

Faculté des sciences de la motricité

Effect of Urolithin A and low frequency Electrical Pulse Stimulation on mitochondrial function in C2C12 myotubes

Auteur : Benjamin Francotte

Promoteurs : Louise Deldicque et Hermann Zbinden-Foncea

Année académique 2023-2024

Master en sciences de la motricité, orientation éducation physique

[120.0] - EDPH2M - Finalité didactique

Je tiens à exprimer ma profonde gratitude et mes sincères remerciements à tous les acteurs ayant joué un rôle dans la réalisation de ce mémoire. Pour ce faire, j'ai rédigé ces quelques mots, dans leur langue respective.

Mille mercis à ma promotrice, Louise Deldicque, pour son aide précieuse dans la rédaction, ses conseils avisés et sa sympathie qui m'a accompagné tout au long de ce travail.

Agradezco profundamente a mi co-tutor, Hermann Zbinden-Foncea, por haberme dado la oportunidad de sumergirme en su laboratorio, por su guía invaluable en esta tesis y durante toda mi experiencia de intercambio en Santiago de Chile.

Agradezco a mi compañero de laboratorio, Ronald Vargas-Foitzick, por enseñarme mi primer Western Blot.

Agradezco a mi compañero de laboratorio, Juan Gutiérrez, por las risas que compartimos y su paciencia en mis aciertos y mis fracasos.

Agradezco a mi compañera de laboratorio, Anita Figueroa Toledo, por su forma de ser y sus preciosos alientos.

Agradezco a mis compañeros de laboratorio, Alfonso, Cristóbal, Mario y German por la buena onda y por siempre mostrarse disponible.

Une pensée particulière à mes deux parents, Fabrice Francotte et Valérie Laroy, pour leur soutien inconditionnel et leur bienveillance tout au long de mes études, de mes projets et de mes aventures.

TABLE OF CONTENTS

ACKNOWLEDGMENT	III
TABLE OF CONTENTS	V
LIST OF ABBREVIATIONS	VI
INTRODUCTION	1
MECHANISM	4
PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA COACTIVATOR 1 ALPHA:	4
AMP-ACTIVATED PROTEIN KINASE ALPHA:	5
UNC-51 LIKE AUTOPHAGY ACTIVATING KINASE 1:	6
MAMMALIAN TARGET OF RAPAMYCIN:	7
TRANSLOCASE OF OUTER MITOCHONDRIAL MEMBRANE 20:	8
ACETYL-CoA CARBOXYLASE:	8
MITOGEN-ACTIVATED PROTEIN KINASE 11:	9
OXIDATIVE PHOSPHORYLATION SYSTEM COMPLEXES:	10
FORMULATION OF A RESEARCH QUESTION AND HYPOTHESIS	12
RESEARCH QUESTION	12
HYPOTHESIS	12
METHODS	15
CELL CULTURE	15
ELECTRIC PULSE STIMULATION	17
UROLITHIN A INCUBATION	17
WESTERN BLOTTING	18
I.1.1 Proteins extraction	18
I.1.2 Proteins concentration assay	18
I.1.3 Running	20
I.1.4 Transfer	21
I.1.5 Blotting	24
STATISTICS	26
RESULTS	28
DISCUSSION	34
MITOCHONDRIAL BIOGENESIS	34
ENERGY REGULATION AND MITOCHONDRIAL QUALITY	34
FATTY ACID SYNTHESIS	34
MITOPHAGY AND PROTEIN SYNTHESIS	35
INFLAMMATION	35
ATP PRODUCTION CAPACITY	35
LIMITS OF THE EXPERIMENTATION	36
CONCLUSION	39
DECLARATION OF INTEREST	39
ANNEXES AND SUPPLEMENTARY FILES	40
REFERENCES	42

LIST OF ABBREVIATIONS

ACC	Acetyl Coenzyme A Carboxylase
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK α	AMP-Activated Protein Kinase α
AP-1	Activator Protein 1
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
C2C12	An Immortalized Mouse Myoblast Cell Line
CPT-1	Carnitine Palmitoyltransferase 1
CRP	C-Reactive Protein
CTRL	Control
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ETC	Electron Transport Chain
EPS	Electrical Pulse Stimulation
ERR	Estrogen-Related Receptor
FUNDC1	FUN14 Domain-Containing 1
G10	Gel With A 10% Acrylamide Concentration
G12	Gel With A 12% Acrylamide Concentration
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
LC3	Microtubule-Associated Protein Light Chain 3
MTOR	Mechanistic Target Of Rapamycin
NRF	Nuclear Respiratory Factor
NF- κ B	Nuclear Factor Kappa B
OXPPOS	Oxidative Phosphorylation Complexes
PARKIN	Parkin Protein
P-ACC	Acetyl Coenzyme A Carboxylase Phosphorylated
P-AMPK α	AMP-Activated Protein Kinase α Phosphorylated
P-MTOR	Mechanistic Target Of Rapamycin Phosphorylated
P-P38	P38 Mitogen-Activated Protein Kinases Phosphorylated
P-ULK	Unc-51-Like Autophagy-Activating Kinases 1 Phosphorylated
P38	P38 Mitogen-Activated Protein Kinases
PGC1 α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 α
ROS	Reactive Oxygen Species
TOM20	Translocase Of Outer Mitochondrial Membrane 20
TBST	Tris-Buffered Saline + Tween 20
UA	Urolithin A
ULK1	Unc-51-Like Autophagy-Activating Kinases 1

INTRODUCTION

In the pursuit of longevity and overall health, the role of mitochondria, the energy-producing powerhouses within our cells, has garnered increasing attention. The intricate relationship between mitochondrial function and age-related decline in skeletal muscle health has been the subject of recent studies, shedding light on potential strategies to combat those degenerative processes (Andreux et al., 2018; Leduc-Gaudet, Hussain, Barreiro, & Gouspillou, 2021; Liu et al., 2022). But why do mitochondria matter, and what can we expect from interventions like urolithin A (UA) supplementation, as well as exercise-induced physiological stress in promoting mitochondrial health and muscle function?

Mitochondria, often referred to as the cellular power station play a crucial role in energy production, apoptosis, and reactive oxygen species (ROS) regulation. Aging is associated with a decline in mitochondrial function, and this decline contributes to a reduced ability for physical activity (Leduc-Gaudet et al., 2021). In fact, impaired mitochondrial function has been linked to age-related muscle health decline, emphasizing the need for strategies that enhance mitochondrial health (Andreux et al., 2018; Leduc-Gaudet et al., 2021). Then, mitophagy, a process by which damaged mitochondria are selectively removed and replaced with healthy ones, represents a promising approach to rejuvenating muscle health in older adults (Leduc-Gaudet et al., 2021). It is important to note that mitochondria are widely distributed in muscle cells, underscoring the connection between muscle health and mitochondrial status (Singh et al., 2022).

Emerging research has spotlighted UA, a natural compound found in pomegranates and available in supplement form, as a potential tool to stimulate mitophagy and improve muscle function (Liu et al., 2022; Ryu et al., 2016). In fact, UA is a metabolite derived from ellagic acid, a polyphenol found in these fruits. Regarding the assimilation mechanism, when humans consume these fruits, the ellagitannins are broken down by gut bacteria into urolithin metabolites. Once formed, urolithin is absorbed through the intestinal lining and enters the bloodstream, where it can exert its effects on various tissues and organs in the body.

Indeed, UA supplementation has shown promising results in recent studies, demonstrating its safety, tolerability, and the ability to increase circulating UA levels in humans (Singh et al., 2022). Additionally, UA has been linked to an increase in mitophagy in human skeletal muscle, offering a potential mechanism through which it benefits muscle health (Singh et al., 2022). High doses of UA have also been associated with improved exercise performance, including enhanced muscle endurance, which may be attributed to its effects on mitochondrial function (Liu et al., 2022).

Furthermore, UA supplementation has been observed to reduce inflammatory markers, such as C-reactive protein (CRP), indicating its potential anti-inflammatory properties (Y. Zhang et al., 2021). These findings suggest that UA may not only contribute to muscle health but also to overall well-being by mitigating chronic inflammation. Such dietary interventions may offer a practical means of preserving mitochondrial health, potentially delaying the age-related decline in muscle mass and strength (Andreux et al., 2018).

On the other hand, exercise-induced physiological stress has been explored as another strategy to promote mitophagy and mitochondrial health. Electric pulse stimulation (EPS) of myotubes serves as an *in vitro* exercise model because studies have shown that it closely mimics the effects of actual exercise on cells, making it a reliable research tool (Evers-van Gogh et al., 2015). Research has shown that EPS in the form of C2C12 myotube contraction, which are cultured muscle cells derived from mouse skeletal muscle tissue, may induce mitophagy and activate the AMP-activated protein kinase - unc-51-like autophagy-activating kinases 1 (AMPK-ULK1) pathway, thereby promoting the expression of FUN14 domain-containing 1 (FUNDC1), a key regulator of mitophagy (Gao, Yu, Wang, Wang, & Liu, 2020). Therefore, the combination of UA supplementation and EPS may hold particular promise in enhancing muscle health and mitochondrial function.

Regarding existing literature, recent studies on UA have shown that it can have effects on mitochondrial function in animals (Ryu et al., 2016) and also in humans (Liu et al., 2022; Vinciguerra, 2022). Indeed, UA can induce an increase in AMPK α phosphorylation as well as upregulate genes involved in lipolysis, beta oxidation, and mitophagy. EPS is a recognized technique for inducing human-like physiological responses to exercise in C2C12 myotubes (Nikolić et al., 2017; Son et al., 2019).

Both EPS and UA are already individually supported by studies: according to Ryu et al. (2016), a concentration of 50 μ M UA for a duration of 24 h was sufficient to induce a significant augmentation of AMPK α phosphorylation in C2C12 myotubes. Evers-van Gogh, (2015) detected a clear augmentation of the P-AMPK α /AMPK α ratio after 24 h of EPS in C2C12 myotubes.

In summary, both EPS and UA, which individually have proven effects on AMPK α *in vitro* in C2C12 myotubes, have never been the subject of a coupled study. The aim of this work is therefore to assess the effects of UA coupled to low-frequency EPS on mitochondrial function in C2C12 myotubes. In addition to AMPK α , we also evaluated other protein expression as PGC1 α as a mitobiogenesis marker, ULK1 as a mitophagy marker, OXPHOS complexes as energy (ATP) production capacity markers, ACC as a fatty acid synthesis marker, mTOR as a protein synthesis marker, TOM20 as a mitochondrial quality and biogenesis marker, P38 as an inflammation marker, and GAPDH as a loading protein.

The choice of each marker will be presented in the next section. By investigating the interplay between these factors, this study aimed to shed light on the potential synergy between UA and EPS in enhancing mitochondrial function and its implications for muscle health.

While our assessment was conducted *in vitro*, the overarching goal was to lay the groundwork for future investigations into these interactions in human subjects. By exploring these dynamics in cell cultures, we aimed to pave the way for potential clinical studies that delve deeper into the synergistic effects and therapeutic applications of combining UA consumption with resistance exercise in human populations.

MECHANISM

In this section, we delve into the physiological mechanisms of the various proteins and markers we studied. The markers under investigation play pivotal roles in mitochondrial function and muscle health, and understanding their expected mechanisms in the context of UA and EPS is crucial.

Peroxisome proliferator-activated receptor-gamma coactivator 1 Alpha:

PGC1 α holds a critical position in regulating **mitochondrial biogenesis**, thereby exerting a profound impact on cellular energy metabolism and homeostasis (Scarpulla, 2011; Vander, Raff, Widmaier, & K., 2013). Acting as a master regulator, PGC1 α governs the transcriptional activation of genes essential for mitochondrial replication, structure, and function (Handschin & Spiegelman, 2006).

Upon activation, PGC1 α initiates a sophisticated cascade of events culminating in the generation of new mitochondria and an increase in cellular energy production capacity (Lin, Handschin, & Spiegelman, 2005). This process involves interactions with various transcription factors and coactivators, including nuclear respiratory factors (NRFs), estrogen-related receptors (ERRs), and peroxisome proliferator-activated receptor gamma (PPAR γ) (Lin et al., 2005). Through these interactions, PGC1 α promotes the expression of genes encoding mitochondrial proteins crucial for respiratory chain complexes, mitochondrial DNA replication and maintenance, and oxidative phosphorylation machinery (Scarpulla, 2011).

Additionally, PGC1 α plays a key role in coordinating mitochondrial dynamics, fusion-fission processes, and quality control mechanisms to maintain a healthy mitochondrial network (Cantó & Auwerx, 2009). By enhancing mitochondrial quantity and function, PGC1 α significantly contributes to cellular energy metabolism, facilitating efficient ATP production to meet cellular energy demands.

In summary, PGC1 α emerges as a central regulator in mitochondrial biogenesis and cellular energy metabolism, orchestrating a complex network of transcriptional events to enhance mitochondrial quantity, function, and adaptability in response to metabolic demands and environmental challenges

AMP-activated protein kinase alpha:

AMPK α emerges as a pivotal kinase with a central role in orchestrating **cellular energy balance and metabolism** (Hardie, 2011; Hardie & Lin, 2017; Vander et al., 2013). Sensitivity to variations in the AMP/ATP (adenosine monophosphate/adenosine triphosphate) and ADP/ATP ratio renders AMPK α a key sensor of the cell's energy status. Upon activation, AMPK α initiates a cascade of signaling events aimed at restoring energy homeostasis and promoting cellular adaptation to metabolic stressors.

Activation of AMPK α occurs in response to energy stress or increased energy demand, leading to a rise in intracellular AMP levels relative to ATP. This activation involves phosphorylation of its catalytic subunit, triggering downstream signaling pathways. Once activated, AMPK α exerts profound effects on cellular metabolism by promoting glucose uptake, enhancing fatty acid oxidation, and stimulating mitochondrial biogenesis (Mihaylova & Shaw, 2011). Additionally, AMPK α plays a critical role in regulating autophagy and mitophagy, processes essential for maintaining cellular homeostasis and eliminating damaged organelles.

One mechanism through which AMPK α influences mitophagy involves the inhibition of mTOR, a key regulator of autophagy. AMPK α phosphorylates mTOR subunits, leading to its deactivation and subsequent promotion of autophagy, including mitophagy. Moreover, AMPK α directly activates ULK1, an essential initiator of autophagy, including mitophagy. Activation of ULK1 by AMPK α facilitates the formation of phagophores and the encapsulation of damaged mitochondria into autophagosomes (Egan et al., 2011).

Furthermore, research indicates that AMPK α selectively regulates mitophagy by modulating the phosphorylation status of key mitophagy regulatory proteins, including Parkin and FUNDC1. For example, a study by Toyama et al. (2016) demonstrated that AMPK α activation enhances Parkin activity by phosphorylating Parkin at specific residues, thereby promoting the ubiquitination and subsequent degradation of damaged mitochondria. Additionally, Egan et al (2011) showed that AMPK α -mediated phosphorylation of FUNDC1 facilitates its interaction with LC3, a critical step in the engulfment of damaged mitochondria by autophagosomes.

In summary, AMPK α serves as a central orchestrator of cellular energy metabolism and homeostasis, regulating a plethora of metabolic processes, including glucose uptake, fatty acid oxidation, and mitochondrial biogenesis. Its role in autophagy and mitophagy underscores its significance in maintaining cellular health and adaptability in response to metabolic challenges. By modulating key signaling pathways and regulatory proteins, AMPK α emerges as a critical mediator of cellular adaptation to changing metabolic demands and stress conditions.

Unc-51 like autophagy activating kinase 1:

ULK1 emerges as a central player in the intricate machinery governing the **initiation of autophagy**, including the selective process of mitophagy (Iorio, Celenza, & Petricca, 2021; Vander et al., 2013). As a key regulator of autophagy initiation, ULK1 phosphorylation serves as a critical switch for triggering the formation of autophagosomes and initiating the engulfment of cytoplasmic cargo, including damaged mitochondria.

Upon phosphorylation, ULK1 exerts pleiotropic effects on the autophagic machinery, facilitating the recruitment of autophagy membranes and coordinating the assembly of the autophagosomal structure (Iorio et al., 2021). This process is essential for sequestering cargo, including dysfunctional mitochondria, within autophagosomes for subsequent degradation and recycling.

Furthermore, ULK1 plays a pivotal role in modulating the activity of other proteins involved in the regulation of mitophagy, thereby adjusting the mitophagy process to meet the metabolic demands and stress conditions of the cell (Egan et al., 2011). Through its interactions with various regulatory factors and downstream effectors, ULK1 ensures the efficient and selective targeting of damaged mitochondria for degradation, thus contributing to the maintenance of mitochondrial quality and cellular energy balance.

In summary, ULK1 stands as a central orchestrator of the initiation of autophagy, including the selective removal of damaged mitochondria through mitophagy. Its phosphorylation-mediated activation triggers a cascade of events leading to the formation of autophagosomal structures and the sequestration of cargo, ultimately culminating in the degradation of dysfunctional mitochondria. By regulating the mitophagic process,

ULK1 plays a crucial role in preserving mitochondrial health and cellular homeostasis, highlighting its significance as a key determinant of cellular adaptation and survival in response to metabolic stressors.

Mammalian target of rapamycin:

mTOR occupies a fundamental position in cellular signaling, exerting extensive control over various cellular processes such as **protein synthesis**, cell growth, metabolism, and autophagy (Saxton & Sabatini, 2017; Vander et al., 2013). Acting as a central regulator of protein synthesis, mTOR governs the intricate balance between anabolic and catabolic pathways, dictating cellular growth and proliferation in response to environmental signals and nutrient availability.

In addition to its pivotal role in protein synthesis and cell growth, mTOR plays a crucial role in regulating autophagy (Kim, Kundu, Viollet, & Guan, 2011). However, the role of mTOR in autophagy is nuanced, as it can act as both a promoter and inhibitor, depending on its activation status.

When mTOR is active, typically phosphorylated, it exerts a suppressive effect on autophagy, including selective forms like mitophagy, which targets damaged mitochondria for degradation and recycling (Saxton & Sabatini, 2017). This inhibition of autophagy by mTOR limits the cell's ability to undergo self-cleaning processes, impairing mitochondrial quality control and leading to the accumulation of dysfunctional mitochondria within the cell. Conversely, inhibition of mTOR unleashes autophagy, facilitating the removal of damaged organelles and protein aggregates, including dysfunctional mitochondria (Kim et al., 2011).

In summary, mTOR emerges as a central hub in cellular signaling, orchestrating a delicate balance between promoting growth and maintaining cellular homeostasis. Its multifaceted roles in protein synthesis, autophagy regulation, and signaling pathways underscore its importance as a key determinant of cellular function, with profound implications for human health.

Translocase of outer mitochondrial membrane 20:

TOM20 plays a central role in the complex process of **importing proteins into mitochondria**, acting as a receptor on the outer mitochondrial membrane to recognize specific targeting signals on precursor proteins (Balsera, Soll, & Bölter, 2009; Vander et al., 2013). This recognition initiates the transport of precursor proteins from the cytosol into the mitochondrial matrix, a crucial step in ensuring the proper functioning of mitochondria in energy production, metabolism, and cellular signaling.

The regulation of TOM20 levels serves as an indicator of mitochondrial health and import efficiency, with increased TOM20 abundance signaling enhanced import of essential proteins into mitochondria (Balsera et al., 2009). This augmentation in TOM20-mediated protein import supports mitochondrial biogenesis and contributes to overall mitochondrial quality maintenance. Moreover, TOM20's role extends beyond protein import, as it is involved in coordinating mitochondrial dynamics and signaling pathways.

Through interactions with other mitochondrial proteins and regulatory factors, TOM20 contributes to maintaining mitochondrial integrity, regulating fusion-fission dynamics, and modulating cellular responses to stress (Suzuki et al., 2022).

Acetyl-CoA carboxylase:

ACC, emerges as a crucial enzyme in the regulation of **fatty acid synthesis**, exerting profound control over cellular lipid metabolism and energy homeostasis (Vander et al., 2013; Wang et al., 2022). As a key regulator of fatty acid biosynthesis, ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, a critical precursor in the de novo synthesis of fatty acids.

However, the activity of ACC is tightly regulated through phosphorylation-mediated mechanisms, which exert allosteric control over its enzymatic activity (Wang et al., 2022). Upon phosphorylation, ACC undergoes conformational changes that inhibit its enzymatic function, thereby reducing the production of malonyl-CoA.

The reduction in malonyl-CoA levels has significant implications for cellular lipid metabolism, particularly in the context of energy production. Malonyl-CoA serves as a potent inhibitor of carnitine palmitoyltransferase I (CPT-I), a key enzyme involved in the

transport of fatty acids into the mitochondria for β -oxidation (Saggerson, 2008). Therefore, the inhibition of ACC by phosphorylation relieves the suppression of CPT-I activity, promoting the entry of fatty acids into the mitochondria for oxidation and subsequent energy production.

This regulatory mechanism ensures the coordination between fatty acid synthesis and degradation, allowing the cell to adapt its lipid metabolism according to its energy requirements (Wang et al., 2022). Under conditions of increased energy demand, such as during prolonged exercise or fasting, phosphorylation-mediated inhibition of ACC promotes fatty acid oxidation, thereby facilitating ATP generation to meet the cell's metabolic needs (Wang et al., 2022).

Conversely, under conditions of ample energy supply, dephosphorylation of ACC allows for the resumption of fatty acid synthesis, ensuring the replenishment of cellular lipid stores and the maintenance of lipid homeostasis (Kastaniotis et al., 2017). This dynamic regulation of ACC activity underscores its pivotal role in governing the balance between fatty acid synthesis and oxidation, thereby contributing to cellular energy metabolism and overall metabolic health.

Mitogen-Activated Protein Kinase 11:

P38, a mitogen-activated protein kinase (MAPK), is a signaling molecule implicated in a myriad of cellular processes, prominently including **inflammation** and **cellular stress responses** (Cuadrado & Nebreda, 2010; Vander et al., 2013). This protein kinase, characterized by its serine/threonine phosphorylation activity, plays a central role in transducing extracellular stimuli into intracellular responses, thereby modulating diverse cellular functions.

Phosphorylation of P38 serves as a critical regulatory mechanism governing its activity and downstream signaling cascades. Upon activation by upstream kinases in response to various extracellular signals such as pro-inflammatory cytokines, oxidative stress, or environmental stressors, P38 becomes phosphorylated at specific threonine and tyrosine residues within its activation loop (Cuadrado & Nebreda, 2010). This phosphorylation event induces a conformational change in P38, leading to its activation and subsequent engagement with downstream effector molecules.

One of the primary functions of phosphorylated P38 is its involvement in the regulation of inflammatory responses. Activated P38 kinase can phosphorylate a plethora of downstream substrates, including transcription factors such as NF- κ B and AP-1, as well as other regulatory proteins involved in cytokine production, chemotaxis, and immune cell activation (Ono & Han, 2000). By modulating the activity of these signaling molecules, phosphorylated P38 orchestrates the expression of pro-inflammatory genes and mediates the inflammatory response, playing a pivotal role in both acute and chronic inflammatory processes.

In addition to its involvement in inflammation, phosphorylated P38 also participates in cellular stress responses and cell survival pathways. Under conditions of cellular stress, such as exposure to ultraviolet radiation, heat shock, or osmotic stress, P38 activation facilitates the induction of stress-responsive genes involved in cellular adaptation and survival (Cuadrado & Nebreda, 2010). By phosphorylating downstream targets involved in stress signaling pathways, phosphorylated P38 promotes cellular resilience and adaptation to adverse environmental conditions, thereby contributing to cell survival and homeostasis maintenance.

Oxidative phosphorylation system complexes:

The OXPHOS complexes represent multi-protein assemblies located within the inner mitochondrial membrane, serving as the primary site for **ATP production** through electron transport chain (ETC) activity (Sousa, D'Imprima, & Vonck, 2018; Vander et al., 2013). Comprising five distinct protein complexes—Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc1 complex), Complex IV (cytochrome c oxidase), and Complex V (ATP synthase)—OXPHOS complexes orchestrate a series of redox reactions and proton translocations to generate ATP, the universal energy currency of the cell.

Complex I, or NADH dehydrogenase, catalyzes the transfer of electrons from NADH to ubiquinone (Coenzyme Q), initiating the flow of electrons through the ETC (Hirst, 2013). This electron transfer generates a proton gradient across the inner mitochondrial membrane, driving the synthesis of ATP by Complex V, ATP synthase.

Complex II, or succinate dehydrogenase, participates in both the citric acid cycle and the ETC, oxidizing succinate to fumarate while transferring electrons to ubiquinone (Sousa et al., 2018). The electrons are subsequently passed to Complex III via ubiquinone, where they are transported to cytochrome c, another mobile electron carrier.

Complex III, or cytochrome bc₁ complex, facilitates the transfer of electrons from ubiquinol to cytochrome c, while also contributing to the proton gradient across the inner mitochondrial membrane (Sousa et al., 2018). This proton pumping activity further contributes to the electrochemical gradient utilized by Complex V for ATP synthesis.

Complex IV, or cytochrome c oxidase, represents the terminal oxidase complex responsible for catalyzing the reduction of molecular oxygen to water, utilizing electrons from cytochrome c (Sousa et al., 2018). This final step in the electron transport chain is coupled with the translocation of protons across the inner mitochondrial membrane, contributing to the proton gradient driving ATP synthesis.

Complex V, or ATP synthase, harnesses the energy stored in the proton gradient to catalyze the synthesis of ATP from ADP and inorganic phosphate (Sousa et al., 2018). This process, known as chemiosmotic coupling, involves the rotary movement of the ATP synthase rotor driven by the flow of protons through the enzyme complex, ultimately leading to ATP production.

In summary, the OXPHOS complexes represent a highly coordinated system of protein assemblies responsible for ATP production through oxidative phosphorylation within the mitochondria. By exploiting the energy derived from electron transfer and proton translocation, these complexes play a central role in cellular energy metabolism, enabling the efficient generation of ATP to meet the dynamic energy demands of the cell.

Transitioning from the exploration of the mechanisms underlying cellular energy metabolism and mitochondrial function, we will now turn our attention to formulating research questions and hypotheses based on the insights gleaned from the literature in the next section.

Formulation of a Research Question and Hypothesis

The present study aims to investigate the effects UA supplementation and EPS on key regulators of cellular energy metabolism and mitochondrial function. Specifically, we will explore the influence of these interventions on the expression and activity of pivotal proteins involved in mitochondrial biogenesis, mitophagy, metabolism, inflammation, and energy regulation.

Research Question

How do UA supplementation and EPS impact the expression and activity of key regulators of cellular energy metabolism and mitochondrial function?

Hypothesis

Drawing upon the relevant literature, our overarching hypothesis posits that UA supplementation and EPS would each individually positively influence the expression and activity of several key proteins involved in cellular energy metabolism and mitochondrial function, as demonstrated in previous studies. Furthermore, we hypothesize that there may be an interaction between UA and EPS, leading to an even greater enhancement of mitochondrial function.

Our first hypothesis suggests that UA supplementation and EPS may enhance the expression of PGC1 α , a master regulator of mitochondrial biogenesis, as indicated by recent literature (Liu et al., 2022; Nikolić et al., 2017; Ryu et al., 2016; Vinciguerra, 2022). Therefore, we anticipated observing an increase in PGC1 α expression following UA supplementation and EPS, leading to improved mitochondrial function and, consequently, enhanced muscle performance.

Furthermore, based on the documented association between UA and AMPK α phosphorylation (Liu et al., 2022; Ryu et al., 2016) and between EPS and AMPK α (Evers-van Gogh et al., 2015; Hu et al., 2018; Nikolić et al., 2017; Son et al., 2019; Tarum, Folkesson, Atherton, & Kadi, 2017), we hypothesized that UA supplementation and EPS would activate AMPK α . This activation was expected to improve energy regulation, glucose metabolism, and mitochondrial quality control, ultimately enhancing mitochondrial function.

Moreover, given the reported potential of UA and EPS to stimulate mitophagy (Gao et al., 2020; Leduc-Gaudet et al., 2021; Liu et al., 2022; Nikolić & Aas, 2019), we hypothesized that UA supplementation and EPS would positively influence ULK1 phosphorylation. This effect was anticipated to enhance mitophagy, thereby improving mitochondrial health and overall function.

Additionally, inspired by findings demonstrating the inhibitory effect of UA on mTOR activity (M. Zhang et al., 2023), we hypothesized that UA supplementation and EPS would similarly inhibit mTOR activity. This inhibition was expected to promote autophagy and mitophagy, contributing to enhanced mitochondrial quality control and function.

Furthermore, we hypothesized that UA supplementation and EPS would positively influence TOM20 expression, indicative of improved mitochondrial protein import and function.

Additionally, considering the known influence of UA on lipid metabolism (Liu et al., 2022; Nikolić & Aas, 2019), we hypothesized that UA supplementation and EPS would lead to decreased ACC phosphorylation as observed by Liu et al., (2022), enhancing fatty acid oxidation and mitochondrial function.

Furthermore, based on evidence of UA's anti-inflammatory effects (Rodriguez, Caille, Ferreira, & Francaux, 2017; Singh et al., 2022; M. Zhang et al., 2022), we hypothesized that UA supplementation would inhibit P38 phosphorylation, while EPS would induce it, aligning with their respective effects on inflammation. On the contrary, EPS inducing physiological stress, is susceptible to increase inflammation (Lambernd et al., 2012). Thus, we expected to see a preventing effect of UA on EPS stress-related inflammation.

Lastly, building on literature regarding UA's influence on mitochondrial function and energy production (Esselun, Theyssen, & Eckert, 2021; Li & Graham, 2012), we hypothesized that UA and EPS would positively impact the quality and quantity of OXPHOS complexes, potentially due to a better turnover of mitochondria. This improvement should increase the ATP production capacity within the mitochondria, thus potentially improving muscle performance.

Conclusively, the elucidation of cellular mechanisms and formulation of hypotheses serve as solid foundations essential for shaping subsequent experimental pursuits. Transitioning to the methods section, these foundational insights seamlessly guide the selection of methodological approaches, ensuring the alignment of theoretical understanding with practical experimentation. This cohesive integration of theoretical knowledge and experimental design is paramount for conducting rigorous scientific investigations and advancing our understanding of complex biological processes.

METHODS

In this section, we will provide a detailed explanation of the methodology employed in our work. This methodological overview is essential for understanding how our research was conducted and the steps taken to gather and analyze the data. We will delve into the experimental procedures of cell culture, EPS protocol, UA incubation and the data collection techniques such as western blotting. By elucidating the methodology, we aim to offer transparency and insight into the robustness of our research approach.

Cell Culture

Mouse skeletal muscle C2C12 cells were maintained in 2mL of growth medium containing : Dulbecco's modified Eagle medium (DMEM) with 4.5g/L glucose (Gibco/Life Technologies, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco/Life Technologies, Canada), 1% of nonessential amino acids (NEAA) (Gibco/Life Technologies, USA), 100µg/mL penicillin and 100µg/mL streptomycin (Gibco/Life Technologies, USA), 0.1% amphotericin B (Gibco/Life Technologies, USA) and the growth medium was changed every 2 days.

Differentiation was induced by switching from growth medium to 2mL of differentiation medium containing : DMEM with 4.5g/L glucose supplemented with 2% horse serum (HS) (Gibco/Life Technologies, USA), 1% of NEAA, 100µg/mL penicillin and 100µg/mL streptomycin, 0,1% of Amphotericin B and the differentiation medium was changed every 2 days.

Differentiation was started when cells reached ~70–80% confluence (at day 0) (Fig.1). All the experiments were performed in triplicates in six-well plates.

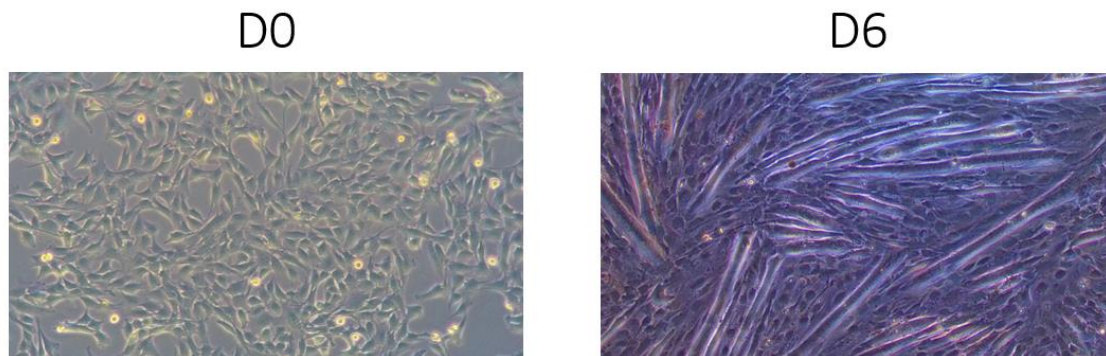


Figure 1: Phase contrast microphotography of C2C12 cells under culture; representative photos from day 0 and day 6 of differentiation; myotubes formation can be seen starting from day 6; magnification 100X;

With regard to the initial number of cells, we start a first proliferation in a 100mm well putting 500 000 cells (previously counted). After 24h proliferation, we moved the cells from the 100mm well to the 30mm six-well plates distributing them equitably (Fig. 2).

To process the manipulation, we followed the outlined protocol as follows:

Firstly, we aspirated the culture medium from the C2C12 culture plate. Following this, the cells were gently rinsed twice with phosphate-buffered saline (PBS) (Gibco/Life Technologies, USA) to ensure the removal of any residual medium components.

Subsequently, we added 1mL of trypsin solution to cover the myotubes completely, and then incubated the culture plate containing trypsin solution at 37°C in a CO₂ incubator for 4 minutes. Throughout the incubation period, we monitored the cells under a microscope to observe detachment progress. Detached myotubes displayed a rounded or loosely attached appearance compared to their adherent state. Once the myotubes had detached, we gently tapped the culture plate to ensure the release of all cells. Following this, we quenched trypsin activity by adding 1mL of serum-containing growth medium. The medium contained serum to neutralize trypsin activity and prevent cell damage.

Using a pipette, we transferred the cell suspension into a sterile Falcon tube. The tube was then centrifuged at 2000rpm for 5min at ambient temperature. Carefully, we removed the supernatant without disturbing the cell pellet. To the tube, we added 12mL of medium and homogenized the cell suspension.

Finally, we distributed 1mL of the cell suspension into each well of the two 30mm six-well plates.



Figure 2: schematic representation of the different experimental conditions;

Immediately before to start the electric pulse stimulation (EPS), differentiation medium was changed to 3mL of conditioned differentiation medium containing whether 50µM

dimethyl sulfoxide (DMSO) (Winkler Ltda, Santiago, Chile) in half of the wells (control, CTRL) or 50 μ M UA (BOC Sciences, NY, USA) in the other half.

During the whole process, cells were kept in a CO₂ incubator (3111 Forma Incubator 6.5-cu ft | 184L, Thermo Scientific, USA), maintaining the followed atmospheric conditions : 37°C; 5% CO₂; 20% O₂.

Electric pulse stimulation

EPS experiments were performed according to Evers-van Gogh (2015).

Myotubes were differentiated until day 6, after which the EPS protocol with the conditions of 1Hz frequency, 2ms pulse duration and 11.5V intensity, was applied using a C-dish with carbon electrodes combined with a pulse generator for 24h (C-Pace 100, IonOptix, Milton, MA, USA).

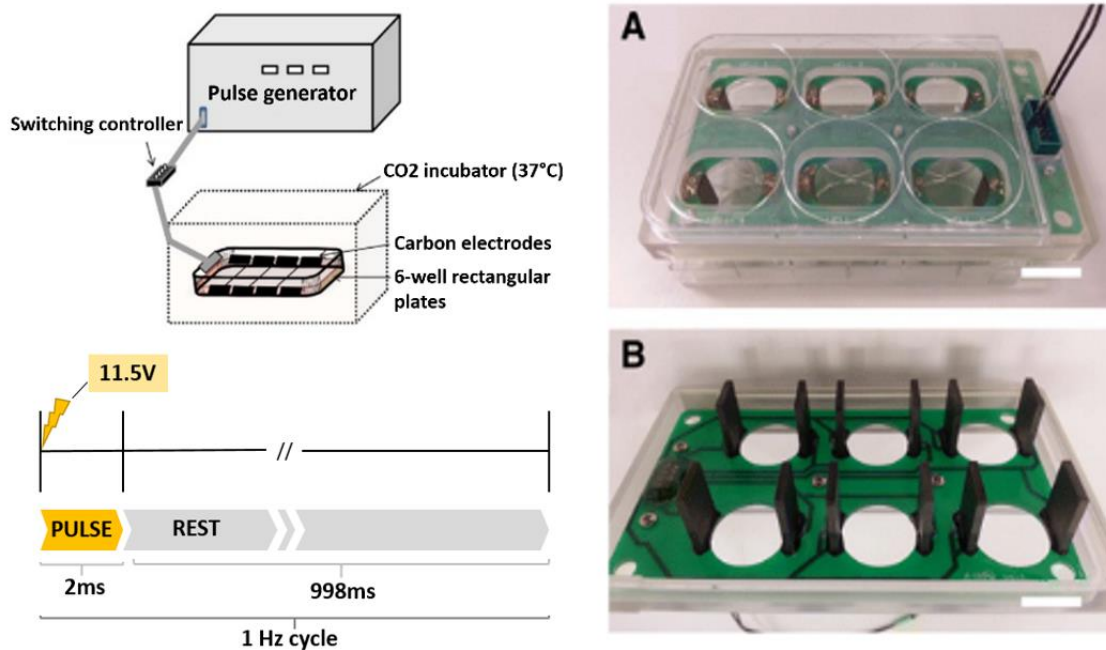


Figure 3: schematic representation of the EPS protocol; **(A)** top view of the carbon electrodes; **(B)** bottom view of the carbon electrodes. Adapted from Evers-van Gogh (2015);

Urolithin A incubation

According to Ryu and al. (2016), a concentration of 50 μ M of UA (Mitopure, Amazentis SA, Ecublens, Switzerland) is enough to increase significantly the phosphorylation of AMPK α in C2C12 cells. Incubation with UA started at the same time as the EPS and

lasted for 24 hours too. The vehicle of UA was DMSO which was added to the control cells as well. UA reserve in lab was guarded at a concentration of 20mg/mL in DMSO at 4°C.

Western blotting

1.1.1 Proteins extraction

To obtain total protein extracts from differentiated myotubes, cells were washed two times with 1mL ice-cold PBS before adding 35µL cold lysis buffer (25mM Tris HCL pH = 7.9, 5mM MgCl₂, 10% glycerol, 100mM KCl; 1% NP40; 0.3mM dithiothreitol, 5mM sodium pyrophosphate, 1mM sodium orthovanadate, 50mM sodium fluoride, containing freshly added protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany)).

Cells were scraped and collected in a microtube. Then homogenized at 3400rpm for 1s with a vortex mixer (Labnet Vortexer 59, Denville Scientific Inc.) and centrifuged at 12.500rpm for 10min at 4°C (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). Supernatants were immediately stored at -80°C.

1.1.2 Proteins concentration assay

Cells lysate proteins concentrations were assayed using the BCA Assay Kit™ (Pierce/ThermoFisher). The BCA Protein Assay integrates the widely recognized process of reducing Cu²⁺ to Cu¹⁺ by proteins in an alkaline environment with the highly sensitive and specific colorimetric detection of Cu¹⁺ (cuprous cation) using bicinchoninic acid (BCA). In the initial stage, copper interacts with proteins in an alkaline setting to generate a light blue complex through chelation. This chemical reaction is commonly referred to as the biuret reaction, where peptides containing three or more amino acid residues form a colorful chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate.

In the second phase of color development, BCA interacts with the reduced cuprous cations produced in the first step. The vivid purple-hued reaction product arises from the chelation of one cuprous ion with two BCA molecules. The BCA-copper complex is soluble in water and exhibits a robust linear absorbance at or near 562 nm, correlating with increasing protein concentrations. Significantly, this complex offers a detection

sensitivity approximately 100 times greater (lower limit of detection) than the initial pale blue color observed in the first reaction.

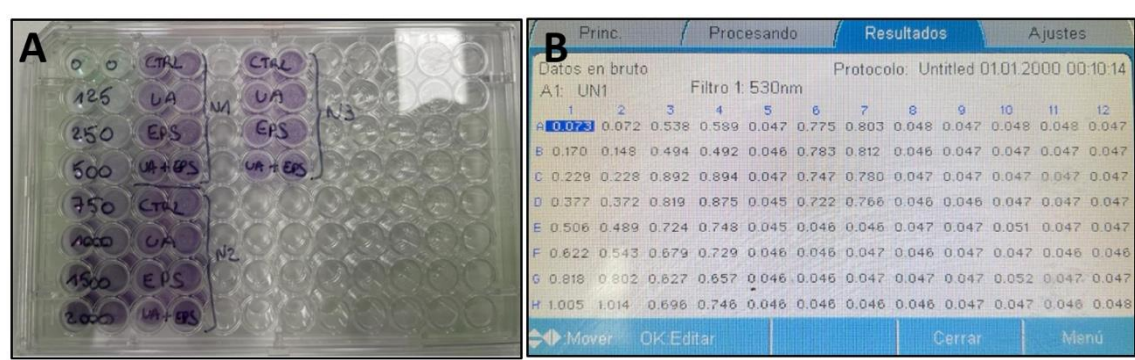


Figure 4: BCA Assay (A) samples wells; (B) spectrophotometer absorbance results;

Concretely, as can be seen in the Figure 4A, we pipetted an albumin standard curve with eight stages of known protein concentration of 0 to 2000µg/L in the reactive solution. This reactive solution was obtained by mixing reagent A and reagent B at a ratio of 1:20. Then we pipetted the experimental samples with a dilution factor of 2 in the reactive solution.

After thorough mixing, the plate was covered and incubated at 37°C for 30 minutes, then cooled to room temperature. Subsequently, we measured absorbance using a spectrophotometer (Multiskan FC, Pierce/Termofisher) at a wavelength of 530nm (Fig. 4B).

Finally, we use the absorbance results to establish a standard curve relating absorbance to protein concentration, which we then used to assay the protein concentration of the experimental samples (Table 1&2).

The BCA Assay protocol was performed as can be seen in Figure 4bis.

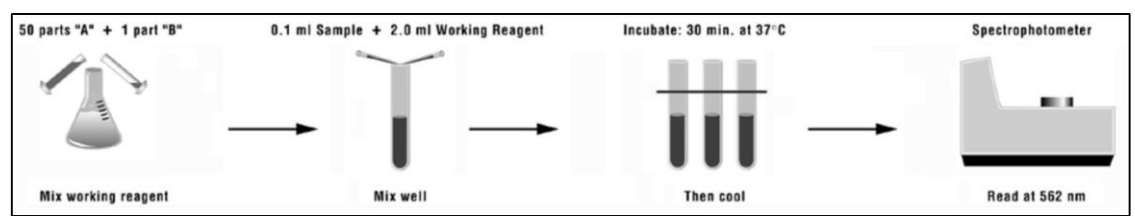
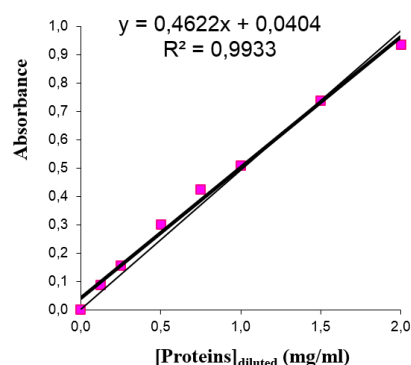


Figure 4bis: BCA Assay protocol, originally published in Smith et al. (1985);

All manipulation were processed twice to reduce human manipulation variability.

Table 1 Absorbance and standard curve establishment.

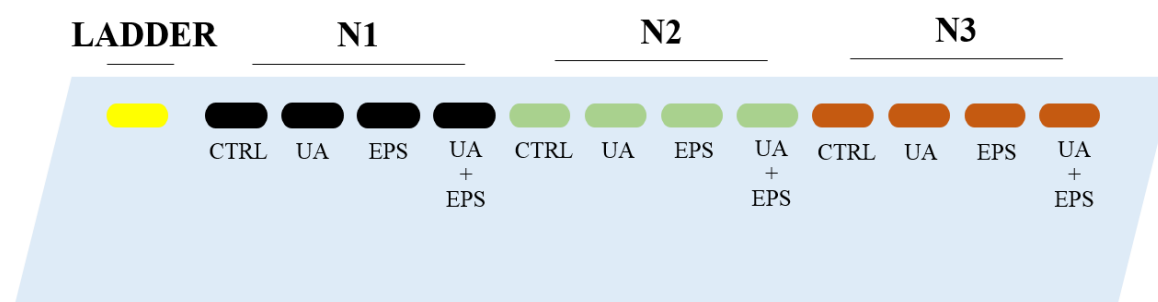
[Albumin] (mg/mL)	Abs. 1	Abs. 2	Abs. Mean	Abs - blank
0	0,073	0,072	0,073	0,000
0,125	0,170	0,148	0,159	0,087
0,250	0,229	0,228	0,229	0,156
0,500	0,377	0,372	0,375	0,302
0,750	0,506	0,489	0,498	0,425
1,000	0,622	0,543	0,583	0,510
1,500	0,818	0,802	0,810	0,738
2	1,005	1,014	1,010	0,937

**Table 2** Protein concentration assay based on samples absorbance.

Sample	Dilution Factor	Abs. 1	Abs. 2	Abs. Mean	Abs - blank	[Protein] _{diluted} (mg/mL)	[Protein] _{real} (mg/mL)
n1 CTRL	2	0,538	0,589	0,564	0,491	0,975	1,950
n1 UA	2	0,494	0,492	0,493	0,421	0,822	1,645
n1 EPS	2	0,892	0,894	0,893	0,821	1,688	3,376
n1 UA+EPS	2	0,819	0,875	0,847	0,775	1,588	3,177
n2 CTRL	2	0,724	0,748	0,736	0,664	1,348	2,696
n2 UA	2	0,679	0,729	0,704	0,632	1,279	2,558
n2 EPS	2	0,627	0,657	0,642	0,570	1,145	2,289
n2 UA+EPS	2	0,696	0,746	0,721	0,649	1,316	2,631
n3 CTRL	2	0,775	0,803	0,789	0,717	1,463	2,926
n3 UA	2	0,783	0,812	0,798	0,725	1,481	2,962
n3 EPS	2	0,747	0,780	0,764	0,691	1,408	2,815
n3 UA+EPS	2	0,722	0,766	0,744	0,672	1,365	2,731

1.1.3 Running

Protein cell lysates were separated by an electrophoretic chamber by loading them into the gels following the pattern shown in figure 5.

**Figure 5:** schematic representation of the protein cell lysates pattern repartition across the gels;

Two polyacrylamide gels were used (recipes in Tables 3). One with a 12% (G12) acrylamide concentration (measuring PGC1 α , P-ULK, ULK, OXPHOS) (Fig. 6A) and the other one with a 10% (G10) acrylamide concentration (measuring P-mTOR, mTOR, P-ACC, ACC, P-AMPK α , AMPK α , GAPDH, P-P38, P38 (Fig. 6B). 20 μ g of proteins were charged for each condition.

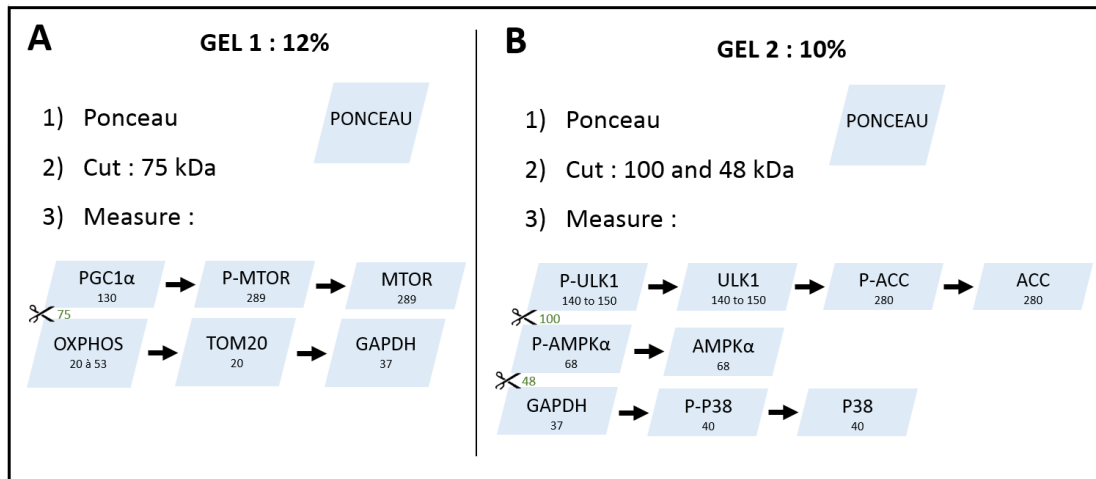


Figure 6: schematic representation of the PVDF membrane cutting planning of **(A)** 12% acrylamide gel; **(B)** 10% acrylamide gel;

Gels were dived into a running buffer (recipe in Table 4) with a voltage of 100V. Then, after the samples passed through the stacking gel (Fig. 7A), the voltage rose to 120V during 1 hour, until the samples have run through the entire gel (Fig. 7B).

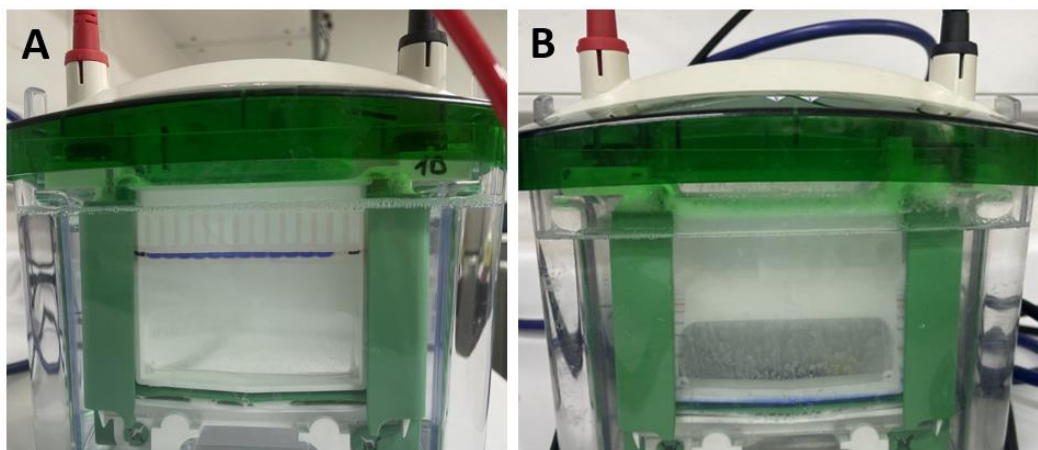


Figure 7: electrophoretic chamber during running protocol of **(A)** the beginning; **(B)** the end;

1.1.4 Transfer

Protein cell lysates were transferred to polyvinylidene difluoride membrane (Immun-Blot® PVDF, Bio-Rad Laboratories, Hercules, California, USA) This process involved

assembling a transfer sandwich with the gel, membrane, and buffer solution (recipe in Table 4). The sandwich was then placed in a transfer apparatus where an electric current facilitated protein migration from the gel to the membrane.

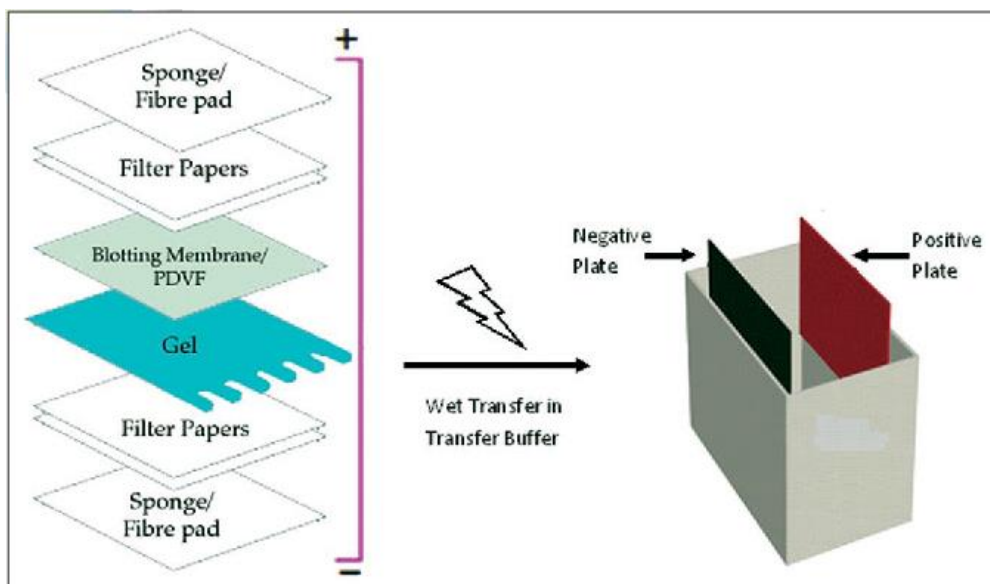


Figure 8: Assembly of a sandwich in western blot originally published in Mahmood & Yang (2012) ;

The electrophoretic transfer was conducted at 100V for a duration of 90min while maintaining the system on ice and in the refrigerator to prevent overheating.

After transfer completion, the membrane was washed to remove buffer residues. Next, the transferred proteins were immobilized on the membrane and blocked to prevent non-specific binding.

Additionally, the membrane was stained with Ponceau to verify the successful execution of the Western blot and to quantify the total protein amount (Fig. 9).

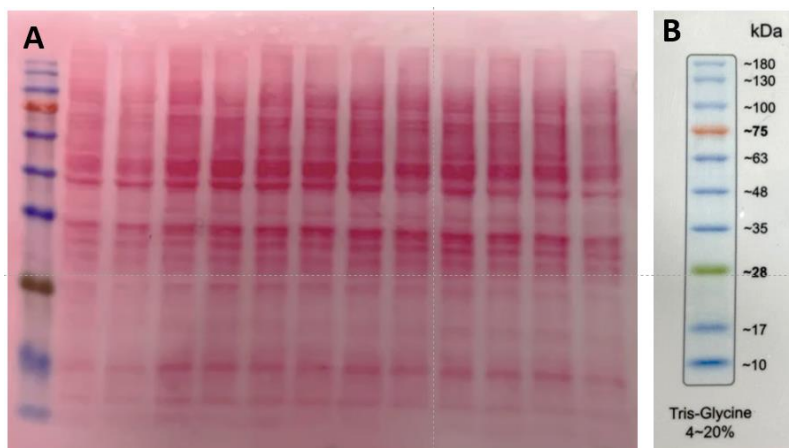


Figure 9: (A) Total proteins on polyvinylidene difluoride membrane stained by PONCEAU and (B) the molecular weight ladder;

After washing the membrane to remove Ponceau residues, it was subsequently then incubated with anti-AMPK α (#2603), anti-phospho-AMPK α (#2535), anti-GAPDH (#97166), anti-PGC1 α (#2178), anti-ACC (#3676), anti-phospho-ACC (#3661), anti-mTOR (#2983), anti-phospho-mTOR (#5536), anti-P38 (#9212), anti-phospho-P38 (#9215), anti-ULK1 (#8054) and anti-phospho-ULK1(#14202) anti-TOM20 (#42406) (all from Cell Signaling Technology, Denvers, MA, USA) and anti-OXPHOS (ab110411) (Abcam, Cambridge, UK) (Table 5). Finally followed by secondary antibodies conjugated with detection molecules.

All the primary antibodies were diluted 1:1000 in 5% skimmed milk in a mixture of tris-buffered saline and tween 20 (TBST), and secondary antibodies were diluted at 1:10000 in 5% skimmed milk in TBST.

Table 3 Recipes for stacking and resolving gels.

	Stacking Gel		Resolving Gel	
	5%		10%	12%
diH₂O	1028		2010	1650
Acrylamide 30%	250		1666	2000
Tris 1.5M, pH 6.8	192		-	-
Tris 1.5M, pH 8.8	-		1250	1250
SDS 10%	15		50	50
APS 10%	15		50	50
TEMED	2		5	5
Total volume (μL)	1500		5000	5000

Table 4 Recipes for transfer and running buffers.

	Transfer Buffer		Running Buffer	
	10x	1x	5x	1x
Tris base	58 g	-	15.1 g	-
Glycine	29 g	-	93.8 g	-
SDS	0.37 g	-	5 g	-
Total volume	1L	-	1L	-
diH₂O	-	0.7L	-	0.8L
Methanol	-	0.2L	-	-
10x/5x Buffer	-	0.1L	-	0.2L
Total volume	-	1L	-	1L

1.1.5 Blotting

Blots were revealed by chemiluminescence (ChemiDOC™ MP, Bio-Rad Laboratories).

Quantification was carried out using Image Lab 6.1. (Tables S6&S7).

Every protein expression was normalized dividing the target phosphorylation status by the total expression of the protein (both phosphorylated and non phosphorylated forms) (P-ULK1/ULK1; P-AMPK α /AMPK α ; P-mTOR/mTOR; P-ACC/ACC; P-P38/P38) or the total form of the target protein by the expression of a loading protein (GADPH) (PGC1 α /GADPH; TOM20/GADPH) or by the total expression of all kinds of proteins (PONCEAU) as it was the case for OXPHOS complexes.

Why using PONCEAU instead of GAPDH :

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme involved in glycolysis, a metabolic process that takes place in the cytoplasm of cells (Vander et al., 2013). It catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate using the cofactor nicotinamide adenine dinucleotide (NAD⁺) as an electron acceptor. Due to its central role in glycolysis, GAPDH is commonly used as a "housekeeping gene or loading protein" in molecular biology studies to normalize gene expression or protein abundance, because its level of expression is generally considered relatively stable in many cells and conditions.

However, GAPDH is mainly found in the cytoplasm, and its role is linked to the metabolic processes that occur in this part of the cell. Mitochondrial proteins, on the other hand, are located in mitochondria, the organelles responsible for ATP production and many metabolic processes related to energy production.

The problem with using GAPDH as a housekeeping gene or loading protein to normalize mitochondrial gene or protein expression is that GAPDH is not a mitochondrial protein and does not necessarily reflect changes in mitochondrial metabolism. The amount of GAPDH in the mitochondria is generally very low, or even negligible, compared to its amount in the cytoplasm.

It is therefore important to use reference proteins adapted to the subcellular region whose expression or abundance you are studying. For mitochondrial proteins, it would be more appropriate to use reference proteins that are specifically localized in mitochondria as

TOM20 or HSP90 (Geng et al., 2010; Ryu et al., 2016). In our case, we finally decided to use Ponceau as the total expression of all kinds of proteins not only including cytoplasm protein. Although we are aware that mitochondrial proteins represent a small percentage of total proteins, this was the most relevant way of normalization that we had available due to the unsatisfactory results following the normalization by TOM20. This issue will be detailed in the results and discussion section.

Then we divided all data by the CTRL mean with the aim of obtaining a mean value equal to 1 for all CTRL conditions. Beside, it is important to note that each culture condition was processed in triplicate. We finally mixed these three copies in the same tube to be further evaluated together by western blotting. Next, we repeated the whole process 3 times (n=3).

Table 5 List of antibodies.

Antibody	Supplier	Reference#	Molecular Weight (kDa)
PGC1α	Cell Signaling	2178	130
ULK1	Cell Signaling	8054	140-150
P-ULK1 (Ser757)	Cell Signaling	14202	140-150
AMPKα	Cell Signaling	2603	68
P-AMPKα (Thr172)	Cell Signaling	2535	68
MTOR	Cell Signaling	2983	289
P-MTOR (Ser2448)	Cell Signaling	5536	289
ACC	Cell Signaling	3676	280
P-ACC (Ser79)	Cell Signaling	3661	280
P38	Cell Signaling	9212	40
P-P38 (Thr180/Tyr182)	Cell Signaling	9215	43
TOM20	Cell Signaling	42406	20
GAPDH	Cell Signaling	97166	37
OXPHOS	Abcam	ab110411	20-56

STATISTICS

In this section, we provide a comprehensive overview of the statistical methodologies utilized to analyze the data obtained in our study. The application of rigorous statistical techniques is essential for deriving meaningful insights and ensuring the reliability of our findings. Through a detailed explanation of our statistical approach we aim to enhance the comprehensibility and validity of our study's conclusions.

The statistical protocol for performing blot quantification was carried out using Image Lab 6.1 involved several steps to ensure accurate and reliable results. Firstly, high-quality images of the Western Blot were captured using a compatible imaging system, such as a chemiluminescent scanner (Bio-Rad Laboratories), ensuring proper exposure settings to avoid saturation. These images were then imported into the Image Lab 6.1 software for analysis, organized into appropriate folders, and labeled for easy reference.

Next, the bands of interest on the Western Blot image were selected using the software's tools, ensuring precise selection to include only the target bands while excluding any background noise or artifacts. The software's quantification tools were then utilized to measure the intensity of the selected bands, determining the pixel density of each band, which correlated with the protein expression level (Fig. 10).

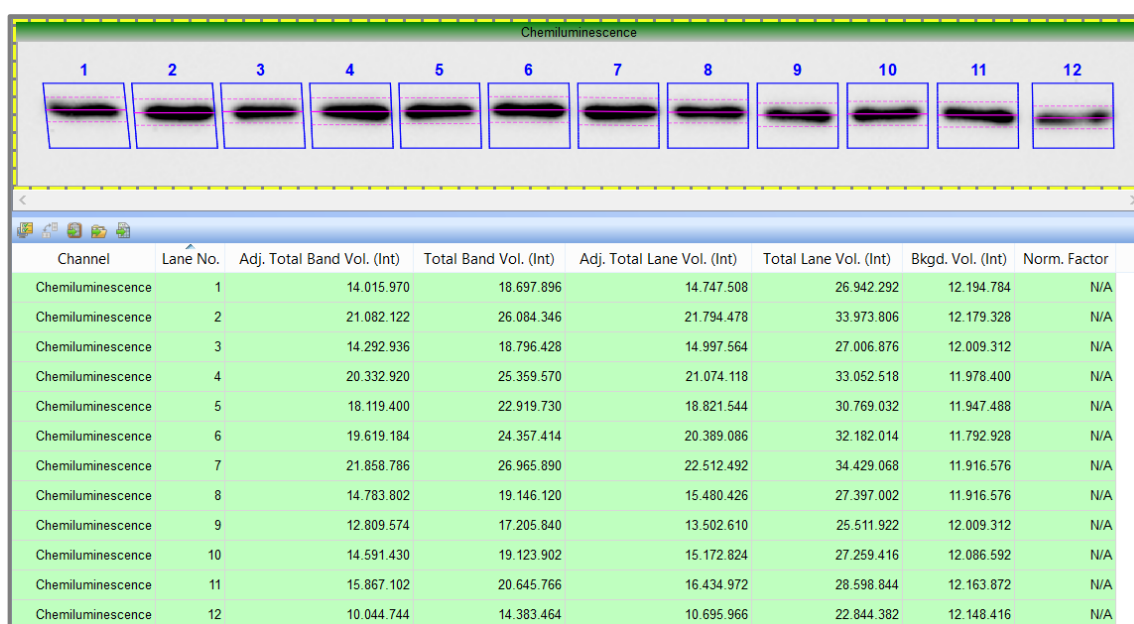


Figure 10: Blots quantification of GAPDH using Image Lab 6.1;

Following quantification, the data were normalized to account for variations in sample loading or protein transfer efficiency. Normalization methods included normalization to total protein loading or to a loading protein. Once normalized, statistical analysis was performed on the quantified data using two-way ANOVA, to assess differences between experimental groups.

All data were considered not normally distributed due to the sample size (n=3). Analyzes were done using GraphPad Prism 8.0.2 (Fig. 11).

Table format: Grouped		Group A			Group B		
		-EPS			+EPS		
		A:1	A:2	A:3	B:1	B:2	B:3
1	-UA	0.84708492	0.87307722	1.27983785	0.99440779	0.93111019	1.00508470
2	+UA	0.90207437	0.73640562	0.93406176	0.75051730	0.91696998	0.88262505

Figure 11: Two-way ANOVA performed with GraphPad Prism 8.0.2;

Two-way ANOVA was used to evaluate potential effects of the different conditions (with and without UA; with and without EPS) on the targeted protein expressions. Not normally distributed variables were log-transformed for analysis to bring the distribution of data closer to normality. Then, variables were back-transformed for presentation to maintain a visual understanding of the data, as demonstrated by Magkos and al. (2016).

Finally, the statistical results such as means, standard deviations, and p-values were presented in graphical representations to illustrate the findings.

Data are expressed as the mean +/- SEM. The significance level was set at $p < 0.05$.

RESULTS

This section provides a comprehensive overview of the results obtained from our investigation into the effects of UA supplementation and EPS on mitochondrial function in C2C12 myotubes. Through meticulous experimental design and rigorous data analysis, we aimed to elucidate the specific impact of these interventions on cellular functions, mitochondrial dynamics, and protein expression profiles.

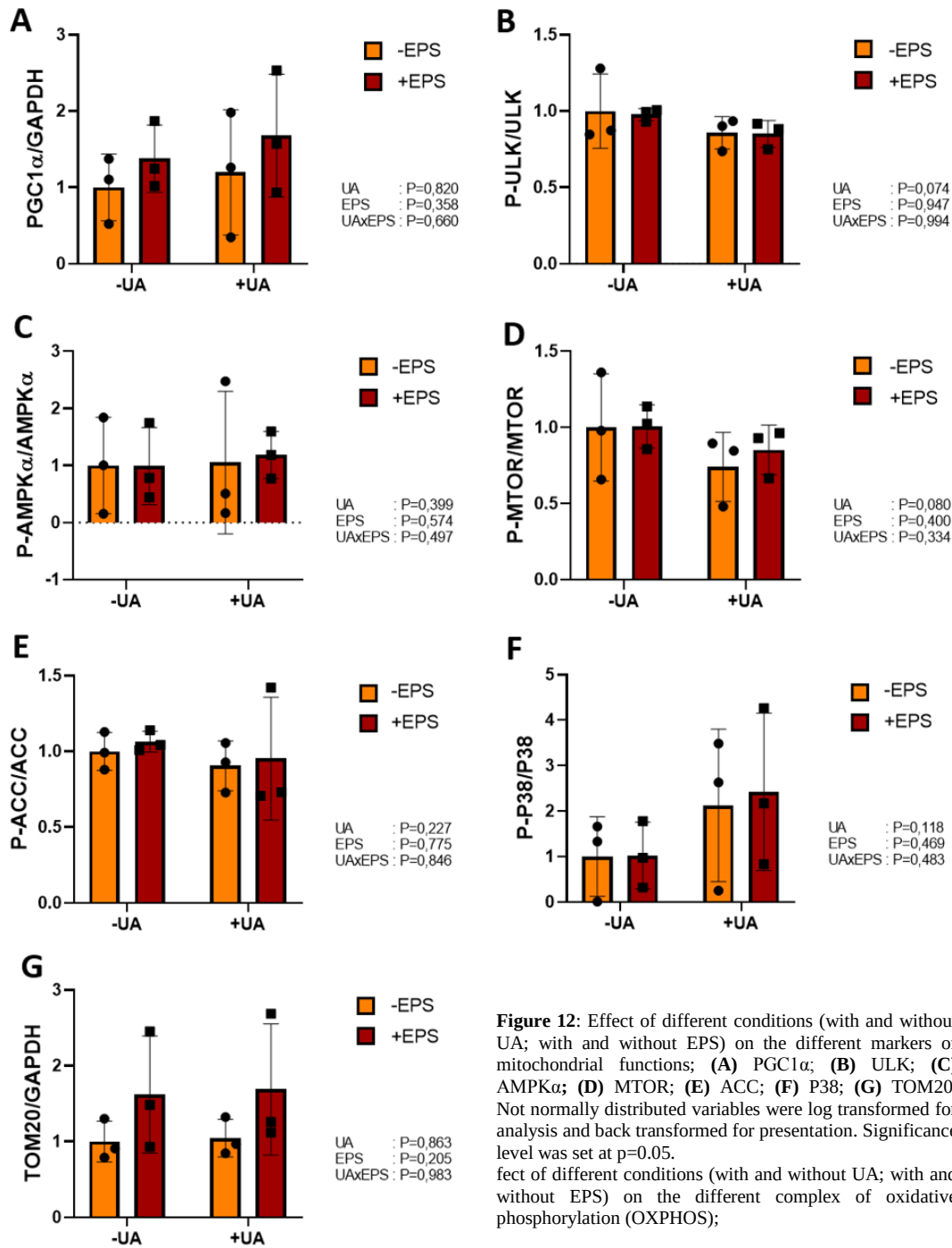


Figure 12: Effect of different conditions (with and without UA; with and without EPS) on the different markers of mitochondrial functions; (A) PGC1 α ; (B) ULK; (C) AMPK α ; (D) MTOR; (E) ACC; (F) P38; (G) TOM20. Not normally distributed variables were log transformed for analysis and back transformed for presentation. Significance level was set at p=0.05.

fect of different conditions (with and without UA; with and without EPS) on the different complex of oxidative phosphorylation (OXPHOS);

As shown in Figure 12, PGC1 α expression was identical across all conditions (Fig. 12A), as was the case for the phosphorylation status of AMPK α and the protein expression of TOM20 (Fig. 12C & 12G). ACC phosphorylation was also observed identical across all conditions (Fig. 12E).

ULK1 and mTOR phosphorylation slightly tended to be decreased with UA treatment (respectively, $P=0.074$ and $P=0.080$) (Fig. 12B & 12D). Otherwise, P-38 phosphorylation slightly tended to be increased with UA treatment ($P=0.118$) (Fig. 12F).

Analyzed blots are shown in Figure 13 and 14.

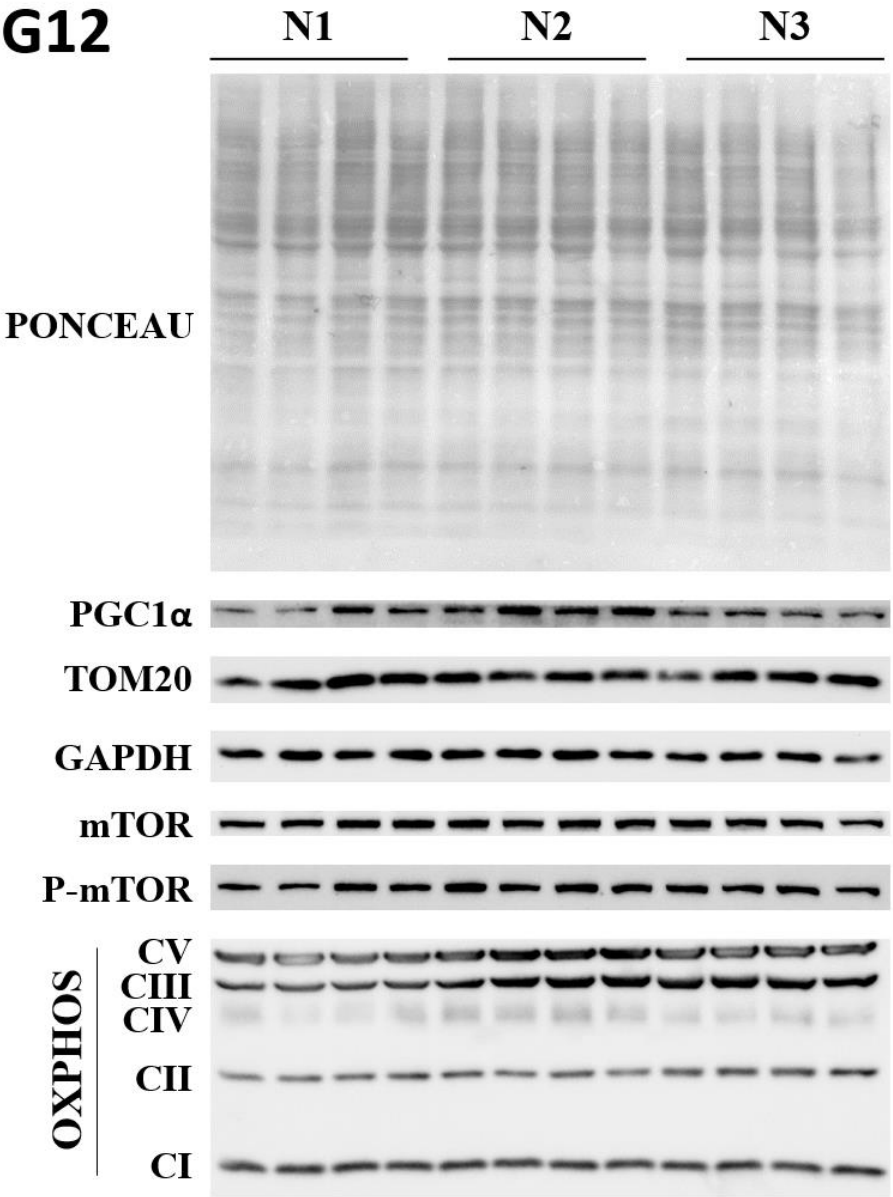


Figure 13: Blots from G12 including PONCEAU; PGC1 α ; TOM20; GAPDH; mTOR; P-MTOR; OXPHOS complexes and (B) G10 including PONCEAU; ULK1; P-ULK1; GAPDH; ACC; P-ACC; AMPK α ; P-AMPK α ; P38; P-P38.

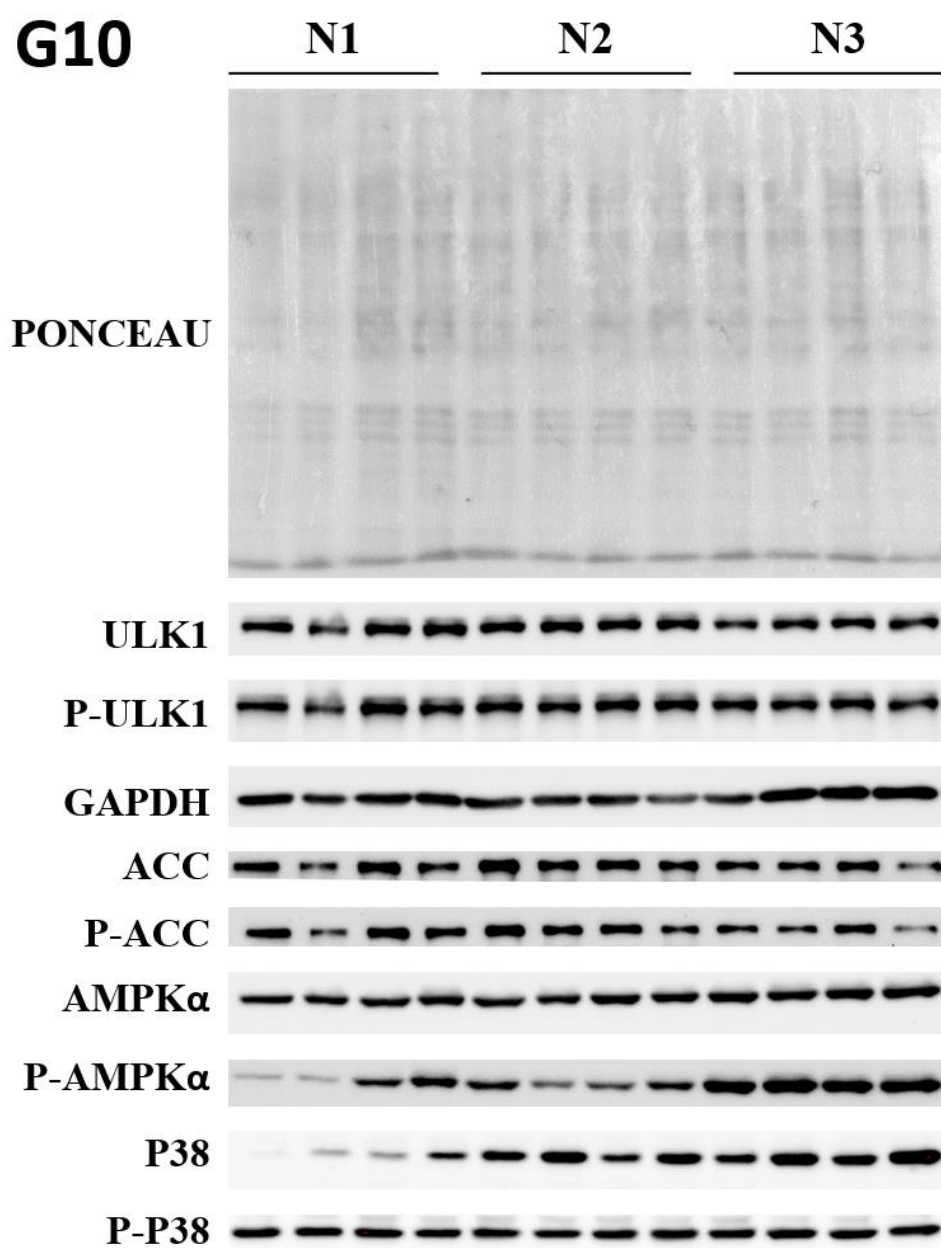


Figure 14: Blots from G10 including PONCEAU; ULK1; P-ULK1; GAPDH; ACC; P-ACC; AMPKα; P-AMPKα; P38; P-P38.

As discussed in the methods, the choice of the loading protein for OXPHOS represented an important point of reflexion. Figures 15, 16 and 17 aimed to illustrate the variability of our results depending on the different standardization. Initially, given that GAPDH was not a sensible way of standardization in mitochondria (Fig. 15), we planned to use TOM20 as loading protein due to the mitochondrial localization of OXPHOS proteins (Fig. 16). Finally, we considered OXPHOS/PONCEAU results (Fig. 17) as our official results because of the senseless tendency of decreasing of OXPHOS/TOM20 expression in each experimental condition relative to control (Fig. 16) which leaves doubt about a possible manipulation error during the measurement of TOM20 expression.

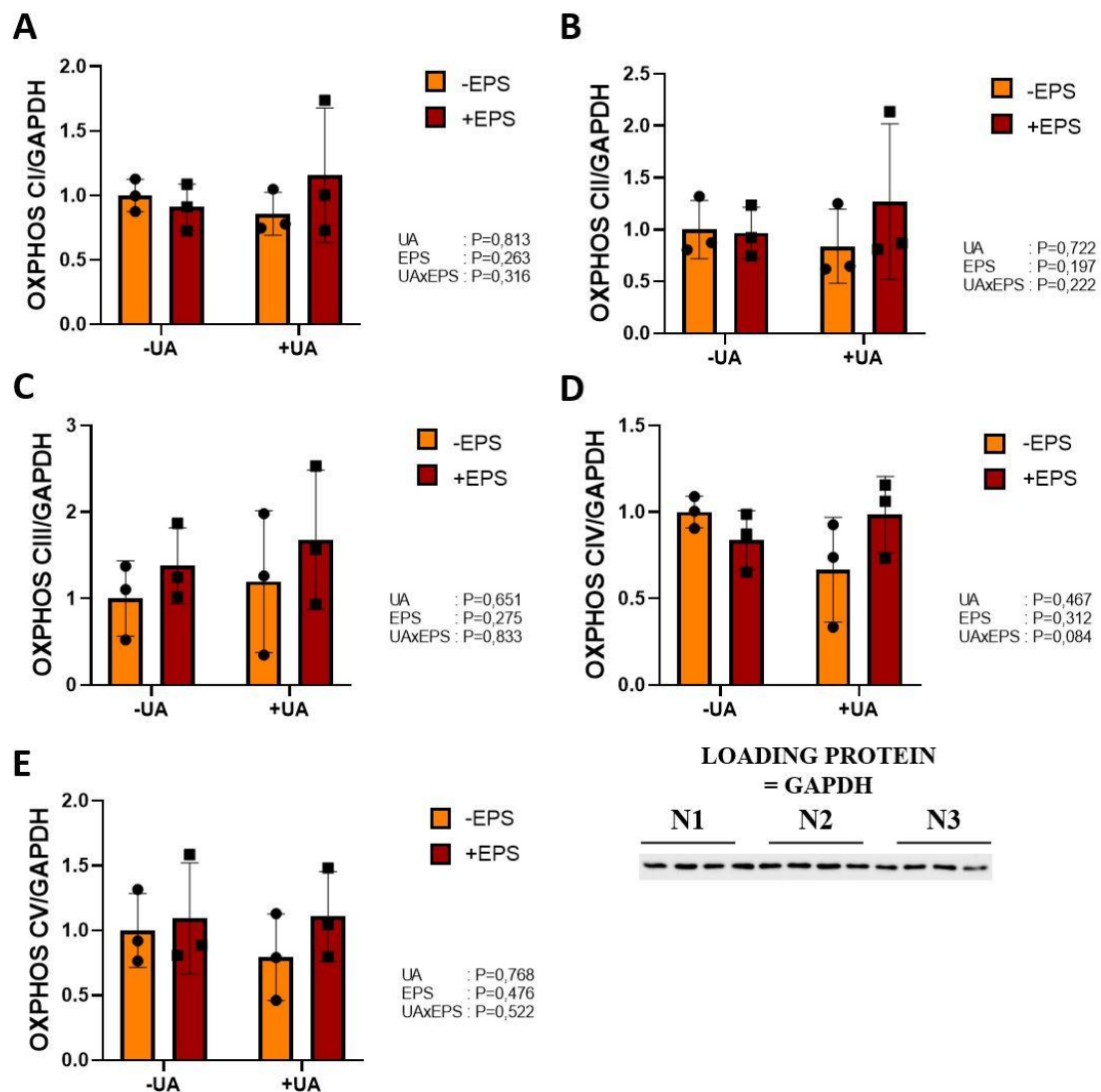


Figure 15: Effect of different conditions (with and without UA; with and without EPS) on the different complex of oxidative phosphorylation (OXPHOS); (A) complex I; (B) complex II; (C) complex III; (D) complex IV; (E) complex V. Not normally distributed variables were log transformed for analysis and back transformed for presentation. Normalization were performed using GAPDH as loading protein. Significance level was fixed to $p=0,05$.

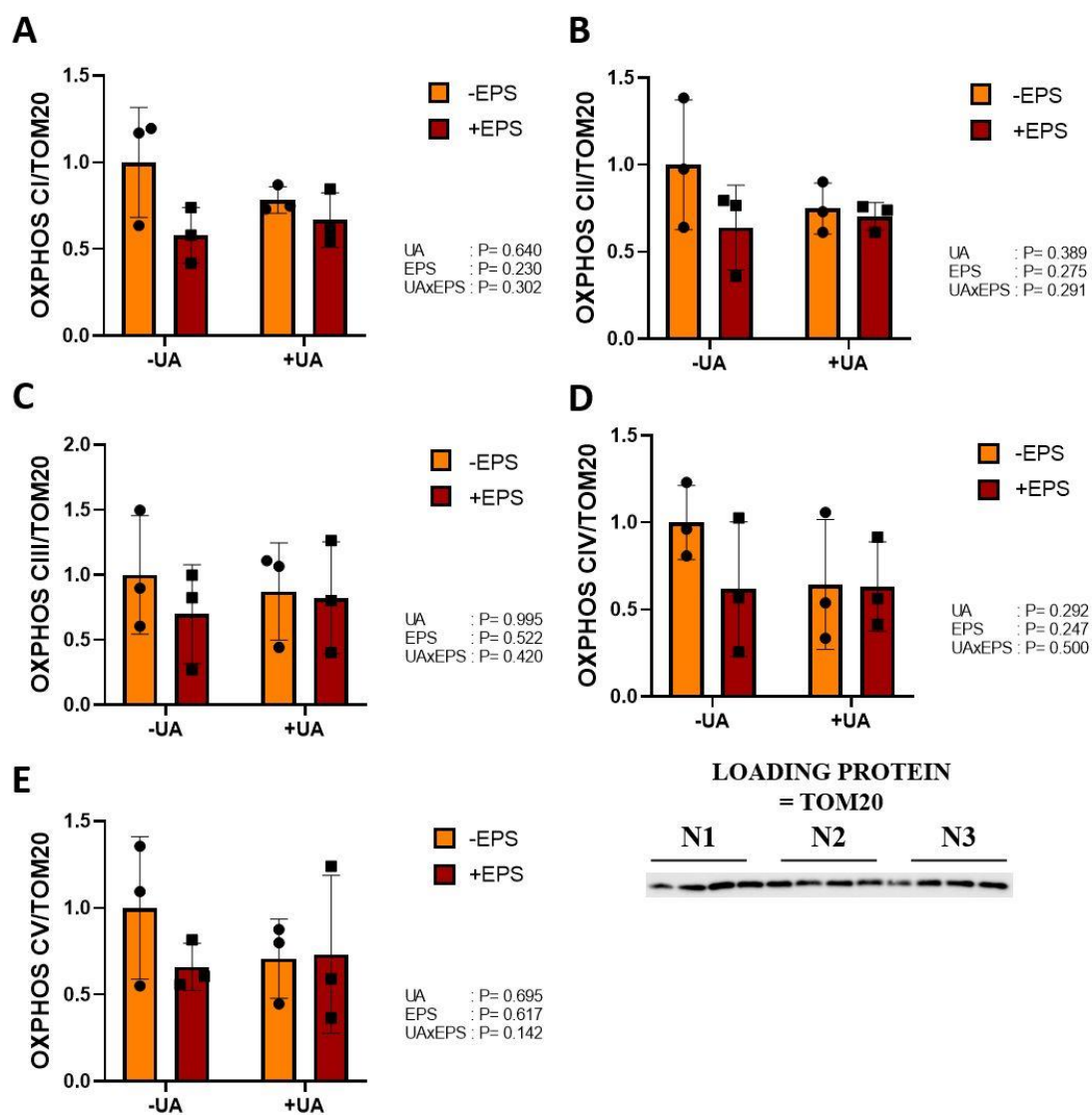


Figure 16: Effect of different conditions (with and without UA; with and without EPS) on the different complex of oxidative phosphorylation (OXPHOS); **(A)** complex I; **(B)** complex II; **(C)** complex III; **(D)** complex IV; **(E)** complex V. Not normally distributed variables were log transformed for analysis and back transformed for presentation. Normalization were performed using TOM20 as loading protein. Significance level was fixed to $p=0,05$.

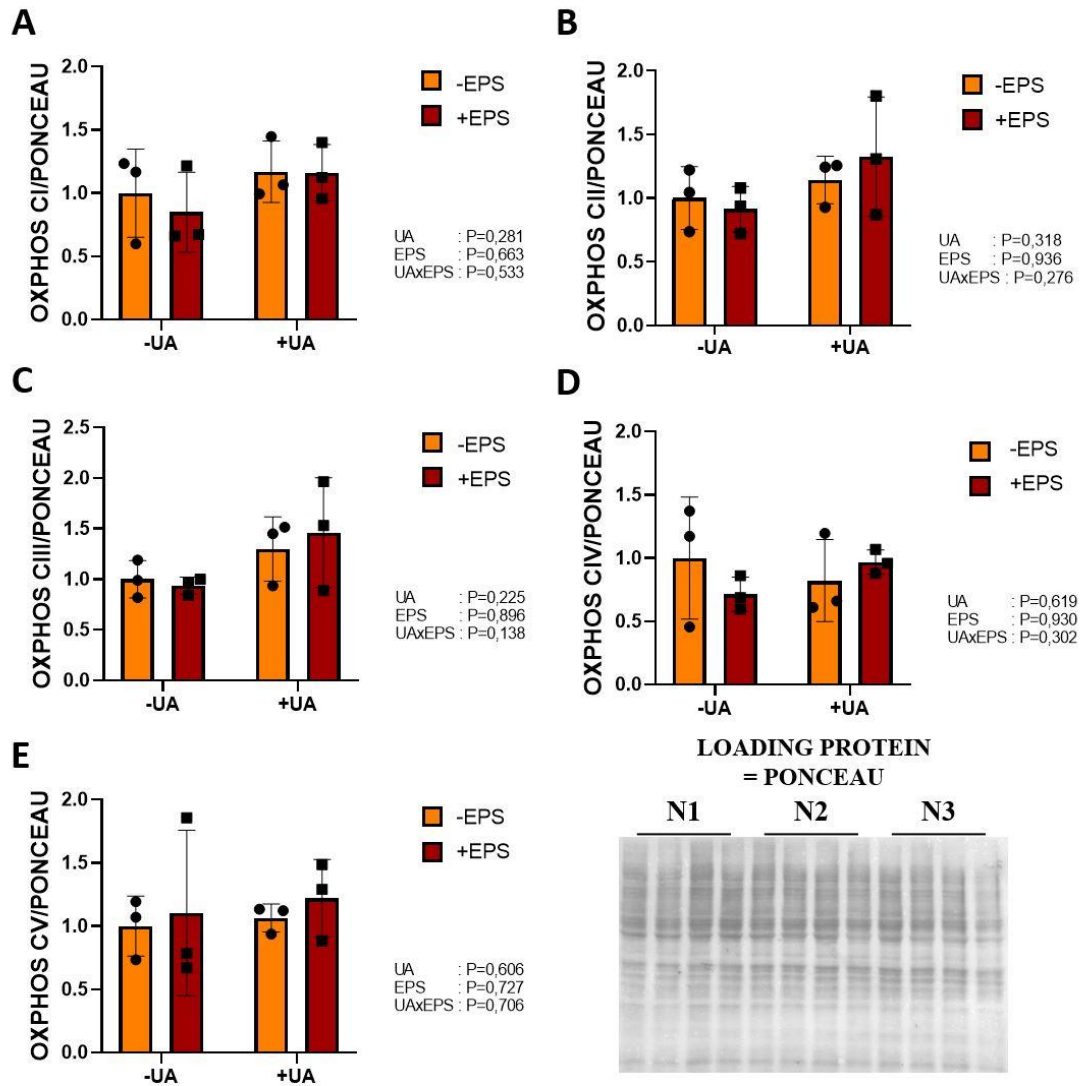


Figure 17: Effect of different conditions (with and without UA; with and without EPS) on the different complex of oxidative phosphorylation (OXPHOS); **(A)** complex I; **(B)** complex II; **(C)** complex III; **(D)** complex IV; **(E)** complex V. Not normally distributed variables were log transformed for analysis and back transformed for presentation. Normalization were performed using PONCEAU as loading protein. Significance level was fixed to $p=0,05$.

As shown in Figure 17, protein expression of the different OXPHOS complexes did not differ significantly between the four conditions in none of the complexes measured.

DISCUSSION

The results obtained in this study have provided insights into the effects of UA supplementation combined with low-frequency EPS on mitochondrial function in C2C12 myotubes. In the context of current literature and our initial hypotheses, the findings, while somewhat unexpected, shed light on the complexity of these interactions. In the forthcoming discussion section, we will illuminate the disparities between our anticipated outcomes and the actual findings about each intracellular process, shedding light on the underlying factors that may have contributed to these discrepancies. Additionally, we will address the limitations inherent in our experimental protocol, providing a comprehensive analysis of the constraints that may have influenced the observed results.

Mitochondrial biogenesis

Our observations have shown that PGC1 α expression, a central regulator of mitochondrial biogenesis, remained unaltered across all conditions was not in line with our initial hypotheses. The literature suggests that UA can upregulate genes involved in mitochondrial biogenesis, and we anticipated an enhancement in this marker (Liu et al., 2022; Nikolić et al., 2017; Vinciguerra, 2022). The lack of significant change in PGC1 α expression may suggest that the biogenetic aspect of mitochondrial function is less influenced by UA and EPS in our experimental model.

Energy regulation and mitochondrial quality

Similarly, the consistent expression of AMPK α and TOM20 across all conditions contradicts our initial hypotheses, as UA has been associated with an increase in AMPK α phosphorylation and mitochondria are central to energy regulation (Liu et al., 2022; Ryu et al., 2016; Vinciguerra, 2022). The absence of significant changes in these markers implies that our interventions did not notably impact the energy regulation or mitochondrial quality within C2C12 myotubes.

Fatty acid synthesis

The maintenance of ACC phosphorylation, a marker of fatty acid synthesis, across conditions also diverges from our expectations. We had anticipated that UA, with its reported influence on lipid metabolism, might lead to changes in this aspect of

mitochondrial function (Liu et al., 2022; Nikolić & Aas, 2019). The lack of change suggests that other factors may be at play, or that the effects of UA and EPS may not extend to fatty acid synthesis in this context.

Mitophagy and protein synthesis

Interestingly, we noted a tendency for a decrease in ULK1 and mTOR phosphorylation, linked to mitophagy and protein synthesis, respectively, with UA treatment. The literature indicates that UA can stimulate mitophagy (Liu et al., 2022; Ryu et al., 2016; Singh et al., 2022), and similar effects have been reported for EPS (Gao et al., 2020). However, it is noteworthy that the effects of EPS on ULK1 and mTOR, as evidenced in the literature, were not replicated in our study. The tendency for a decrease, while not statistically significant, raises questions about the specific impact of UA on these markers in our model. These results suggest that the influence of UA and EPS on mitophagy and protein synthesis may not be as straightforward as previously thought.

Inflammation

The observation that P38 phosphorylation, an inflammation marker, tended to increase with UA treatment is intriguing. While unexpected, this may indicate that UA has an amplifying role in modulating inflammation, which could be of significance for further investigations. It is worth noting that there have been studies that suggested a potential anti-inflammatory effect of UA (Lambernd et al., 2012; Rodriguez et al., 2017; Y. Zhang et al., 2021). One notable observation is the reduction of inflammatory markers, including CRP, suggesting that UA may have anti-inflammatory properties. Additionally, UA appears to influence specific cellular pathways related to inflammation, with evidence of modulation in the activation of kinases crucial to inflammatory responses. Furthermore, UA's impact on the production of pro-inflammatory cytokines has been suggested, although the exact mechanisms underlying this relationship require further exploration. Knowing this, it is all the more surprising to observe a trend of increase.

ATP production capacity

In light of the observation that none of the mitochondrial factors were significantly affected by the experimental protocol, our initial expectations regarding the impact on

OXPHOS complexes, representing the capacity for ATP production, were revised. Given the consistent findings across mitochondrial markers, it was hypothesized that there would be no discernible alterations in the expression levels of OXPHOS complexes in response to UA supplementation and low-frequency EPS. This adjustment in our hypothesis was based on the premise that the overall mitochondrial function, as reflected by the expression of various mitochondrial factors, remained unaffected by the experimental interventions.

Indeed, the initial anticipation of significant alterations in OXPHOS complex expression levels was not observed in the results of our study independently of the way of standardizing.

This discrepancy raises questions about the appropriateness of the normalization strategy employed for OXPHOS protein expression. While we utilized PONCEAU staining to assess total protein expression, the decision to use this method as a standardization way was necessitated by challenges encountered with the normalization by TOM20, a specific mitochondrial marker typically used to normalize mitochondrial proteins, such as OXPHOS, in the literature (Geng et al., 2010; Ryu et al., 2016). Although PONCEAU staining allows for the assessment of overall protein abundance, it may not provide an accurate reflection of mitochondrial-specific changes. As such, the lack of significant alterations in OXPHOS complex expression may be attributed, in part, to the limitations of the normalization approach.

Furthermore, the eventual utilization of inappropriate standardization strategy introduces potential confounders that could obscure true biological effects. Future studies may benefit from employing a more appropriate normalization methods tailored to the subcellular localization and functional relevance of each protein target. This approach would enable a more precise assessment of mitochondrial protein expression and function, thereby enhancing the reliability and interpretability of the results.

Limits of the experimentation

With regards to the references of this thesis, our research did not obtain most of the results already shown in the current literature in term of *UA only* effects or *EPS only* effects, which leads us to question the methods and materials of our study.

Firstly, it is important to note that C2C12 cells were not differentiated as much as we can see in Ryu (2016) and Evers-Van-Glow (2015) studies. Lower amount of myotubes could have led to a weaker response to myotubes contractions and to a weaker protein expression in our experiments.

Moreover, it is essential to acknowledge the inherent learning curve associated with conducting experimental manipulations, particularly when performed by an investigator for the first time. While rigorous adherence to established protocols and procedures was maintained throughout the study, the execution of experimental techniques may have been subject to variability or suboptimal performance due to inexperience. Over time, with increased familiarity and expertise, manipulation techniques are likely to become more refined, thereby enhancing the precision and reliability of experimental outcomes.

Furthermore, the relatively small sample size employed in our study, with only three replicates per condition, represents another significant limitation. While the determination of sample size was guided by practical considerations and resource constraints, such as availability of cells and reagents, it nonetheless poses challenges in terms of statistical power and robustness of the findings. A larger sample size would not only increase the sensitivity of the analyses but also improve the reliability and generalizability of the results.

In addition to the challenges mentioned above, it's crucial to address an issue of cell contamination that we encountered during the course of our experimentation. This contamination significantly impeded our progress and necessitated increased surveillance and vigilance, particularly under the microscope outside of the incubator, to verify that our cells remained uncontaminated. The heightened attention to contamination may have inadvertently introduced variability into the protocol beyond our control. Despite our efforts to maintain sterility and integrity, the presence of contaminants posed a challenge and may have influenced the outcomes of our experiments. This unexpected complication underscores the importance of stringent quality control measures in laboratory settings to mitigate the risk of contamination and ensure the reliability of experimental results.

In summary, it is crucial to recognize and address the limitations encountered. Future research should focus on optimizing cell differentiation protocols, refining experimental

techniques, and increasing sample sizes to enhance the validity and comprehensiveness of our findings. By systematically addressing these limitations, we can advance our understanding of the complex interplay between UA, EPS, and mitochondrial function, thereby paving the way for more robust and impactful scientific discoveries.

CONCLUSION

The investigation into the combined effects of UA and low-frequency EPS on mitochondrial function in C2C12 myotubes, has provided valuable insights, although the results did not align with our initial hypotheses.

It is clear that the absence of significant changes in most markers challenges our initial hypothesis and highlights the complexity of the interactions between these interventions and mitochondrial health.

These unexpected findings offer a valuable foundation for future investigations. Further research is needed to elucidate the specific molecular mechanisms underlying the observed tendencies and their potential implications for muscle health, mitochondrial dynamics, and overall cellular function.

More concretely, future investigations should aim to exploring the specific molecular pathways through which UA interacts with ULK1 and mTOR phosphorylation, to gain a comprehensive understanding of its impact on mitophagy and protein synthesis; to determine the contexts in which UA and EPS may or may not influence markers like PGC1 α , AMPK α , and TOM20, essential for mitochondrial function and cellular energy regulation; and to elucidate the precise mechanisms through which UA influences inflammation markers, despite its known anti-inflammatory potential.

In conclusion, while the results did not meet our initial hypotheses, they open doors to further research and a deeper understanding of the molecular mechanisms at play in the context of UA supplementation and EPS. Such findings, though unexpected, contribute to the growing body of knowledge on mitochondrial function and muscle health. Moreover, they pave the way for future research to evaluate the combined effects of UA and EPS, providing insight into their synergistic or antagonistic interactions.

DECLARATION OF INTEREST

No conflict of interest declaration.

ANNEXES AND SUPPLEMENTARY FILES

Table S6 Adjusted total blot volume of the G12.

Blot	PONCEAU	CI	CII	CIII	CIV	CV
n1 CTRL	174068784	14303378	6919698	12316234	7708896	13961046
n1 UA	166943568	16101343	7984299	11169901	3850720	10507872
n1 EPS	196504824	15906048	8062139	11829734	5097600	24516666
n1 UA+EPS	202241760	15156282	10069716	12833175	8167264	17532072
n2 CTRL	208320660	16199199	9676485	17720832	10792288	14973552
n2 UA	219887958	15610117	7760370	22823522	9935168	16749198
n2 EPS	362676006	16228667	9968941	25155664	11797152	19111344
n2 UA+EPS	237242286	15136405	7842936	25981880	8586016	23683146
n3 CTRL	369848004	14767916	10352303	21697344	6356448	18245118
n3 UA	236932890	15693795	11209238	25661207	5910464	17868654
n3 EPS	336569442	14799191	11980966	24106353	7568640	15167580
n3 UA+EPS	192492474	17956875	13205898	27054397	6393408	11435646

Table S6bis Adjusted total blot volume of G12 (suite)

Blot	PGC1 α	GAPDH	mTOR	P-mTOR	TOM20
n1 CTRL	1413566	14054196	13379184	5858748	8660157
n1 UA	1414388	21108066	14370552	4589046	15937305
n1 EPS	5162297	14322468	20701044	11793222	27486824
n1 UA+EPS	3668997	20385222	19901196	8811180	20086452
n2 CTRL	3859701	18122574	19210086	17401230	18457086
n2 UA	7497599	19615596	14806512	8345106	12971721
n2 EPS	5253676	21887490	17716986	13411062	15888395
n2 UA+EPS	7227572	14802018	16631496	10661616	12959340
n3 CTRL	3403765	12837864	15810732	10305288	9133200
n3 UA	3563096	14657946	15324624	9133236	15172593
n3 EPS	3122230	15890700	14133294	9640134	18424893
n3 UA+EPS	3067430	10121188	10224900	6322428	21252928

Table S7 Adjusted total blot volume of G10

Blot	PONCEAU	GAPDH	ULK1	P-ULK1	ACC	P-ACC
n1 CTRL	98382074	23929200	25566846	30775434	12280968	9232650
n1 UA	91193112	15260256	16144344	20694876	4431798	4053798
n1 EPS	115990624	22831419	27704328	39148238	16657578	12401172
n1 UA+EPS	126380113	23404176	29076876	31010483	11239200	6711138
n2 CTRL	98825812	21634464	26597844	32998878	18658836	15963948
n2 UA	82888455	17609616	26555064	27788463	11536686	9273726
n2 EPS	101740081	19189296	24496656	32412159	12763926	10396638
n2 UA+EPS	96048830	12965040	24407922	31804312	6661242	7994070
n3 CTRL	100534343	16802496	16454154	29924754	7548660	7279650
n3 UA	115860703	32108392	21607350	28679870	5925276	6900768
n3 EPS	115043458	30996864	21494880	30699957	11047932	9290232
n3 UA+EPS	89815670	44882805	22063854	27673093	3376296	3919230

Table S7bis Adjusted total blot volume of G10 (suite)

Blot	AMPKα	P-AMPKα	P38	P-P38
n1 CTRL	21763700	1383138	155420200	86817
n1 UA	19754000	1384299	150318870	7792116
n1 EPS	21798420	7073199	158507180	10550265
n1 UA+EPS	24266200	11861937	138610420	26266851
n2 CTRL	22417640	9306423	135956340	50250918
n2 UA	19380340	4061178	124866170	94649493
n2 EPS	27720980	5070603	132765490	29457021
n2 UA+EPS	25286520	8064306	146555890	69664773
n3 CTRL	31029600	23594012	139617140	41952606
n3 UA	26958540	27573560	162121570	91772148
n3 EPS	31185980	22483458	152351290	60679407
n3 UA+EPS	35330100	23252171	156087490	141160701

REFERENCES

- Andreux, P. A., van Diemen, M. P. J., Heezen, M. R., Auwerx, J., Rinsch, C., Groeneveld, G. J., & Singh, A. (2018). Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. *Scientific Reports*, 8(1), 8548. doi:10.1038/s41598-018-26944-x
- Balsera, M., Soll, J., & Bölter, B. (2009). Protein import machineries in endosymbiotic organelles. *Cell Mol Life Sci*, 66(11-12), 1903-1923. doi:10.1007/s00018-009-8644-2
- Cantó, C., & Auwerx, J. (2009). PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Curr Opin Lipidol*, 20(2), 98-105. doi:10.1097/MOL.0b013e328328d0a4
- Cuadrado, A., & Nebreda, A. R. (2010). Mechanisms and functions of p38 MAPK signalling. *Biochem J*, 429(3), 403-417. doi:10.1042/bj20100323
- Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., . . . Shaw, R. J. (2011). Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science*, 331(6016), 456-461. doi:10.1126/science.1196371
- Esselun, C., Theyssen, E., & Eckert, G. P. (2021). Effects of Urolithin A on Mitochondrial Parameters in a Cellular Model of Early Alzheimer Disease. *Int J Mol Sci*, 22(15). doi:10.3390/ijms22158333
- Evers-van Gogh, I. J., Alex, S., Stienstra, R., Brenkman, A. B., Kersten, S., & Kalkhoven, E. (2015). Electric Pulse Stimulation of Myotubes as an In Vitro Exercise Model: Cell-Mediated and Non-Cell-Mediated Effects. *Sci Rep*, 5, 10944. doi:10.1038/srep10944
- Gao, J., Yu, L., Wang, Z., Wang, R., & Liu, X. (2020). Induction of mitophagy in C2C12 cells by electrical pulse stimulation involves increasing the level of the mitochondrial receptor FUNDC1 through the AMPK-ULK1 pathway. *Am J Transl Res*, 12(10), 6879-6894. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7653589/pdf/ajtr0012-6879.pdf>
- Geng, T., Li, P., Okutsu, M., Yin, X., Kwek, J., Zhang, M., & Yan, Z. (2010). PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. *Am J Physiol Cell Physiol*, 298(3), C572-579. doi:10.1152/ajpcell.00481.2009
- Handschin, C., & Spiegelman, B. M. (2006). Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr Rev*, 27(7), 728-735. doi:10.1210/er.2006-0037
- Hardie, D. G. (2011). AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev*, 25(18), 1895-1908. doi:10.1101/gad.17420111
- Hardie, D. G., & Lin, S. C. (2017). AMP-activated protein kinase - not just an energy sensor. *F1000Res*, 6, 1724. doi:10.12688/f1000research.11960.1
- Hirst, J. (2013). Mitochondrial complex I. *Annu Rev Biochem*, 82, 551-575. doi:10.1146/annurev-biochem-070511-103700
- Hu, F., Li, N., Li, Z., Zhang, C., Yue, Y., Liu, Q., . . . Niu, W. (2018). Electrical pulse stimulation induces GLUT4 translocation in a Rac-Akt-dependent manner in C2C12 myotubes. *FEBS Lett*, 592(4), 644-654. doi:10.1002/1873-3468.12982
- Iorio, R., Celenza, G., & Petricca, S. (2021). Mitophagy: Molecular Mechanisms, New Concepts on Parkin Activation and the Emerging Role of AMPK/ULK1 Axis. *Cells*, 11(1). doi:10.3390/cells11010030
- Kastaniotis, A. J., Autio, K. J., Kerätär, J. M., Monteuuis, G., Mäkelä, A. M., Nair, R. R., . . . Hiltunen, J. K. (2017). Mitochondrial fatty acid synthesis, fatty acids and mitochondrial physiology. *Biochim Biophys Acta Mol Cell Biol Lipids*, 1862(1), 39-48. doi:10.1016/j.bbalip.2016.08.011
- Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*, 13(2), 132-141. doi:10.1038/ncb2152

- Lambernd, S., Taube, A., Schober, A., Platzbecker, B., Görgens, S. W., Schlich, R., . . . Eckel, J. (2012). Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia*, 55(4), 1128-1139. doi:10.1007/s00125-012-2454-z
- Leduc-Gaudet, J. P., Hussain, S. N. A., Barreiro, E., & Gouspillou, G. (2021). Mitochondrial Dynamics and Mitophagy in Skeletal Muscle Health and Aging. *Int J Mol Sci*, 22(15). doi:10.3390/ijms22158179
- Li, Z., & Graham, B. H. (2012). Measurement of mitochondrial oxygen consumption using a Clark electrode. *Methods Mol Biol*, 837, 63-72. doi:10.1007/978-1-61779-504-6_5
- Lin, J., Handschin, C., & Spiegelman, B. M. (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab*, 1(6), 361-370. doi:10.1016/j.cmet.2005.05.004
- Liu, S., D'Amico, D., Shankland, E., Bhayana, S., Garcia, J. M., Aebischer, P., . . . Marcinek, D. J. (2022). Effect of Urolithin A Supplementation on Muscle Endurance and Mitochondrial Health in Older Adults: A Randomized Clinical Trial. *JAMA Netw Open*, 5(1), e2144279. doi:10.1001/jamanetworkopen.2021.44279
- Magkos, F., Fraterrigo, G., Yoshino, J., Luecking, C., Kirbach, K., Kelly, Shannon C., . . . Klein, S. (2016). Effects of Moderate and Subsequent Progressive Weight Loss on Metabolic Function and Adipose Tissue Biology in Humans with Obesity. *Cell Metabolism*, 23(4), 591-601. doi:10.1016/j.cmet.2016.02.005
- Mahmood, T., & Yang, P.-C. (2012). Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences*, 4, 429 - 434.
- Mihaylova, M. M., & Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol*, 13(9), 1016-1023. doi:10.1038/ncb2329
- Nikolić, N., & Aas, V. (2019). Electrical Pulse Stimulation of Primary Human Skeletal Muscle Cells. *Methods Mol Biol*, 1889, 17-24. doi:10.1007/978-1-4939-8897-6_2
- Nikolić, N., Görgens, S. W., Thoresen, G. H., Aas, V., Eckel, J., & Eckardt, K. (2017). Electrical pulse stimulation of cultured skeletal muscle cells as a model for in vitro exercise - possibilities and limitations. *Acta Physiol (Oxf)*, 220(3), 310-331. doi:10.1111/apha.12830
- Ono, K., & Han, J. (2000). The p38 signal transduction pathway: activation and function. *Cell Signal*, 12(1), 1-13. doi:10.1016/s0898-6568(99)00071-6
- Rodriguez, J., Caille, O., Ferreira, D., & Francaux, M. (2017). Pomegranate extract prevents skeletal muscle of mice against wasting induced by acute TNF- α injection. *Mol Nutr Food Res*, 61(4). doi:10.1002/mnfr.201600169
- Ryu, D., Mouchiroud, L., Andreux, P. A., Katsyuba, E., Moullan, N., Nicolet-Dit-Félix, A. A., . . . Auwerx, J. (2016). Urolithin A induces mitophagy and prolongs lifespan in *C. elegans* and increases muscle function in rodents. *Nat Med*, 22(8), 879-888. doi:10.1038/nm.4132
- Saggerson, D. (2008). Malonyl-CoA, a key signaling molecule in mammalian cells. *Annu Rev Nutr*, 28, 253-272. doi:10.1146/annurev.nutr.28.061807.155434
- Saxton, R. A., & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, 168(6), 960-976. doi:10.1016/j.cell.2017.02.004
- Scarpulla, R. C. (2011). Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta*, 1813(7), 1269-1278. doi:10.1016/j.bbamcr.2010.09.019
- Singh, A., D'Amico, D., Andreux, P. A., Fouassier, A. M., Blanco-Bose, W., Evans, M., . . . Rinsch, C. (2022). Urolithin A improves muscle strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. *Cell Reports Medicine*, 3(5). doi:10.1016/j.xcrm.2022.100633
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., . . . Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem*, 150(1), 76-85. doi:10.1016/0003-2697(85)90442-7

- Son, Y. H., Lee, S. M., Lee, S. H., Yoon, J. H., Kang, J. S., Yang, Y. R., & Kwon, K. S. (2019). Comparative molecular analysis of endurance exercise in vivo with electrically stimulated in vitro myotube contraction. *J Appl Physiol* (1985), 127(6), 1742-1753. doi:10.1152/japplphysiol.00091.2019
- Sousa, J. S., D'Imprima, E., & Vonck, J. (2018). Mitochondrial Respiratory Chain Complexes. *Subcell Biochem*, 87, 167-227. doi:10.1007/978-981-10-7757-9_7
- Suzuki, J., Liu, D., Yang, F., Zuo, M. Q., Li, C., Dong, M. Q., . . . Sui, S. F. (2022). Structural basis of Tom20 and Tom22 cytosolic domains as the human TOM complex receptors. *Proc Natl Acad Sci U S A*, 119(26), e2200158119. doi:10.1073/pnas.2200158119
- Tarum, J., Folkesson, M., Atherton, P. J., & Kadi, F. (2017). Electrical pulse stimulation: an in vitro exercise model for the induction of human skeletal muscle cell hypertrophy. A proof-of-concept study. *Exp Physiol*, 102(11), 1405-1413. doi:10.1113/ep086581
- Toyama, E. Q., Herzig, S., Courchet, J., Lewis, T. L., Jr., Losón, O. C., Hellberg, K., . . . Shaw, R. J. (2016). Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science*, 351(6270), 275-281. doi:10.1126/science.aab4138
- Vander, A., Raff, H., Widmaier, E., & K., S. (2013). *Physiologie humaine : Les mécanismes du fonctionnement de l'organisme* (C. E. Maloine Ed. 6ème ed.).
- Vinciguerra, M. (2022). Urolithin A improves physical performance in middle age. *Nature Aging*, 2(6), 460-460. doi:10.1038/s43587-022-00239-3
- Wang, Y., Yu, W., Li, S., Guo, D., He, J., & Wang, Y. (2022). Acetyl-CoA Carboxylases and Diseases. *Front Oncol*, 12, 836058. doi:10.3389/fonc.2022.836058
- Zhang, M., Cui, S., Mao, B., Zhang, Q., Zhao, J., Zhang, H., . . . Chen, W. (2022). Ellagic acid and intestinal microflora metabolite urolithin A: A review on its sources, metabolic distribution, health benefits, and biotransformation. *Crit Rev Food Sci Nutr*, 1-23. doi:10.1080/10408398.2022.2036693
- Zhang, M., Tang, X., Mao, B., Zhang, Q., Zhao, J., Chen, W., & Cui, S. (2023). Inhibition of the NF- κ B and mTOR targets by urolithin A attenuates D-galactose-induced aging in mice. *Food Funct*, 14(23), 10375-10386. doi:10.1039/d3fo03847e
- Zhang, Y., Aisker, G., Dong, H., Halemahebai, G., Zhang, Y., & Tian, L. (2021). Urolithin A suppresses glucolipotoxicity-induced ER stress and TXNIP/NLRP3/IL-1 β inflammation signal in pancreatic β cells by regulating AMPK and autophagy. *Phytomedicine*, 93, 153741. doi:10.1016/j.phymed.2021.153741

In the ever-ascending pursuit of longevity and vibrant health, the microscopic power station known as mitochondria have taken center stage. These cellular powerhouses are inextricably linked to skeletal muscle vitality and have become the focal point of recent investigations.

This study investigates the combined impact of urolithin A (UA), a compound found in pomegranates and health/sport supplements, and electrical pulse stimulation (EPS), a recognized technique for inducing human-like physiological responses to exercise *in vitro*, on mitochondrial function in C2C12 myotubes.

The results presented unexpected findings, challenging the initial hypotheses. Several markers exhibited similar expressions across all conditions, including PGC1 α , AMPK α , TOM20, ACC, and the OXPHOS complexes. Tendencies for reduced ULK1 and mTOR phosphorylation with UA treatment could mean that UA decrease mitophagy and protein synthesis in these experimental conditions, which are results contrary to the current literature. Surprisingly, P38 phosphorylation showed a tendency to increase with UA treatment, suggesting an amplifying role in modulating inflammation, also contrary to the current literature.

The study emphasizes on the expression and the phosphorylation stage of various mitochondrial proteins, paving the way for future research to uncover the underlying mechanisms and their implications for muscle health and cellular function.