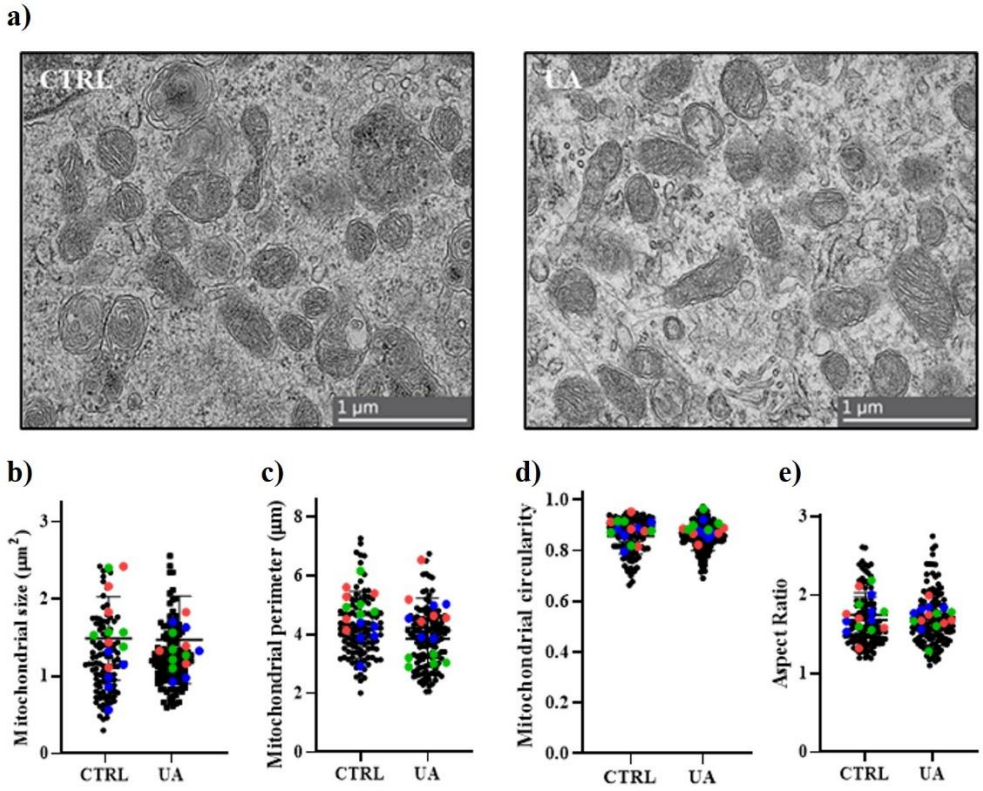


Figure 5. Urolithin A does not induce ultrastructural changes in mitochondrial morphology in differentiated C2C12 myotubes. C2C12 myotubes were treated with 2 µM Urolithin A (UA) for 72 h starting on day 3 of differentiation (days 3–6). (a) Representative transmission electron microscopy (TEM) images of CTRL and UA-treated samples (scale bar: 1 µm). (b) Quantitative analysis of mitochondrial area, (c) perimeter, (d) circularity, and (e) aspect ratio. Data are presented as median with interquartile range (Mann–Whitney U test). Quantification was performed from n = 5 myotubes per condition, with ≥50 mitochondria analyzed per group. Black dots represent individual mitochondrial measurements, and colored dots represent the mean value for each condition.

Discussion

The principal finding of this study is that Urolithin A (UA) modulates mitochondrial biogenesis-related markers and maximal respiratory capacity in differentiated C2C12 myotubes (days 3–6), a phase characterized by myotube maturation and predominant mitochondrial biogenesis (8, 63), without altering steady-state levels of mitophagy/autophagy markers or mitochondrial ultrastructure. This pattern indicates that UA amplifies the mitochondrial remodeling that naturally accompanies myogenic differentiation, resulting in myotubes with a higher oxidative reserve. However, the consequences for long-term contractile performance and stress resistance remain to be determined.

In the present study, 2 μM UA was selected based on its lack of cytotoxic effects in C2C12 myotubes and its alignment with plasma concentrations reported after oral UA supplementation in humans (26, 29). While 2 μM UA had no effect on cell viability, higher concentrations applied for 72 h significantly reduced survival, suggesting that excessive exposure is detrimental during late-stage C2C12 differentiation (days 3–6). This is consistent with reports showing that high UA concentrations can reduce viability in human muscle *in vitro* models (74), and may trigger pro-apoptotic signaling (75). Importantly, our H&E analysis showed that 2 μM UA increased myotube diameter, which is consistent with an effect on myotube maturation. Accordingly, all subsequent experiments were conducted using 2 μM UA to model a non-cytotoxic and physiologically relevant exposure condition.

Although UA increased mitochondrial biogenesis related-markers and respiratory capacity, no overt ultrastructural changes were detected by TEM. This apparent discrepancy between biochemical/functional adaptations and mitochondrial morphology is consistent with previous reports describing mitochondrial optimization without gross structural alterations detectable by conventional TEM (19). Notably, TEM samples in this study were prepared from centrifuged cell pellets rather than in situ-embedded adherent myotubes, a preparation that may disrupt myotube

architecture, mix differentiated myotubes with residual myoblasts, and reduce the sensitivity to detect subtle network remodeling (76). Together, these findings suggest that UA primarily promotes mitochondrial functional optimization rather than overt morphological remodeling or turnover during late-stage C2C12 differentiation.

In our C2C12 differentiation model (days 3–6), UA did not significantly alter mitophagy-related markers. However, a limitation of the present study is that autophagic/mitophagic flux was not directly assessed. Therefore, our data indicate only that UA did not change the steady-state levels of these markers under the tested conditions, rather than demonstrating the absence of mitophagy altogether. This observation agrees with reports that UA can activate autophagy without inducing mitophagy in ischemic neuronal models (56). By contrast, Ryu et al. (2016) showed that 5–10 μM UA applied for 12–24 h induces mitophagy in adult C. elegans and fully differentiated C2C12 myotubes (32), while Andreux et al. (2019) reported a mitophagy-related molecular signature in human skeletal muscle after oral UA supplementation (29). These differences likely reflect the distinct experimental context, since those studies used mature systems and either higher doses or shorter exposure times, whereas our model involved 2 μM UA for 72 h during an active differentiation phase (days 3–6). At this stage, mitochondrial biogenesis and oxidative adaptation may predominate over mitochondrial turnover (8) which could account for the lack of detectable mitophagy activation in our conditions.

We observed activation of the AMPKα–PGC-1α axis. AMPKα is a central energy sensor that can promote mitochondrial biogenesis through direct and indirect regulation of PGC-1α, and its activation has been linked to improved mitochondrial function in differentiating myotubes (23). In human skeletal muscle, UA has been reported to increase PGC-1α and mitochondrial gene expression (29), as well as respiratory chain components, including Complex II (26). In our model, however, UA did not significantly alter TOM20, OPA1 or MFN2, indicating that the observed

functional effects were not accompanied by detectable changes in mitochondrial content or mitochondrial fusion markers. However, because direct measurements of mitochondrial biogenesis were not performed, our data should be interpreted as evidence of biogenesis-related signaling and mitochondrial functional adaptation rather than definitive proof of enhanced mitochondrial biogenesis. Notably, UA increased the abundance of OXPHOS Complex I and Complex II, which may help explain the higher maximal respiratory capacity observed after treatment, as both complexes represent major electron-entry sites into the respiratory chain and may support greater OXPHOS flux (77). Functionally, UA was associated with increased maximal respiratory capacity (FCCP) and spare respiratory capacity, consistent with improvements reported in skeletal muscle and cardiac models following UA treatment (50 μM for 24 h) (29, 53). Importantly, basal respiration remained unchanged, suggesting that UA enhances mitochondrial reserve capacity rather than resting energy demand, an adaptation consistent with increased metabolic flexibility during muscle maturation. Although mitochondrial ROS production was not directly assessed in the present study, the dissociation between increased maximal respiration and unchanged basal respiration suggests improved mitochondrial efficiency rather than increased oxidative burden (78). Together, these findings suggest that UA enhances mitochondrial function during late-stage C2C12 differentiation without evidence of concomitant mitophagy activation or overt structural remodeling.

A key limitation of our study is that UA was administered only during days 3–6 of C2C12 differentiation, precluding evaluation of potential effects during earlier stages, when autophagy and mitophagy are more prominent. Moreover, we did not assess contractile function, fatigue resistance, or long-term myotube survival, so the impact of UA on muscle performance remains to be determined. These limitations highlight the need for future studies spanning the full myogenic program and incorporating functional outcomes in more physiologically relevant models. In this context, our findings help address an important gap in the literature by showing that UA can enhance

mitochondrial respiratory capacity during late-stage C2C12 differentiation without detectable changes in steady-state mitophagy markers or mitochondrial ultrastructure. Collectively, these results suggest that UA may support mitochondrial oxidative adaptation during myogenic differentiation and provide a rationale for future *in vivo* studies.

Finally, our findings provide a mechanistic rationale to evaluate UA in *in vivo* models of muscle regeneration (e.g., cardiotoxin injury, denervation, or hindlimb unloading), where the translational goal would be to determine whether UA-enhanced mitochondrial oxidative capacity in differentiating fibers translates to improved contractile recovery and functional outcomes. Moreover, our results help to expand current knowledge on the effects of urolithin A by identifying a previously unrecognized temporal window during differentiation in which it exerts its action, highlighting its relevance not only as a therapeutic approach but also as a potential preventive strategy in populations susceptible to muscle damage, such as athletes at risk of sports injuries and aging individuals.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

RV-F contributed to the study conceptualization, methodology, investigation, original draft preparation, review and editing of the manuscript, and visualization, and performed all experiments except respirometry assays. DI contributed to the mitochondrial function experiments and data analysis. MV-B contributed to the study conceptualization, methodology, and manuscript writing. EB-C contributed to the study conceptualization, results analysis, and manuscript review and

editing. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Faculty of Medicine, Universidad Finis Terrae, and the PhD Program in Exercise Science, Universidad Finis Terrae.

Acknowledgments

The authors would like to sincerely thank Francisco Diaz for technical support and Paola Haeger for her assistance during the final writing of the manuscript.

Reference

1. Sin J, Andres AM, Taylor DJ, Weston T, Hiraumi Y, Stotland A, et al. Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. Autophagy. 2016;12(2):369-80.

2. Remels AH, Langen RC, Schrauwen P, Schaart G, Schols AM, Gosker HR. Regulation of mitochondrial biogenesis during myogenesis. Mol Cell Endocrinol. 2010;315(1-2):113-20.

3. Wang X, Feng Z, Wang X, Yang L, Han S, Cao K, et al. O-GlcNAcase deficiency suppresses skeletal myogenesis and insulin sensitivity in mice through the modulation of mitochondrial homeostasis. Diabetologia. 2016;59(6):1287-96.

4. Serova M, Didry-Barca B, Deloux R, Foucault AS, Veillet S, Lafont R, et al. BIO101 stimulates myoblast differentiation and improves muscle function in adult and old mice. J Cachexia Sarcopenia Muscle. 2024;15(1):55-66.

5. Tezze C, Romanello V, Desbats MA, Fadini GP, Albiero M, Favaro G, et al. Age-Associated Loss of OPA1 in Muscle Impacts Muscle Mass,

Metabolic Homeostasis, Systemic Inflammation, and Epithelial Senescence. Cell Metab. 2017;25(6):1374-89.e6.

6. Sebastián D, Sorianello E, Segalés J, Irazoki A, Ruiz-Bonilla V, Sala D, et al. Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway. Embo j. 2016;35(15):1677-93.

7. Rochard P, Rodier A, Casas F, Cassar-Malek I, Marchal-Victorion S, Daury L, et al. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. J Biol Chem. 2000;275(4):2733-44.

8. Gonzalez-Jamett A, Vasquez W, Cifuentes-Riveros G, Martinez-Pando R, Saez JC, Cardenas AM. Oxidative Stress, Inflammation and Connexin Hemichannels in Muscular Dystrophies. Biomedicines. 2022;10(2).

9. Tomás-Barberán FA, García-Villalba R, González-Sarrías A, Selma MV, Espín JC. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. J Agric Food Chem. 2014;62(28):6535-8.

10. Singh A, D'Amico D, Andreux PA, Fouassier AM, Blanco-Bose W, Evans M, et al. Urolithin A improves muscle strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. Cell Rep Med. 2022;3(5):100633.

11. Tang L, Mo Y, Li Y, Zhong Y, He S, Zhang Y, et al. Urolithin A alleviates myocardial ischemia/reperfusion injury via PI3K/Akt pathway. Biochem Biophys Res Commun. 2017;486(3):774-80.

12. Andreux PA, Blanco-Bose W, Ryu D, Burdet F, Ibberson M, Aebischer P, et al. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. Nat Metab. 2019;1(6):595-603.

13. Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Félix AA, et al. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. Nat Med. 2016;22(8):879-88.

14. Ono Y, Sakamoto K. Lipopolysaccharide inhibits myogenic differentiation of C2C12 myoblasts through the Toll-like receptor 4-nuclear factor-κB signaling pathway and myoblast-derived tumor necrosis factor-α. PLoS One. 2017;12(7):e0182040.

15. Castro-Sepulveda M, Tuñón-Suárez M, Rosales-Soto G, Vargas-Foitzick R, Deldicque L, Zbinden-Foncea H. Regulation of mitochondrial morphology and cristae architecture by the TLR4 pathway in human skeletal muscle. Front Cell Dev Biol. 2023;11:1212779.

16. Zhao H, Song G, Zhu H, Qian H, Pan X, Song X, et al. Pharmacological Effects of Urolithin A and Its Role in Muscle Health and Performance: Current Knowledge and Prospects. Nutrients. 2023;15(20).

17. El-Wetidy MS, Ahmad R, Rady I, Helal H, Rady MI, Vaali-Mohammed MA, et al. Urolithin A induces cell cycle arrest and apoptosis by inhibiting Bcl-2, increasing p53-p21 proteins and reactive oxygen species production in colorectal cancer cells. Cell Stress Chaperones. 2021;26(3):473-93.

18. Hinton A, Jr., Katti P, Christensen TA, Mungai M, Shao J, Zhang L, et al. A Comprehensive Approach to Sample Preparation for Electron Microscopy and the Assessment of Mitochondrial Morphology in Tissue and Cultured Cells. Adv Biol (Weinh). 2023;7(10):e2200202.

19. Ahsan A, Zheng YR, Wu XL, Tang WD, Liu MR, Ma SJ, et al. Urolithin A-activated autophagy but not mitophagy protects against ischemic neuronal injury by inhibiting ER stress *in vitro* and *in vivo*. CNS Neurosci Ther. 2019;25(9):976-86.

20. Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A. 2007;104(29):12017-22.

21. Maekawa S, Takada S, Furihata T, Fukushima A, Yokota T, Kinugawa S. Mitochondrial respiration of complex II is not lower than that of complex I in mouse skeletal muscle. Biochem Biophys Rep. 2020;21:100717.

22. Rodriguez J, Pierre N, Naslain D, Bontemps F, Ferreira D, Priem F, et al. Urolithin B, a newly identified regulator of skeletal muscle mass. J Cachexia Sarcopenia Muscle. 2017;8(4):583-97.

23. Jones AE, Sheng L, Acevedo A, Veliova M, Shirihai OS, Stiles L, et al. Forces, fluxes, and fuels: tracking mitochondrial metabolism by integrating measurements of membrane potential, respiration, and metabolites. American Journal of Physiology-Cell Physiology. 2021;320(1):C80-C91.

ARTICLE 2 – FIRST DRAFT / MANUSCRIPT IN PREPARATION

This chapter is based on the following original research manuscript, currently in the final stages of preparation for submission to a peer-reviewed scientific journal.

Urolithin A modulates mTOR signaling and enhances baseline protein synthesis during late-stage C2C12 myogenesis

Background: Terminal myotube maturation requires a coordinated balance between structural assembly, translational control, and metabolic adaptation. Urolithin A (UA) is known to enhance mitochondrial fitness, but its role in regulating translational machinery during late-stage myogenesis remains unclear. **Methods:** C2C12 myoblasts were differentiated into myotubes and treated with a physiological dose of UA (2 μM) during late differentiation (days 3–6). mTOR related-pathway components (AKT, mTOR, p70S6K, S6, and 4E-BP1) were analyzed by Western blot and baseline de novo protein translation was quantified using the non-radioactive SUnSET assay. **Results:** Physiological UA exposure induced

selective remodeling of translational signaling rather than generalized pathway activation. UA increased total mTOR abundance and phosphorylation at Ser2448, together with elevated total p70S6K protein levels. In contrast, the phospho/total p70S6K ratio was reduced, while AKT, S6, and 4E-BP1 remained unchanged. Importantly, these molecular changes were accompanied by a significant increase in global baseline protein synthesis, as shown by enhanced puromycin incorporation into nascent polypeptide chains. **Conclusions:** UA influences translational signaling dynamics during late-stage C2C12 differentiation and supports global protein synthesis to meet the biosynthetic demands of myotube maturation. Together with our previous evidence of mitochondrial adaptation, these findings suggest that UA coordinates metabolic remodeling and translational control during the terminal myogenic program.

Keywords: Urolithin A; Myogenesis; mTOR signaling; Protein synthesis; SUnSET; C2C12 myotubes.

Introduction

Skeletal muscle regeneration, maintenance, and remodeling depend on a highly coordinated sequence of cellular events driven by muscle satellite cells (1). Upon activation, these quiescent cells proliferate as myoblasts and sequentially express key myogenic regulatory factors (MRFs), such as MyoD and myogenin, which orchestrate exit from the cell cycle and induce fusion into multinucleated, mature myotubes characterized by myosin heavy chain (MHC) expression (79). As we recently demonstrated (80), late-stage myogenic differentiation (days 3–6) involves a critical metabolic shift from glycolysis to oxidative phosphorylation (OXPHOS), accompanied by comprehensive mitochondrial functional adaptation and network remodeling.

While mitochondrial fitness provides the ATP required to support differentiation, the anabolic and structural growth of nascent myotubes is fundamentally governed by the mechanistic target of rapamycin (mTOR) signaling pathway (8, 41). Activation of the AKT/mTOR axis and its downstream effectors, such as p70S6K and 4E-BP1, is essential for de novo protein synthesis and myofibrillar maturation (42). Notably, mTOR signaling is blunted in muscle-wasting conditions and aging, contributing to reduced translation and impaired myotube formation (81), which underscores the relevance of understanding how nutraceuticals such as UA modulate this pathway during differentiation.

Urolithin A (UA), a gut microbiota-derived metabolite of ellagitannins and ellagic acid, has emerged as a potent nutraceutical capable of enhancing skeletal muscle health. Previous clinical and preclinical studies have documented that UA stimulates mitophagy and mitochondrial biogenesis, thereby restoring muscle function in models of muscular dystrophy and age-related decline (28, 29, 32, 33). Moreover, recent evidence suggests that urolithins can accelerate myocyte fusion and increase the transcription of specific myosin isoforms. Nonetheless, contradictory findings persist regarding their direct anabolic effects, whereas urolithin B has been identified as a regulator of muscle mass via mTOR activation, earlier work reported that urolithin A did not trigger direct protein synthesis pathways under basal, non-stimulated conditions (53). Consequently, the molecular crosstalk between UA-mediated mitochondrial adaptation and the mTOR-driven translational machinery during late myogenesis remains poorly understood.

To address this gap, the present study investigates the effect of a physiologically relevant dose of Urolithin A on mTOR pathway dynamics and global protein synthesis during the late maturation stage (days 3–6) of C2C12 differentiation. We hypothesized that UA modulates translational signaling and supports the biosynthetic demands of terminal myotube maturation, complementing the mitochondrial adaptation we previously described in the same model. Accordingly, this study characterizes how UA

affects the abundance and phosphorylation of key mTOR-pathway components (AKT, mTOR, p70S6K, S6, and 4E-BP1) and global de novo protein synthesis, providing mechanistic insight into the role of UA during myogenic differentiation.

Materials and Methods

Cell Culture

The murine C2C12 cell line (ATCC) was used. For maintenance of C2C12 myoblasts, cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (GIBCO), 2% penicillin/streptomycin, 1% NEAA and 0.1% Amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C. When cells reached 70% confluence, the proliferation medium was replaced by a differentiation medium containing 2% horse serum (GIBCO), 2% penicillin/streptomycin, 1% NEAA, and 0.1% Amphotericin B. The differentiation medium was replaced every two days. On the third day of differentiation, cells were treated with Urolithin A (2 μM) (BOCScience, USA) for 72 h, with the treatment replaced every 24 h.

Cell viability

The cellular viability was determined using the MTT assay (Invitrogen) following the manufacturer's instructions. C2C12 myotubes were incubated on the third day of differentiation with various concentrations of Urolithin A (0, 0.2, 2, 5, 10, and 20 μM) for 72 h. At the end of the experiment, 1.2 mM MTT solution was added. After 4 hours at 37°C, blue formazan crystals were dissolved in 100 μl of 0.1% SDS-HCL solution and incubated for 18 hours in a humidified chamber. Absorbance was measured at 570 nm.

Experimental design

Based on prior viability screening, 2 μM UA was selected as a non-cytotoxic concentration. Differentiating C2C12 myotubes were assigned to two groups and treated from day 3 to day 6 of differentiation: Control (CTRL), cultured in standard differentiation medium with vehicle (0.1% DMSO); and

Urolithin A (UA), treated with 2 μM UA. Treatment was refreshed every 24 h. All experiments were performed in four independent biological replicates (n = 4).

Protein extraction

Myotubes were rinsed once with PBS and harvested in an ice-cold lysis buffer containing 20 mM Tris, pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT (1,4 dithiothreitol), and a protease inhibitor cocktail containing 1 mM EDTA (Roche Applied Science, Belgium). The homogenates were centrifuged at 12,500 rpm for 10 min at 4 °C. The supernatants were stored at −80◦C. Protein concentration was determined using the DC protein assay kit (Bio-Rad).

Western Blot Analyses

Cell lysates (18 μg) were combined with Laemmli sample buffer and warmed for 5 min at 95◦C before being loaded on gels. Samples were separated by SDS/PAGE (12%). After electrophoretic separation at 120 V for 1.5 h, the proteins were transferred to PVDF membranes at 80 V for 1.5 h for western blot analysis. Membranes were blocked for 60 min in Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% milk. Subsequently, membranes were incubated with the following antibodies overnight at 4◦C:, rabbit anti-phospho-mTOR (1:1000, 5536S; Cell Signaling, Danvers, MA, USA), rabbit anti-mTOR (1:1000, 2983S; Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-p70S6K (1:1000, 9206S; Cell Signaling, Danvers, MA, USA), rabbit anti-p70S6K (1:1000, 2708S; Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-S6 ribosomal (1:1000, 4856S; Cell Signaling, Danvers, MA, USA), rabbit anti- S6 ribosomal (1:1000, 2317S; Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-4E-BP1 (1:1000, 2855S; Cell Signaling, Danvers, MA, USA), rabbit anti-4E-BP1 (1:1000, 9644S; Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-AKT

(1:1000, 4060S; Cell Signaling, Danvers, MA, USA), rabbit anti-AKT (1:1000, 9272S; Cell Signaling, Danvers, MA, USA). The membranes were then incubated with an appropriate secondary antibody: goat anti-mouse IgG-HRP (1:10000; 31430, Invitrogen, USA) or goat anti-rabbit IgG-HRP (1:10000; 7074P2, Cell Signaling, Danvers, MA, USA). After three additional washes, chemiluminescence detection was carried out using the ECL kit (Bio-Rad). The membranes were scanned on ChemidocMP™ (Bio-Rad) and quantified with ImageJ software (NIH, Bethesda, MD, USA). Equal protein loading (18 µg per lane) was verified by Ponceau S staining, which served as the loading control for densitometric normalization.

SUrface SEnsing of Translation (SUnSET) assay

Protein synthesis was measured as previously described using the SUrface SEnsing of Translation (SUnSET) assay, which measures the incorporation of puromycin (P8833; Sigma-Aldrich, St. Louis, MO, USA) into nascent peptide chains (82), detectable by Western blot. Puromycin was added to the media of both conditions (myotubes with and without 2 µM of UA) at a final concentration of 1 µM, 60 min before cell lysis using the buffer for Western blot (83).

Statistical analysis

Statistically significant differences in viability assay were determined by Kruskal-Wallis for 3 or more independent groups followed by Dunn´s multiple comparison test. Statistically significant differences between 2 independent groups were determined with the Mann-Whitney test. Data in the text and figures are presented as the mean ± standard deviation (SD). All statistical analyses were performed with Prism 8.0 analysis software (GraphPad Software, San Diego, CA, USA). The p-values<0.05 were considered statistically significant.

Results

Urolithin A remodels mTOR pathway signaling in maturing myotubes

To elucidate the molecular networks underpinning the homeostatic adjustments between mitochondrial remodeling, as established in our previous study (Vargas-Foitzick et al., 2026), and the translational machinery, we systematically analyzed the components of the mechanistic target of rapamycin (mTOR) pathway.

To assess the effect of UA on mTOR-related signaling during late-stage C2C12 differentiation, we analyzed the abundance of key proteins within the pathway after 72 h of treatment with 2 μM UA. (Figure 1). We found a significant increase of 42% of mTOR phosphorylation (Ser2448) with UA (1.42 ± 0.17 AU, P = 0.01) as compared to the control condition (CTRL 1.00 ± 0.15 AU) (Fig. 1E). UA significantly increased total-mTOR expression by 53% (1.53 ± 0.25 AU; p=0.0143) respect to control (1.00 ±0.19 AU) (Fig 1F). Also, we found that UA increased total-p70S6K expression (1.29 ± 0.30 AU; equal to 29%; p=0.02) as compared to CTRL (1.00 ± 0.05 AU) (Fig 1I) and decreased the relation between phospho/total p70S6K (1.00 ± 0.09 AU and 0.76 ± 0.13 AU; CTRL and UA; p=0.02) (Fig 1J). Expression in protein mass of phospho-AKT increased around 32% with UA stimulus but is not significative (1.32 ± 0.22 AU; p=0.057) as compared to control condition (1.00 ± 0.21 AU) (Fig. 1B) and relation between phospho/total AKT increased around 24% without being significant when comparing UA (1.24 ± 0.22 AU; p=0.057) versus the CTRL (1.00 ± 0.15 AU) (Fig. 1D). We did not observe changes in the expression of the other proteins in the mTOR pathway, such as 4E-BP1 (Fig. 1K, 1L and 1M) and ribosomal protein S6 (rp S6) (Fig. 1N, 1O and 1P) (Data available in supplementary Table 1).

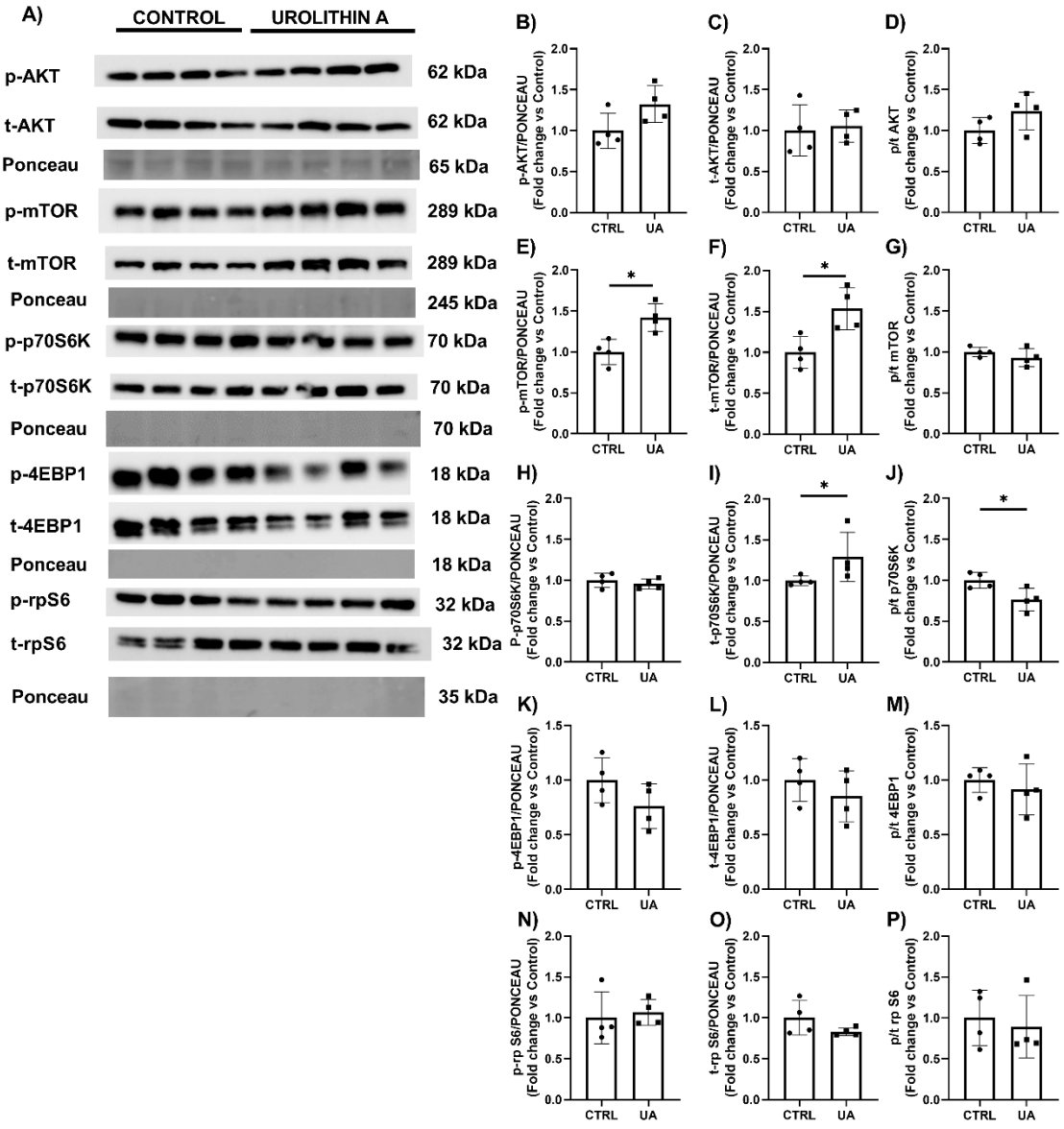


Figure 1. UA shows changes in mTOR phosphorylation state but not in other proteins in the pathway. UA exposition increased protein mass in mTOR and p70S6K in cells that were treated for 72 h. A) Different labels proteins were detected by western blot analysis, using Ponceau as the loading control. Densitometry analysis for B) phospho-AKT C) total-AKT, D) p/t-AKT, E) phospho- mTOR, F) total-mTOR, G) p/t-mTOR, H) phospho-p70S6K, I)total-p70S6K, J) p/t p70S6K, K)phospho-4EBP1, L)total-4EBP1, M) p/t-4EBP1, N)phospho-rp S6, O) total-rp S6, and P) p/t-rp S6 is represented as a fold of change expressed as the mean ± SD of four

independent experiments (Mann-Whitney analysis, *p < 0.05 with respect to the control group).

Urolithin A increases the protein synthesis in myotubes

To determine whether UA affected global translation during late-stage C2C12 differentiation, protein synthesis was evaluated by puromycin incorporation. UA treatment significantly increased the puromycin/Ponceau signal relative to control, indicating higher translational activity in UA-treated myotubes ($p < 0.05$). (Fig 2B).

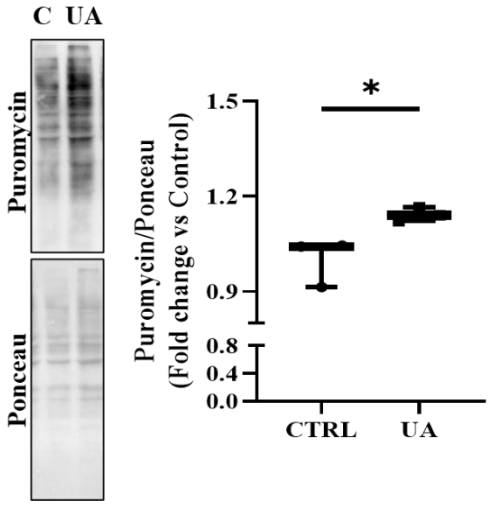


Figure 2. UA increases global protein synthesis in differentiating C2C12 myotubes. Puromycin incorporation, normalized to Ponceau staining, was higher in UA-treated cells than in CTRL ($p < 0.05$).

Discussion

Late-stage of myogenic differentiation requires a finely balanced coordination between structural assembly and metabolic adaptation. In the present study, UA modulated the mTOR signaling axis and was accompanied by an increase in basal global protein synthesis in differentiating C2C12 myotubes. This finding is consistent with the high biosynthetic demand of late-stage differentiation, a phase in which newly formed myotubes must sustain elevated translation to support structural maturation.

The canonical control of muscle anabolism is mediated by the AKT/mTORC1 pathway, where phosphorylation of mTOR and its

downstream effectors regulates translation initiation and protein synthesis (17-19). In our study, UA increased total mTOR and phospho-mTOR abundance, as well as total p70S6K protein levels. However, the phosphorylation ratio of p70S6K was reduced, suggesting that the pathway was not uniformly activated at the level of downstream catalytic output. This pattern is consistent with a selective remodeling of translational signaling rather than a generalized activation of the canonical AKT/mTOR cascade.

Importantly, the increase in puromycin incorporation indicates that these changes in pathway composition were accompanied by a functional rise in basal protein synthesis (82). This is a relevant observation because protein synthesis is one of the most energy-demanding cellular processes, and its regulation is tightly coupled to differentiation status, nutrient availability, and metabolic state (84, 85). In this context, the increase in translational activity observed with UA suggests that the compound may support the biosynthetic demands associated with late-stage differentiation.

These findings also differ from reports describing anabolic effects of other urolithins, particularly urolithin B, which has been linked to enhanced muscle mass-related signaling (53). Such differences are not unexpected, given the distinct chemical structures and bioactivities of these metabolites. In addition, many prior *in vitro* studies of UA used higher, acute doses that may not be directly comparable to the sustained physiological exposure used here. By contrast, the present study examined a prolonged 2 μM treatment during late differentiation, which may better reflect a steady-state adaptation during myotube maturation.

These findings should be considered alongside our previous work in the same differentiation window, where UA enhanced mitochondrial functional adaptation, including increased PGC-1α expression, AMPKα phosphorylation, OXPHOS complex abundance, and maximal respiratory capacity (Vargas-Foitzick et al., 2026). Together, the two studies suggest that UA acts on complementary aspects of myogenic maturation, linking metabolic remodeling with translational control (8). Rather than implying a

single linear mechanism, the data are more consistent with a supportive role for UA in the late differentiation program.

Several limitations should be noted. First, the study was performed in an *in vitro* murine C2C12 model, which does not fully reproduce the complexity of skeletal muscle *in vivo*. Second, the present data establishes association rather than causality between changes in mTOR signaling and protein synthesis. Third, the effect of UA under inflammatory or stress conditions remains to be tested directly (72). Future studies using pathway inhibition, polysome profiling, and inflammatory challenges will be useful to clarify the functional meaning of these observations.

Overall, the present findings would indicate that UA influences translational signaling during late-stage myogenic differentiation, while our previous work shows that it also promotes mitochondrial adaptation in the same model (3). Together, these data suggest that UA may support myotube maturation through coordinated regulation of metabolic and anabolic processes.

Bibliography

1. Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. Physiol Rev. 2013;93(1):23-67.

2. Pownall ME, Gustafsson MK, Emerson CP, Jr. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol. 2002;18:747-83.

3. Vargas-Foitzick R, Irribarra-Tapia D, Valero-Breton M, Balboa E. Urolithin A enhances mitochondrial biogenesis-related markers and maximal respiratory capacity during C2C12 differentiation. Frontiers in Cell and Developmental Biology. 2026;Volume 14 - 2026.

4. Sin J, Andres AM, Taylor DJ, Weston T, Hiraumi Y, Stotland A, et al. Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. Autophagy. 2016;12(2):369-80.

5. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007;450(7170):736-40.

6. Morita M, Gravel SP, Chénard V, Sikström K, Zheng L, Alain T, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. Cell Metab. 2013;18(5):698-711.

7. Fukada S-i, Ito N. Regulation of muscle hypertrophy: Involvement of the Akt-independent pathway and satellite cells in muscle hypertrophy. Experimental Cell Research. 2021;409(2):112907.

8. Andreux PA, Blanco-Bose W, Ryu D, Burdet F, Ibberson M, Aebischer P, et al. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. Nat Metab. 2019;1(6):595-603.

9. Liu S, D'Amico D, Shankland E, Bhayana S, Garcia JM, Aebischer P, et al. Effect of Urolithin A Supplementation on Muscle Endurance and Mitochondrial Health in Older Adults: A Randomized Clinical Trial. JAMA Netw Open. 2022;5(1):e2144279.

10. Luan P, D'Amico D, Andreux PA, Laurila PP, Wohlwend M, Li H, et al. Urolithin A improves muscle function by inducing mitophagy in muscular dystrophy. Sci Transl Med. 2021;13(588).

11. Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Félix AA, et al. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. Nat Med. 2016;22(8):879-88.

12. Rodriguez J, Pierre N, Naslain D, Bontemps F, Ferreira D, Priem F, et al. Urolithin B, a newly identified regulator of skeletal muscle mass. J Cachexia Sarcopenia Muscle. 2017;8(4):583-97.

13. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009;6(4):275-7.

14. Crossland H, Smith K, Atherton PJ, Wilkinson DJ. A novel puromycin decorporation method to quantify skeletal muscle protein breakdown: A proof-of-concept study. Biochem Biophys Res Commun. 2017;494(3-4):608-14.

15. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol. 2001;3(11):1009-13.

16. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science. 2005;307(5712):1098-101.

17. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960-76.

18. Buttgereit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. Biochemical Journal. 1995;312(1):163-7.

19. Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews. 1997;77(3):731-58.

20. Ono Y, Sakamoto K. Lipopolysaccharide inhibits myogenic differentiation of C2C12 myoblasts through the Toll-like receptor 4-nuclear factor-κB signaling pathway and myoblast-derived tumor necrosis factor-α. PLoS One. 2017;12(7):e0182040.

Supplementary table 1

Densitometric quantification of mTOR-pathway components in control (CTRL) and Urolithin A (UA)–treated C2C12 myotubes after 72 h of treatment (days 3–6 of differentiation).

Protein	Control (AU)	Urolithin A (AU)	p value
phospho AKT	1.00 ± 0.21	1.32 ± 0.22	0.05
total AKT	1.00 ± 0.31	1.05 ± 0.19	0.34
p/t AKT	1.00 ± 0.15	1.24 ± 0.22	0.05
phospho mTOR	1.00 ± 0.15	1.42 ± 0.17	**0.01**
total mTOR	1.00 ± 0.19	1.53 ± 0.25	**0.01**
p/t mTOR	1.00 ± 0.05	0.93 ± 0.10	0.17
phospho p70S6K	1.00 ± 0.08	0.95 ± 0.06	0.34
total p70S6k	1.00 ± 0.05	1.29 ± 0.30	**0.02**
p/t p70S6K	1.00 ± 0.09	0.76 ± 0.13	**0.02**
phospho 4EBP1	1.00 ± 0.20	076. ± 0.20	0.10
total 4EBP1	1.00 ± 0.19	0.85 ± 0.23	0.24
p/t 4EBP1	1.00 ± 0.11	0.91 ± 0.23	0.34
phospho prS6	1.00 ± 0.31	1.06 ± 0.15	0.17
total prS6	1.00 ± 0.21	0.82 ± 0.04	0.10
p/t prS7	1.00 ± 0.33	0.89 ± 0.38	0.44

Note: Values correspond to the total abundance, the phosphorylated form, and the phospho/total ratio of AKT, mTOR (Ser2448), p70S6K, ribosomal protein S6, and 4E-BP1, detected by Western blot and normalized to total protein (Ponceau S staining). Data are expressed as fold change relative to the control condition (arbitrary units, AU) and presented as the mean ± standard deviation (SD) of four independent experiments (n = 4). Differences between groups were assessed by the Mann-Whitney test; $p < 0.05$ was considered statistically significant.

CHAPTER 4 — CONCLUSIONS

4.1 Study Limitations and Future Directions.

Several limitations should be considered when interpreting the findings of this thesis. First, all experiments were conducted in the murine C2C12 cell line, an in vitro model that, although well established and reproducible, does not fully recapitulate the structural, metabolic, and systemic complexity of skeletal muscle in vivo; consequently, the present results should be regarded as a first mechanistic step, whose extrapolation to human muscle and to clinical or exercise settings will require validation in more physiological and *in vivo* models. Second, Urolithin A was administered only during the late-differentiation window (days 3-6), so its potential effects during earlier stages of myogenesis, when autophagy and mitophagy are more prominent, were not evaluated. Third, autophagic and mitophagic flux were not assessed directly; the data therefore reflect the steady-state levels of the corresponding markers rather than dynamic organelle turnover. Fourth, the analyses establish association rather than causality, as the study did not include pharmacological or genetic inhibition of the mTOR and mitochondrial pathways to confirm the mechanistic contribution of each component. Finally, the effect of Urolithin A under inflammatory or stress conditions was not tested experimentally in the present work. Future studies spanning the complete myogenic program and incorporating pathway inhibition, flux assays, inflammatory challenges, and *in vivo* models will be necessary to confirm and extend these observations.

4.2 General Conclusion.

This doctoral thesis supports the general hypothesis that Urolithin A modulates late-stage C2C12 myogenic differentiation through coordinated effects on mitochondrial adaptation and translational signaling. Across the studies included in this work, UA increased myogenic marker expression, enhanced mitochondrial functional parameters, modulated mTOR-related signaling, and increased global protein synthesis during the late phase of differentiation. These findings indicate that UA influences key cellular processes involved in myotube maturation. Rather than acting through a single isolated mechanism, UA appears to participate in the integration of anabolic and metabolic responses that accompany terminal muscle differentiation. Together, the results provide evidence that UA contributes to the regulation of myogenic remodeling in C2C12 cells under physiological experimental conditions.

4.3 Specific Conclusions.

Urolithin A at 2 μM was non-cytotoxic during late-stage C2C12 differentiation and increased the expression of myogenic differentiation markers, including MyoD and MHC.

1. Urolithin A enhanced mitochondrial functional adaptation during C2C12 differentiation, increasing AMPKα phosphorylation, PGC-1α expression, OXPHOS complex abundance, and maximal respiratory capacity.

2. Urolithin A modulated mTOR-related signaling during late-stage C2C12 differentiation, increased total mTOR, phosphorylated mTOR, and total p70S6K abundance, while AKT, S6, and 4E-BP1 remained unchanged.

3. Urolithin A treatment increased global protein synthesis, as shown by enhanced puromycin incorporation in the SUnSET assay.

4. The combined findings indicate that Urolithin A influences both metabolic remodeling and translational activity during late myotube maturation.

4.4 Scientific Contribution.

This thesis provides evidence that Urolithin A acts during a late and developmentally relevant window of C2C12 differentiation, a stage that has been less explored in previous studies.

1. The work contributes new information on the relationship between Urolithin A and translational signaling in skeletal muscle cells, particularly through its effects on the mTOR pathway and global protein synthesis.

2. The thesis integrates mitochondrial functional data with translational signaling results, offering a broader view of how Urolithin A may influence muscle cell maturation.

3. The findings support the use of C2C12 cells as a useful model for studying the effects of Urolithin A on myogenic differentiation and metabolic adaptation.

4. This work provides a foundation for future studies aimed at evaluating whether these effects are maintained or modified under pathological or inflammatory conditions.

CHAPTER 5 — BIBLIOGRAPHY

1. Yin H, Price F, Rudnicki MA. Satellite cells and the muscle stem cell niche. Physiol Rev. 2013;93(1):23-67.
2. CHARGÉ SBP, RUDNICKI MA. Cellular and Molecular Regulation of Muscle Regeneration. Physiological Reviews. 2004;84(1):209-38.
3. Wang YX, Rudnicki MA. Satellite cells, the engines of muscle repair. Nat Rev Mol Cell Biol. 2011;13(2):127-33.
4. White RB, Biérinx AS, Gnocchi VF, Zammit PS. Dynamics of muscle fibre growth during postnatal mouse development. BMC Dev Biol. 2010;10:21.
5. Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. Eur J Histochem. 2004;48(3):223-33.
6. Buckingham M, Rigby PW. Gene regulatory networks and transcriptional mechanisms that control myogenesis. Dev Cell. 2014;28(3):225-38.
7. Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. J Cell Biol. 1998;142(6):1447-59.
8. Sin J, Andres AM, Taylor DJ, Weston T, Hiraumi Y, Stotland A, et al. Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. Autophagy. 2016;12(2):369-80.
9. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem. 2010;47:69-84.
10. Scarpulla RC. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochim Biophys Acta. 2011;1813(7):1269-78.
11. Chen TH, Koh KY, Lin KM, Chou CK. Mitochondrial Dysfunction as an Underlying Cause of Skeletal Muscle Disorders. Int J Mol Sci. 2022;23(21).
12. Hood DA, Tryon LD, Carter HN, Kim Y, Chen CC. Unravelling the mechanisms regulating muscle mitochondrial biogenesis. Biochem J. 2016;473(15):2295-314.
13. Lei Y, Gan M, Qiu Y, Chen Q, Wang X, Liao T, et al. The role of mitochondrial dynamics and mitophagy in skeletal muscle atrophy: from molecular mechanisms to therapeutic insights. Cell Mol Biol Lett. 2024;29(1):59.
14. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. The Biochemical journal. 2012;441(2):523-40.
15. Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, et al. Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science. 2002;296(5566):349-52.
16. Laplante M, Sabatini David M. mTOR Signaling in Growth Control and Disease. Cell. 2012;149(2):274-93.

17. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science. 2005;307(5712):1098-101.
18. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960-76.
19. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol. 2001;3(11):1009-13.
20. Sakamoto K, Aschenbach WG, Hirshman MF, Goodyear LJ. Akt signaling in skeletal muscle: regulation by exercise and passive stretch. Am J Physiol Endocrinol Metab. 2003;285(5):E1081-8.
21. Goodman CA, Mayhew DL, Hornberger TA. Recent progress toward understanding the molecular mechanisms that regulate skeletal muscle mass. Cell Signal. 2011;23(12):1896-906.
22. Drake JC, Yan Z. Mitophagy in maintaining skeletal muscle mitochondrial proteostasis and metabolic health with ageing. J Physiol. 2017;595(20):6391-9.
23. Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A. 2007;104(29):12017-22.
24. Tomás-Barberán FA, García-Villalba R, González-Sarrías A, Selma MV, Espín JC. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. J Agric Food Chem. 2014;62(28):6535-8.
25. Lorenzo JM, Munekata PE, Putnik P, Kovačević DB, Muchenje V, Barba FJ. Chapter 6 - Sources, Chemistry, and Biological Potential of Ellagitannins and Ellagic Acid Derivatives. In: Atta ur R, editor. Studies in Natural Products Chemistry. 60: Elsevier; 2019. p. 189-221.
26. Singh A, D'Amico D, Andreux PA, Fouassier AM, Blanco-Bose W, Evans M, et al. Urolithin A improves muscle strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. Cell Rep Med. 2022;3(5):100633.
27. Singh A, D'Amico D, Andreux PA, Dunngalvin G, Kern T, Blanco-Bose W, et al. Direct supplementation with Urolithin A overcomes limitations of dietary exposure and gut microbiome variability in healthy adults to achieve consistent levels across the population. Eur J Clin Nutr. 2022;76(2):297-308.
28. Liu S, D'Amico D, Shankland E, Bhayana S, Garcia JM, Aebischer P, et al. Effect of Urolithin A Supplementation on Muscle Endurance and Mitochondrial Health in Older Adults: A Randomized Clinical Trial. JAMA Netw Open. 2022;5(1):e2144279.
29. Andreux PA, Blanco-Bose W, Ryu D, Burdet F, Ibberson M, Aebischer P, et al. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. Nat Metab. 2019;1(6):595-603.

30. Jiménez-Loygorri JI, Villarejo-Zori B, Viedma-Poyatos Á, Zapata-Muñoz J, Benítez-Fernández R, Frutos-Lisón MD, et al. Mitophagy curtails cytosolic mtDNA-dependent activation of cGAS/STING inflammation during aging. Nat Commun. 2024;15(1):830.
31. Abdelazeem KNM, Kalo MZ, Beer-Hammer S, Lang F. The gut microbiota metabolite urolithin A inhibits NF-κB activation in LPS stimulated BMDMs. Sci Rep. 2021;11(1):7117.
32. Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Félix AA, et al. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. Nat Med. 2016;22(8):879-88.
33. Luan P, D'Amico D, Andreux PA, Laurila PP, Wohlwend M, Li H, et al. Urolithin A improves muscle function by inducing mitophagy in muscular dystrophy. Sci Transl Med. 2021;13(588).
34. Tapia-Curimil G, Valero-Breton M, Deldicque L, Zbinden-Foncea H. Effects of Urolithin A on Mitochondrial Homeostasis Disruption by LPS in C2C12 Myotubes. Cell Physiol Biochem. 2026;60(3):260-9.
35. Wilhelmsen A, Karagounis LG, Bennett AJ, D'Amico D, Fouassier AM, Jones SW, et al. The polyphenol metabolite urolithin A suppresses myostatin expression and augments glucose uptake in human skeletal muscle cells. Nutr Metab (Lond). 2025;22(1):12.
36. Yaffe D, Saxel O. A Myogenic Cell Line with Altered Serum Requirements for Differentiation. Differentiation. 1977;7(1):159-66.
37. Philp A, Belew MY, Evans A, Pham D, Sivia I, Chen A, et al. The PGC-1α-related coactivator promotes mitochondrial and myogenic adaptations in C2C12 myotubes. Am J Physiol Regul Integr Comp Physiol. 2011;301(4):R864-72.
38. Pollard HJ, Willett M, Morley SJ. mTOR kinase-dependent, but raptor-independent regulation of downstream signaling is important for cell cycle exit and myogenic differentiation. Cell Cycle. 2014;13(16):2517-25.
39. Shu L, Houghton PJ. The mTORC2 complex regulates terminal differentiation of C2C12 myoblasts. Mol Cell Biol. 2009;29(17):4691-700.
40. Ogasawara R, Fujita S, Hornberger TA, Kitaoka Y, Makanae Y, Nakazato K, et al. The role of mTOR signalling in the regulation of skeletal muscle mass in a rodent model of resistance exercise. Sci Rep. 2016;6:31142.
41. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007;450(7170):736-40.
42. Morita M, Gravel SP, Chénard V, Sikström K, Zheng L, Alain T, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. Cell Metab. 2013;18(5):698-711.
43. Sebastián D, Hernández-Alvarez MI, Segalés J, Sorianello E, Muñoz JP, Sala D, et al. Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. Proceedings of the National Academy of Sciences. 2012;109(14):5523-8.

44. Tezze C, Romanello V, Desbats MA, Fadini GP, Albiero M, Favaro G, et al. Age-Associated Loss of OPA1 in Muscle Impacts Muscle Mass, Metabolic Homeostasis, Systemic Inflammation, and Epithelial Senescence. Cell Metab. 2017;25(6):1374-89.e6.
45. Noone J, O'Gorman DJ, Kenny HC. OPA1 regulation of mitochondrial dynamics in skeletal and cardiac muscle. Trends Endocrinol Metab. 2022;33(10):710-21.
46. McMillan Elliott M, Quadrilatero J. Autophagy is required and protects against apoptosis during myoblast differentiation. Biochemical Journal. 2014;462(2):267-77.
47. Hood DA, Uguccioni G, Vainshtein A, D'Souza D. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle: implications for health and disease. Compr Physiol. 2011;1(3):1119-34.
48. D'Amico D, Olmer M, Fouassier AM, Valdés P, Andreux PA, Rinsch C, et al. Urolithin A improves mitochondrial health, reduces cartilage degeneration, and alleviates pain in osteoarthritis. Aging Cell. 2022;21(8):e13662.
49. Nuñez-Sánchez MA, García-Villalba R, Monedero-Saiz T, García-Talavera NV, Gómez-Sánchez MB, Sánchez-Álvarez C, et al. Targeted metabolic profiling of pomegranate polyphenols and urolithins in plasma, urine and colon tissues from colorectal cancer patients. Molecular Nutrition & Food Research. 2014;58(6):1199-211.
50. García-Villalba R, Espín JC, Tomás-Barberán FA. Chromatographic and spectroscopic characterization of urolithins for their determination in biological samples after the intake of foods containing ellagitannins and ellagic acid. J Chromatogr A. 2016;1428:162-75.
51. Singh A, D'Amico D, Andreux PA, Fouassier AM, Blanco-Bose W, Evans M, et al. Urolithin A improves muscle strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. Cell Reports Medicine. 2022;3(5):100633.
52. Zhao H, Zhu H, Yun H, Liu J, Song G, Teng J, et al. Assessment of Urolithin A effects on muscle endurance, strength, inflammation, oxidative stress, and protein metabolism in male athletes with resistance training: an 8-week randomized, double-blind, placebo-controlled study. J Int Soc Sports Nutr. 2024;21(1):2419388.
53. Rodriguez J, Pierre N, Naslain D, Bontemps F, Ferreira D, Priem F, et al. Urolithin B, a newly identified regulator of skeletal muscle mass. J Cachexia Sarcopenia Muscle. 2017;8(4):583-97.
54. Faitg J, D'Amico D, Rinsch C, Singh A. Mitophagy Activation by Urolithin A to Target Muscle Aging. Calcif Tissue Int. 2024;114(1):53-9.
55. Tao H, Li W, Zhang W, Yang C, Zhang C, Liang X, et al. Urolithin A suppresses RANKL-induced osteoclastogenesis and postmenopausal osteoporosis by, suppresses inflammation and downstream NF-κB activated pyroptosis pathways. Pharmacol Res. 2021;174:105967.
56. Ahsan A, Zheng YR, Wu XL, Tang WD, Liu MR, Ma SJ, et al. Urolithin A-activated autophagy but not mitophagy protects against ischemic neuronal injury by inhibiting ER stress in vitro and in vivo. CNS Neurosci Ther. 2019;25(9):976-86.

57. Ghosh N, Das A, Biswas N, Gnyawali S, Singh K, Gorain M, et al. Urolithin A augments angiogenic pathways in skeletal muscle by bolstering NAD+ and SIRT1. Scientific Reports. 2020;10(1):20184.
58. Toney AM, Fan R, Xian Y, Chaidez V, Ramer-Tait AE, Chung S. Urolithin A, a Gut Metabolite, Improves Insulin Sensitivity Through Augmentation of Mitochondrial Function and Biogenesis. Obesity. 2019;27(4):612-20.
59. O'Neil TK, Duffy LR, Frey JW, Hornberger TA. The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. The Journal of Physiology. 2009;587(14):3691-701.
60. Ge Y, Wu AL, Warnes C, Liu J, Zhang C, Kawasome H, et al. mTOR regulates skeletal muscle regeneration in vivo through kinase-dependent and kinase-independent mechanisms. Am J Physiol Cell Physiol. 2009;297(6):C1434-44.
61. Song H, Yun C, Choi Y, Jeong W, Kim Y, Kim J, et al. Urolithin A activates mitophagy via the AMPK-mTOR axis and modulates the gut-ceramide axis to ameliorate cardiac remodeling in HFpEF. Exp Mol Med. 2026.
62. Zhang B, Zhang X, Treebupachatsakul W, Pantan R, Kampan N, Phatsara M, et al. Neuroprotective effect of Urolithin A via downregulating VDAC1-mediated autophagy in Alzheimer's disease. Acta Histochem. 2025;127(4):152290.
63. Remels AH, Langen RC, Schrauwen P, Schaart G, Schols AM, Gosker HR. Regulation of mitochondrial biogenesis during myogenesis. Mol Cell Endocrinol. 2010;315(1-2):113-20.
64. Snijders T, Nederveen JP, McKay BR, Joanisse S, Verdijk LB, van Loon LJ, et al. Satellite cells in human skeletal muscle plasticity. Front Physiol. 2015;6:283.
65. Waltz TB, Fivenson EM, Morevati M, Li C, Becker KG, Bohr VA, et al. Sarcopenia, Aging and Prospective Interventional Strategies. Curr Med Chem. 2018;25(40):5588-96.
66. Wang X, Feng Z, Wang X, Yang L, Han S, Cao K, et al. O-GlcNAcase deficiency suppresses skeletal myogenesis and insulin sensitivity in mice through the modulation of mitochondrial homeostasis. Diabetologia. 2016;59(6):1287-96.
67. Serova M, Didry-Barca B, Deloux R, Foucault AS, Veillet S, Lafont R, et al. BIO101 stimulates myoblast differentiation and improves muscle function in adult and old mice. J Cachexia Sarcopenia Muscle. 2024;15(1):55-66.
68. Sebastián D, Sorianello E, Segalés J, Irazoki A, Ruiz-Bonilla V, Sala D, et al. Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway. Embo j. 2016;35(15):1677-93.
69. Rochard P, Rodier A, Casas F, Cassar-Malek I, Marchal-Victorion S, Daury L, et al. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. J Biol Chem. 2000;275(4):2733-44.

70. Gonzalez-Jamett A, Vasquez W, Cifuentes-Riveros G, Martinez-Pando R, Saez JC, Cardenas AM. Oxidative Stress, Inflammation and Connexin Hemichannels in Muscular Dystrophies. Biomedicines. 2022;10(2).
71. Tang L, Mo Y, Li Y, Zhong Y, He S, Zhang Y, et al. Urolithin A alleviates myocardial ischemia/reperfusion injury via PI3K/Akt pathway. Biochem Biophys Res Commun. 2017;486(3):774-80.
72. Ono Y, Sakamoto K. Lipopolysaccharide inhibits myogenic differentiation of C2C12 myoblasts through the Toll-like receptor 4-nuclear factor-κB signaling pathway and myoblast-derived tumor necrosis factor-α. PLoS One. 2017;12(7):e0182040.
73. Castro-Sepulveda M, Tuñón-Suárez M, Rosales-Soto G, Vargas-Foitzick R, Deldicque L, Zbinden-Foncea H. Regulation of mitochondrial morphology and cristae architecture by the TLR4 pathway in human skeletal muscle. Front Cell Dev Biol. 2023;11:1212779.
74. Zhao H, Song G, Zhu H, Qian H, Pan X, Song X, et al. Pharmacological Effects of Urolithin A and Its Role in Muscle Health and Performance: Current Knowledge and Prospects. Nutrients. 2023;15(20).
75. El-Wetidy MS, Ahmad R, Rady I, Helal H, Rady MI, Vaali-Mohammed MA, et al. Urolithin A induces cell cycle arrest and apoptosis by inhibiting Bcl-2, increasing p53-p21 proteins and reactive oxygen species production in colorectal cancer cells. Cell Stress Chaperones. 2021;26(3):473-93.
76. Hinton A, Jr., Katti P, Christensen TA, Mungai M, Shao J, Zhang L, et al. A Comprehensive Approach to Sample Preparation for Electron Microscopy and the Assessment of Mitochondrial Morphology in Tissue and Cultured Cells. Adv Biol (Weinh). 2023;7(10):e2200202.
77. Maekawa S, Takada S, Furihata T, Fukushima A, Yokota T, Kinugawa S. Mitochondrial respiration of complex II is not lower than that of complex I in mouse skeletal muscle. Biochem Biophys Rep. 2020;21:100717.
78. Jones AE, Sheng L, Acevedo A, Veliova M, Shirihai OS, Stiles L, et al. Forces, fluxes, and fuels: tracking mitochondrial metabolism by integrating measurements of membrane potential, respiration, and metabolites. American Journal of Physiology-Cell Physiology. 2021;320(1):C80-C91.
79. Pownall ME, Gustafsson MK, Emerson CP, Jr. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol. 2002;18:747-83.
80. Vargas-Foitzick R, Irribarra-Tapia D, Valero-Breton M, Balboa E. Urolithin A enhances mitochondrial biogenesis-related markers and maximal respiratory capacity during C2C12 differentiation. Frontiers in Cell and Developmental Biology. 2026;Volume 14 - 2026.
81. Fukada S-i, Ito N. Regulation of muscle hypertrophy: Involvement of the Akt-independent pathway and satellite cells in muscle hypertrophy. Experimental Cell Research. 2021;409(2):112907.
82. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009;6(4):275-7.

83. Crossland H, Smith K, Atherton PJ, Wilkinson DJ. A novel puromycin decorporation method to quantify skeletal muscle protein breakdown: A proof-of-concept study. Biochem Biophys Res Commun. 2017;494(3-4):608-14.
84. Buttgereit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. Biochemical Journal. 1995;312(1):163-7.
85. Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews. 1997;77(3):731-58.

SUBMISSION CHECKLIST

The student must verify all items below before submitting the thesis draft to the committee. The draft must be submitted at least two weeks before the scheduled committee meeting.

#	Item	Done
1	Document submitted at least 2 weeks before the committee meeting	☐
2	Written entirely in English; formatted as a monograph	☐
3	Arial 12 pt; 1.5 line spacing; A4; 3 cm left margin	☐
4	Body text of appropriate length for the scope of the research (typically 80–150 pages)	☐
5	Abstract: structured format (Background / Aim / Methods / Results / Conclusion / Keywords), maximum 300 words	☐
6	Hypothesis formally stated and falsifiable	☐
7	Specific objectives numbered, ordered logically, and matched to results in Chapter 5	☐
8	Literature review: minimum 20 pages, minimum 50 references	☐
9	Methods: positive and negative controls defined and justified for every assay	☐
10	Methods: blinding procedure stated for all imaging-based analyses; if absent, declared as a limitation	☐
11	Methods: dose or intervention selection justified with reference to published data	☐
12	Results: viability or tolerability data presented for all treatment conditions	☐
13	Discussion: limitations section present with a comprehensive and critical discussion of study limitations	☐
14	Discussion: integrative interpretation and novelty statement included	☐
15	References: minimum 100, Vancouver format, numbered sequentially by order of first citation	☐

16	Appendix E: at least one manuscript derived from the thesis has been submitted to or published in a peer-reviewed indexed journal (required for private defence)	☐
17	All figures: minimum 300 dpi, numbered per chapter, self-explanatory captions	☐
18	All tables: numbered per chapter, complete titles, units specified in all column headers	☐
19	List of abbreviations: complete and alphabetical	☐