Desminopathy-Associated TLR4 Dysregulation and Mitochondrial Alterations: Potential Effects of Natural Compounds in a Translational Model

Germán Tapia-Curimil

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Thesis defense commission

President: Robert Hardwick

Secretary: Jean-Marc Raymackers

Member committee: Nicolas Pierre

Promotor: Louise Deldicque

Co-promotor: Salomé Mariotti

External members:

Jocelyn Fuentes-García

Diego García-Diaz

Mauricio Castro-Sepúlveda

Esta trabajo científico y tesis de PhD está dedicado a mi Padre

Pablo Alfredo Tapia Guerra

Papá, te extraño y junto a mi madre Jacqueline Curimil Pedraza son mi fuente de inspiración.

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Finalmente, aprendí que los genes pueden determinar nuestra naturaleza, pero nuestra conducta estará determinada por la experiencia en el camino de la vida.

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Abbreviations

AP-1: Activator protein 1

BECN: Beclin-1

BNIP3: BCL2 and adenovirus E1B 19-kDa-interacting protein 3

DAMPs: Damage-associated molecular pattern

DAPI: 4′,6-diamidino-2-phenylindole

DES: Desmin

DMD: Duchenne muscular dystrophy

DRP1: Dynamin-related protein 1

EPI: Epicatechin

ERK: Extracellular signal-regulated kinase

IKK: IκB-kinase

IL-6: Interleukin-6

IκBα: Inhibitor of nuclear factor kappa-B alpha

JNK: c-Jun N-terminal kinases

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MEF2a: Myocyte enhancer factor 2A

MFN1: Mitofusin 1

MFN2: Mitofusin 2

MRC: Medical Research Council scale

MyD88: Myeloid differentiation primary response 88

Myf5: Myogenic factor 5

MyoD: Myogenic differentiation 1

NF-κB: Nuclear factor-kappa B

OPA1: Optic atrophy 1

OXPHOS: Oxidative phosphorylation

P38: p38 mitogen-activated protein kinase (p38 MAPK)

PAMPs: Pathogen-associated molecular patterns

PARK2: Parkin

PBMC: Peripheral blood mononuclear cells

PINK1: PTEN-induced kinase 1

SQSTM1: Sequestosome 1

TAK1: Transforming growth factor-β-activated kinase 1

TLR4: Toll-like receptor 4

TOM20: Translocase of the outer mitochondrial membrane, 20 kDa subunit

UA: Urolithin A

Abstract thesis

This thesis presents the first exploration of cellular inflammation via the TLR4 pathway, together with mitochondrial dynamics and morphology, in the genetic muscle disease desminopathy—a condition that currently has no cure, lacks disease-specific treatments, and is marked by gaps in our understanding of its pathophysiology. To address these gaps, this thesis specifically investigates TLR4 inflammatory signaling in skeletal muscle (the primary tissue affected by desmin mutations) and in peripheral blood mononuclear cells (PBMCs) as a model of systemic immune response. In addition, we characterize the clinical and functional phenotype of patients with desminopathy to integrate molecular findings with disease presentation.

Additionally, this thesis includes the first case report in the scientific literature suggesting potential modulation of the TLR4 pathway and mitochondrial dynamics/morphology in desminopathy by a cocoa-derived natural compound. Consistent with this translational direction, we also conducted in vitro experimental work using a C2C12 cell culture model to determine the effect of another natural compound, i.e. urolithin A, on TLR4 signaling and mitochondrial dynamics/morphology. The selected compound was chosen because prior evidence has shown effects on health markers (from higher expression of PINK1, AMPKα, PGC1α until higher skeletal muscle endurance) in humans, as well as in animal and cellular models.

Taken together, this work outlines a potential research avenue involving natural-compound–based interventions aimed at investigating—and ultimately limiting—the progression of desminopathy.

Chapter I: Introduction

Desminopathy is a genetically determined myopathy arising from pathogenic variants in DES gene, which encodes desmin, the intermediate filament that scaffolds myofibrils, links the sarcomere to the sarcolemma and costameres, and anchors mitochondria within skeletal muscle (Capetanaki, 2002). Disruption of this cytoskeletal network leads to myofibrillar disarray, protein aggregation, impaired mitochondrial positioning and quality control (integrated cellular mechanisms that preserve a healthy mitochondrial population) and, clinically, progressive weakness with frequent cardiac involvement. Despite advances in gene discovery, the cellular signaling mechanisms that couple desmin dysfunction to muscle degeneration remain incompletely defined—particularly the intersection of cytoskeletal stress with mitochondrial bioenergetics, proteostasis (autophagy/mitophagy), and inflammatory pathways (Clemen et al., 2013).

To address those gaps, this thesis adopts a translational design spanning human tissue to complementary in vitro systems. In muscle samples from patients and matched controls, we quantified clinical markers of the pathology alongside targeted readouts of inflammatory signaling and mitochondrial dynamics and morphology. In parallel, we employed C2C12 myotubes to facilitate causal interrogation and pathway perturbation, testing candidate modulators of inflammation as well as mitochondrial dynamics and morphology.

What is Desmin?

Desmin is a structural protein belonging to the type III intermediate filament family, playing a key role in maintaining the structural and functional integrity of skeletal and cardiac muscle Capetanaki et al. (2015). The synthesis of the desmin protein is encoded by the DES gene, which is located on chromosome 2q35 in the human genome. The DES gene is transcribed into messenger RNA (mRNA) in the nucleus, and the mRNA is subsequently translated in the cytoplasm to produce the desmin protein. This allows the protein to assemble into intermediate filaments, integrating into the cytoskeletal network of the muscle fiber that connects the sarcoplasmic membrane, sarcomere, nucleus, and mitochondria. The gene DES comprises nine exons (8 coding exons) (Figure 1.1)

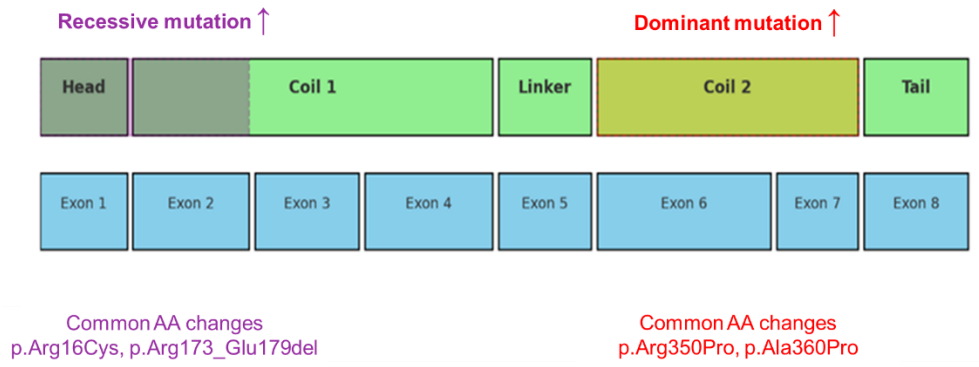


Figure 1.1 DES gene: exons, desmin protein domains, and frequent mutation sites. Domain encoded by each region: Exon 1 – head domain (N-terminal head), necessary for the initiation of filament assembly. Exons 2–4 – coil 1 domain (α-helical coiled-coil), involved in polymerization. Exon 5 – linker region, providing a flexible connection between coils. Exons 6–7 – coil 2 domain (C-terminal coiled-coil), a critical region for filament stability (the site where most dominant mutations are concentrated). Exon 8 – tail domain (C-terminal tail), interacts with other proteins and regulates final filament assembly.

Desmin provides stability by connecting the sarcolemma to the Z-disc in the sarcomere, thus enabling efficient force transmission during skeletal muscle contractions (Hughes et al., 2015). It constitutes of the muscle cytoskeleton and

actively participates in the organization and mechanical stability of myofibrils by integrating various cellular structures such as the sarcolemma, Z-discs, nuclei, and mitochondria (Clemen et al., 2013) (Figure 1.2).

Regarding its structural role, desmin is involved in critical processes such as efficient force transmission from sarcomeres to cellular membranes and the extracellular matrix during skeletal muscle contraction, as well as in regulating intracellular signaling pathways, calcium homeostasis, and modulation of apoptotic processes (Capetanaki et al., 2015).

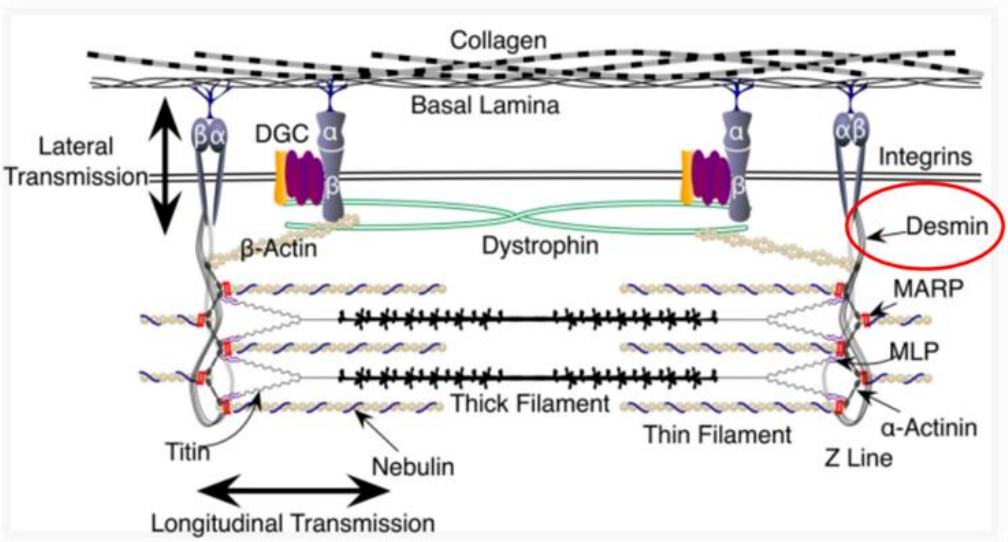


Figure 1.2. Sarcomeric structural proteins. The diagram illustrates the complex network of structural and contractile proteins within the muscle fiber. The red circle highlights desmin, which links the Z-line to the sarcolemma (via the DGC or dystrophin-glycoprotein complex) and the basal lamina, facilitating lateral force transmission. The contractile apparatus consists of thick filaments (myosin), centered by titin, and thin filaments (actin) stabilized by nebulin. Thin filaments anchor at the Z-line alongside β-actinin, MLP, and MARP proteins. Integration with the extracellular matrix, including collagen, is achieved through integrins, dystrophin, and β-actin, ensuring both longitudinal and lateral stability during skeletal muscle contraction.

From skeletal muscle fiber to clinical manifestations of desminopathy

Desmin mutations produce alterations in the structure and function of the desmin protein that can lead to desminopathies, a group of hereditary myopathies characterized by progressive muscle weakness, cardiac insufficiency, and in some cases, systemic involvement associated with pathological desmin aggregation (Goldfarb & Dalakas, 2009). The aggregation of this protein reflects its suboptimal distribution within the muscle fiber, leading to its accumulation in regions such as the subsarcolemma and the sarcoplasm of the muscle cell (Clemen et al., 2013) (Figure 1.3). Understanding the molecular mechanisms underlying desmin aggregation and cytoskeletal disruption is therefore essential for elucidating disease pathogenesis and improving clinical management strategies. (Goldfarb & Dalakas, 2009).

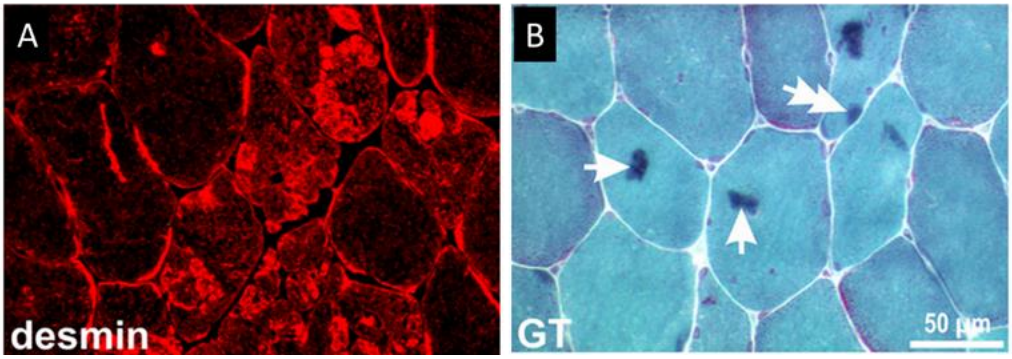


Figure 1.3 Desmin protein aggregates in skeletal muscle fibers. A) Immunofluorescence staining of the desmin protein shows its accumulation in regions of the subsarcolemma and within the sarcoplasm of the muscle fiber. (B) Gomori trichrome (GT) reveals protein accumulations and abnormal fibers. Figure adapted from Clemen et al. (2013).

Desminopathy is typically a genetic disorder characterized by autosomal dominant inheritance, To date, multiple mutations have been reported in the literature at various specific sites of the desmin protein, the most common being located in coil 2 (exon 6) (Clemen et al., 2013). The clinical manifestations

including skeletal muscle atrophy, progressive loss of mobility of extremities, and strength reduction, resulting in limitations in daily activities (Figure 1.4) (Kley et al., 2016).

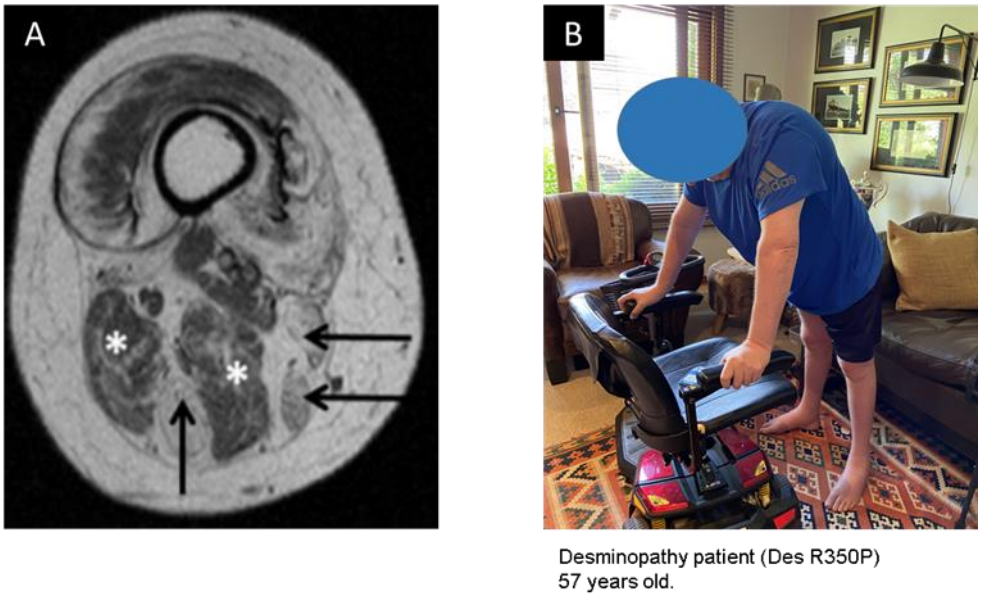


Figure 1.4 Skeletal muscle characteristics of desminopathy and clinical traits. A. Representative skeletal muscle by magnetic resonance in DES patient, arrows show the fat mass infiltration in hamstring represented by asterisk. B. DES patient in stand-up position with some limitations: bipodal contact with skeletal muscle activity in hip but poor skeletal muscle contraction in lower limb (MRC=2), limitation to walk and use of an electric wheelchair.

One of the first reported cases providing evidence of desminopathy was published in 1998 (Lobrinus et al., 1998), demonstrating that desminopathy leads to desmin aggregation and alterations in the ultrastructure of both cardiac and skeletal muscle. These findings are consistent with the clinical manifestations of the disease, which include heart failure, skeletal muscle atrophy, and weakness. Currently, desminopathy lacks a specific treatment; clinical management is focused solely on maintaining functional capacity for daily activities (Monje et al., 2020). In summary, desminopathy is caused by a mutation in the DES gene that impairs the function of the desmin protein.

Although the protein continues to be produced, it aggregates within skeletal muscle, ultimately leading to the clinical manifestations of muscle weakness, atrophy, and cardiac complications. However, current evidence reveals a knowledge gap regarding physiological processes in skeletal muscle that may be associated with the maintenance of skeletal muscle homeostasis. The molecular mechanisms linking desmin aggregation to clinical progression remain poorly understood.

Roles of the TLR4 pathway in skeletal muscle and potential implication in desminopathy

Inflammation is a process closely associated with muscle homeostasis, and one key inflammatory pathway involved in skeletal muscle homeostasis is mediated by Toll-like receptor 4 (TLR4), a membrane-bound pattern recognition receptor that responds to endogenous and exogenous danger-associated molecular patterns, which induces gene expression involved in inflammatory responses; is activated by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), with one of the best-known activators being lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria (Ono et al., 2020; Ono & Sakamoto, 2017). Upon activation, TLR4 first activates myeloid differentiation primary response 88 (MyD88), initiating downstream signaling via the IKK complex, leading to the activation of Nuclear factor-kappa B (NF-κB), or through mitogen-activated protein kinases (MAPK), including p38, JNK, and ERK (Lu et al., 2008). This culminates in NF-κB activation, interleukin transcription, and the generation of the systemic inflammatory response in cells (Li et al., 2019; Rossol et al., 2011). The myokine interleukin (IL-6) is produced in response to inflammation and other stressors, such as skeletal muscle damage (Welc & Clanton, 2013) (Figure 1.5).

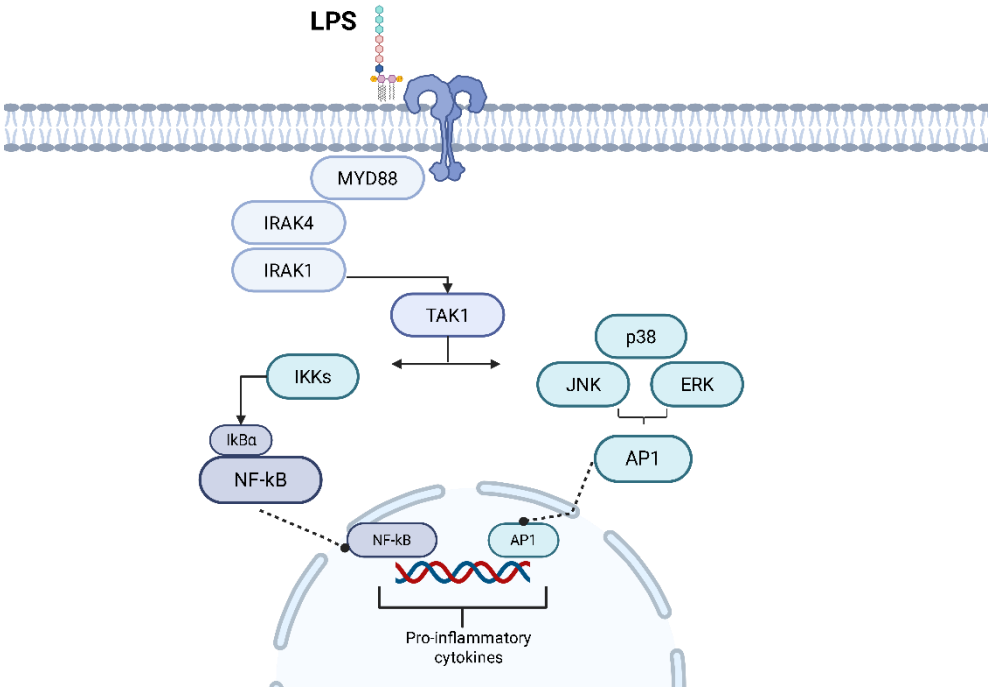


Figure 1.5 TLR4 signaling pathway. TLR4 is located on the cell membrane. Upon activation, TLR4 initiates downstream signaling, primarily through a MyD88-dependent pathway. MyD88, situated just below the membrane, recruits and activates interleukin-1 receptor-associated kinase (IRAK). Activated IRAK subsequently triggers TAK1, which can regulate inflammation via two main routes: 1) the IKK complex pathway, which phosphorylates and degrades IκBα, allowing NF-κB to translocate to the nucleus and promote the transcription of proinflammatory cytokine genes. 2) the MAPK pathway, involving kinases such as JNK, p38, and ERK, which activate the transcription factor AP-1, further regulating proinflammatory gene expression. The final cellular outcome of this signaling cascade is the production of inflammatory cytokines. Illustration created in Biorender. Adapted from Lu et al. (2008).

Ono et al. (2020) demonstrated, using both C2C12 myotubes and an in vivo mouse model, that activation of TLR4 signaling through lipopolysaccharide (LPS) increases the expression of pro-inflammatory cytokines and upregulates muscle-specific atrogenes associated with skeletal muscle atrophy. Inhibition of this pathway using TAK-242 attenuated these effects, supporting a mechanistic role for TLR4 in muscle catabolism. In humans, increased TLR4 signaling has been associated with reduced mobility and inflammatory states (Drummond et al., 2013). Thus, the inflammatory pathway plays a key role in the regulation of

skeletal muscle mass, where elevated levels of inflammation can contribute to muscle atrophy. In other clinical conditions as well, such as chronic kidney disease, elevated expression of TLR4 in skeletal muscle has been shown to exacerbate sarcopenia (Verzola et al., 2017). Also, the activation of TLR4 could decrease protein expression in relation to skeletal muscle repair in stem cells (Tao et al., 2022). This suggests that the TLR4 pathway could represent a potential therapeutic target to modulate the progression of desminopathy, particularly with respect to skeletal muscle atrophy and functional decline. Therefore, investigating TLR4 signaling in skeletal muscle of patients with desminopathy may provide mechanistic insight into inflammation-associated muscle degeneration and functional decline in this condition.

Roles of mitochondrial dynamics in skeletal muscle and implication in desminopathy

In this context, mitochondrial dynamics is a continuous and regulated process of mitochondrial fusion and fission. The fusion process involves mitochondrial merging to form elongated mitochondria, whereas fission involves mitochondrial division into smaller units (Giacomello et al., 2020), These mitochondrial dynamics determine mitochondrial morphology.

The mitochondrial fusion process is regulated by key proteins such as Optic atrophy 1 (OPA1), located in the inner mitochondrial membrane, and mitofusin 1 (MFN1) and mitofusin 2 (MFN2), located in the outer mitochondrial membrane. Fusion is essential for maintaining mitochondrial function and supporting various metabolic processes (Tabara et al., 2025). In contrast, the fission process is primarily regulated by the dynamin-related protein 1 (DRP1), which plays a critical role in mitochondrial distribution during cell division and in the removal of damaged mitochondria through quality control mechanisms (Figure 1.6) (Youle & van der Bliek, 2012). Mitochondrial dynamics proteins play a key role in determining mitochondrial morphology, including elongation

(resulting in more elongated mitochondria) and fragmentation (resulting in more circular and smaller mitochondria (Tabara et al., 2025).

The mitochondrial dynamics in the skeletal muscle is a crucial process to maintain the muscle mass and function (Romanello & Sandri, 2015). Additionally, an imbalance in mitochondrial dynamics may lead to skeletal muscle atrophy (Lei et al., 2024).

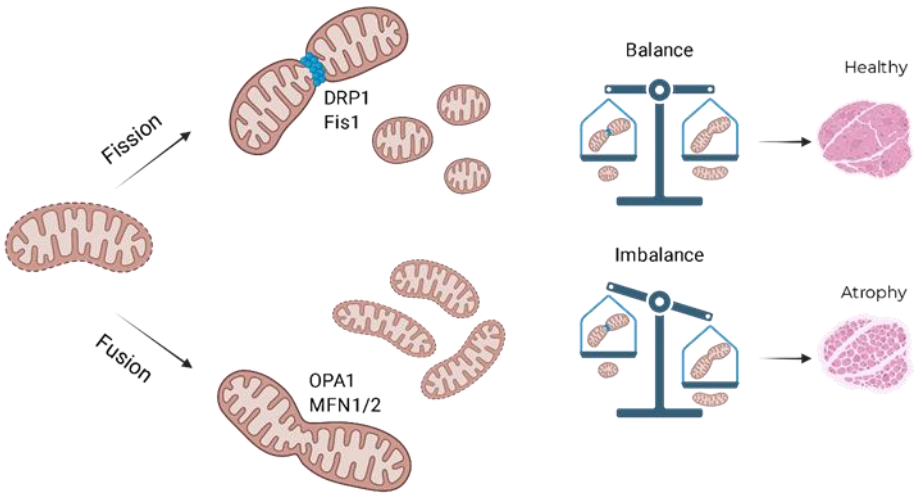


Figure 1.6 Mitochondrial dynamics and skeletal muscle mass. Mitochondria can undergo structural changes, primarily through the processes of fission and fusion. Fission involves the division of mitochondria into smaller organelles and is regulated by key proteins such as DRP1 and Fis1. In contrast, fusion is the merging of mitochondria, resulting in elongated mitochondrial structures, and is mediated by proteins including OPA1, MFN1, and MFN2. Mitochondrial dynamics proteins play a crucial role in maintaining skeletal muscle homeostasis. An optimal balance between fission and fusion is associated with skeletal muscle health, whereas an imbalance in these processes contributes to muscle atrophy (Giacomello et al., 2020) Illustration created in Biorender.

Beyond mitochondrial dynamics, alterations in mitochondrial morphology have been reported in several pathological conditions. Mitochondrial fragmentation is frequently associated with impaired oxidative phosphorylation (OXPHOS), bacterial infections, cancer, and apoptotic cell death. In contrast, elongated mitochondrial networks have been observed under conditions of nutrient deprivation, endoplasmic reticulum stress, and viral infection (Wai, 2024). In the context of desminopathy, disruption of the intermediate filament network compromises cytoskeletal organization and organelle positioning, including mitochondrial anchoring to the Z-disc. This structural disorganization may interfere with mitochondrial fusion–fission balance, particularly through dysregulation of DRP1-mediated fission, ultimately contributing to mitochondrial dysfunction. Indeed, experimental models carrying desmin mutations exhibit abnormal mitochondrial morphology and reduced respiratory capacity (Alam et al., 2018). Although mitochondrial abnormalities have been described in desminopathy, the mechanistic interplay between mitochondrial dynamics and inflammatory signaling pathways remains incompletely understood. (Winter et al., 2016).

Desminopathy is clinically characterized by progressive skeletal muscle weakness and atrophy. However, there is currently limited evidence regarding the relationship between mitochondrial morphology and dynamics in this condition, as well as the potential interactions between inflammation and mitochondrial processes. In this context, mitochondrial dynamics are known to play a key role in maintaining skeletal muscle homeostasis. In desminopathy, mitochondrial abnormalities have been observed, yet the precise role of fission–fusion imbalance remains unclear.

Relationship between TLR4, mitochondrial dynamics and mitochondrial morphology

Emerging evidence suggests that mitochondrial morphology and the ultrastructure of cristae may be influenced by innate immune signaling pathways, including TLR4 activation (Castro-Sepulveda et al., 2023). This receptor, classically involved in recognizing pathogen-associated molecular patterns and initiating inflammatory responses, has been implicated in the regulation of mitochondrial integrity. Specifically, downstream effectors of TLR4, such as the mitogen-activated protein kinase MAPK family, including ERK, appear to modulate mitochondrial dynamics (Favaro et al., 2019).

ERK signaling regulates mitochondrial fission by modulating the phosphorylation of DRP1 at serine 616, enhancing DRP1 activity and facilitating mitochondrial fragmentation, in skeletal muscle, pharmacological inhibition of ERK was associated with a reduction in autophagic activity, potentially due to decreased mitochondrial fission mediated through lower levels of phosphorylated DRP1 and altered expression of fusion proteins like MFN2 (Yuan et al., 2023).

Additionally, proinflammatory cytokines such as interleukin-6 (IL-6) have been reported to upregulate ERK expression in myogenic cells. For instance, studies in C2C12 myotubes have demonstrated that IL-6 stimulation enhances ERK activation, which correlates with increased expression of mitochondrial fission-related proteins including DRP1 and Fis1 (Fix et al., 2019). Collectively, these findings support the hypothesis that inflammatory signaling pathways, especially those involving TLR4 and ERK, are closely linked to mitochondrial morphology and dynamics in skeletal muscle cells.

Inflammatory signaling is known to directly modulate mitochondrial dynamics, promoting remodeling through redox-dependent mechanisms and regulation of fission–fusion proteins. Therefore, the observed association is consistent with established links between inflammatory activation and altered mitochondrial

morphology, potentially exacerbating muscle pathology in conditions such as desminopathy.

However, to date, there is no available scientific literature reporting muscle-specific findings on inflammation, morphology and mitochondrial dynamics in desminopathy, particularly in relation to their potential role in clinical progression.

Potential key roles of peripheral blood mononuclear cells in desminopathy

PBMCs include lymphocytes (T cells, B cells, and NK cells) and monocytes, while NK cells belong to the lymphoid lineage, they are considered part of the innate immune response, whereas T and B lymphocytes are components of adaptive immunity (Verhoeckx et al., 2015). PBMCs are particularly valuable in research focused on peripheral immune responses, including chronic systemic inflammation (Alexovic et al., 2022). Recent evidence suggests that in older adults, PBMC composition may be negatively associated with skeletal muscle health. Specifically, a higher proportion of circulating monocytes has been linked to lower appendicular skeletal muscle mass index (ASMI) and reduced gait speed (de Sousa et al., 2023).

Studies from the 1980s highlighted the potential key role of mononuclear cells in various myopathies, particularly emphasizing the extent of their presence within skeletal muscle fibers (Engel & Arahata, 1986), (Kalovidouris et al., 1989). Within PBMCs, monocytes could potentially infiltrate damaged skeletal muscles, differentiating into macrophages to perform specific functions during injury, an activity regulated by cytokines (Theret et al., 2022).

In children with juvenile dermatomyositis, a myopathy, PBMCs display very high expression of genes associated with the type I interferon pathway as well as inflammatory signals mediated by IL-1, IL-19, and NF-κB (Roberson et al.,

2022). Moreover, the level of gene expression of cytokines in PBMCs correlated with clinical features in both skin and skeletal muscle, suggesting that PBMCs may serve as a peripheral biomarker of inflammatory activity to be explored in diseases affecting skeletal muscles.

It has been shown that PBMCs promote myogenic processes in 3D models. Specifically, in a three-dimensional system combining mesenchymal stem cells (hBM-MSCs) and myoblasts, PBMCs stimulated the expression of myogenic genes such as MyoD and desmin (Scala et al., 2022). PBMCs have not been extensively investigated in patients with desminopathy. However, growing evidence indicates that circulating immune cells can reflect systemic inflammatory signatures associated with muscle pathology. Given that skeletal muscle injury can release damage-associated molecular patterns (DAMPs), including HMGB1 and other alarmins capable of activating TLR4 signaling, it is biologically plausible that systemic immune cells may exhibit parallel inflammatory activation. Based on this rationale, we hypothesized that in desminopathy patients, inflammatory signaling pathways (particularly TLR4-mediated activation of NF-κB and MAPK cascades) may be upregulated both in skeletal muscle and in PBMCs. While this parallel activation has not been previously demonstrated in desminopathy, similar immune–muscle crosstalk mechanisms have been described in other chronic muscle disorders. Furthermore, activation of TLR4 signaling has been shown in experimental models to influence mitochondrial dynamics through ERK-mediated phosphorylation of DRP1 at Ser616, promoting mitochondrial fission. Therefore, systemic inflammatory activation could potentially contribute to mitochondrial remodeling in affected skeletal muscle. The proposed hypothesis is illustrated in Figure 1.7.

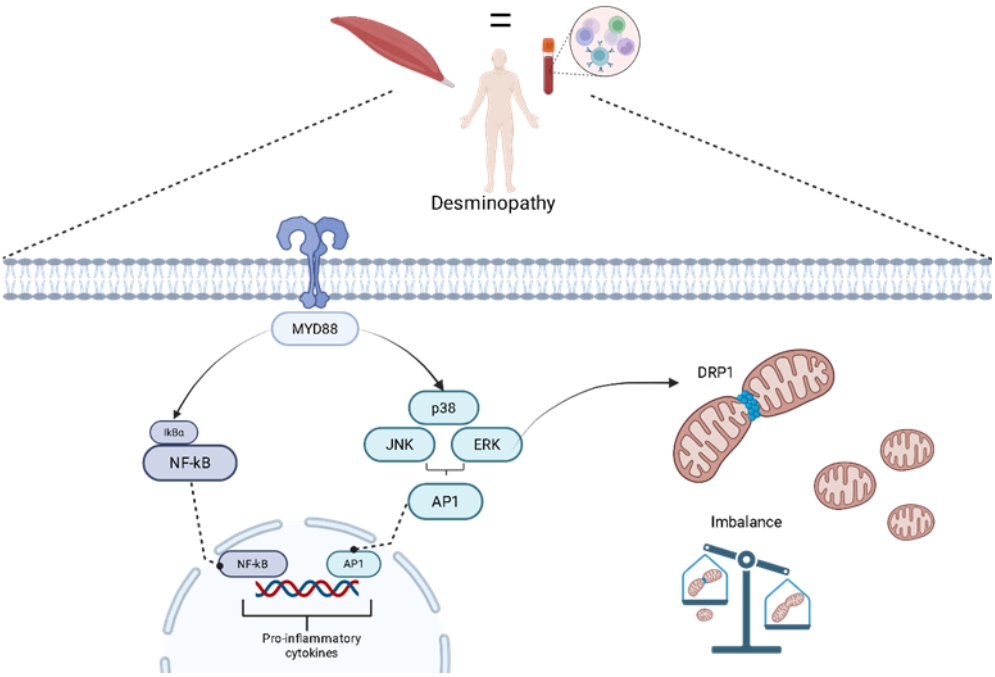


Figure 1.7 Illustrative hypothesis. In desminopathy patients, the inflammatory response is similar in both PBMCs and skeletal muscle, characterized by elevated activation of the TLR4 signaling pathway. This includes increased expression and activation of the MAPK and NF-κB pathway components, leading to enhanced cytokine production. In parallel, mitochondrial fission would be upregulated due to ERK-mediated phosphorylation of DRP1 at serine 616 (p-DRP1 S616), resulting in a greater proportion of smaller, fragmented mitochondria. Illustration created in Biorender.

Natural compounds as potential treatments for myopathies

Currently, desminopathy lacks a specific treatment to control its clinical progression. Moreover, there is a significant gap in knowledge regarding the characterization of TLR4 signaling, mitochondrial morphology, and dynamics in the context of desminopathy.

In addition, natural compounds often present favorable safety profiles and translational potential, making them attractive candidates for therapeutic exploration in chronic neuromuscular disorders where long-term intervention is required.

In the present work, we selected two specific natural compounds, namely urolithin A (UA) and epicatechin (EPI), as they have already been investigated intensively and have robust anti-oxidant and anti-inflammatory properties in other contexts than desminopathy(Denk et al., 2025; Ding et al., 2020; Prince et al., 2017; Ramirez-Sanchez et al., 2013). it is tempting to hypothesize that certain myopathies, amongst which desminopathy, could benefit from the action of those natural compounds.

Urolithin A

The first compound is UA, a natural metabolite generated by the gut microbiota from dietary ellagitannins and ellagic acid. These polyphenols are abundant in foods such as pomegranate, berries, and nuts. Accumulating evidence indicates that UA exerts beneficial effects on muscle and mitochondrial health. However, only approximately 40% of the global population has the microbial capacity to produce UA following the intake of ellagitannin-rich foods. This interindividual variability is attributed to differences in gut microbiota composition, although the precise mechanisms remain incompletely understood. Based on this limitation, direct UA supplementation has emerged as a translational strategy to bypass microbial dependency (D'Amico et al., 2021), (Figure 1.8).

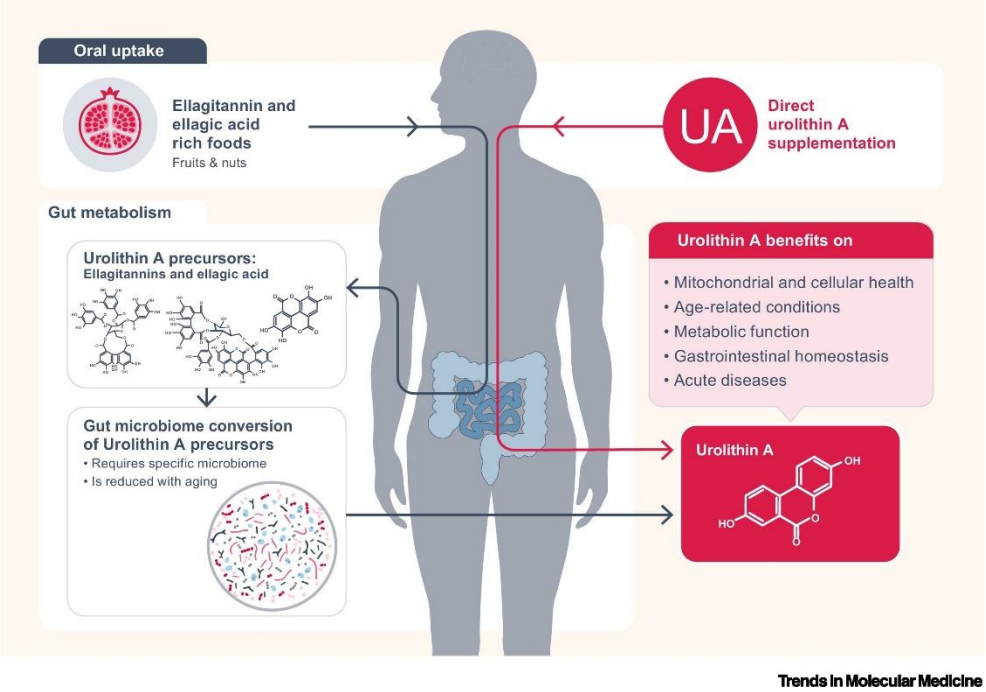


Figure 1.8 Urolithin A metabolism. The intake of foods rich in ellagic acid and ellagitannins can lead to the production of urolithin A (UA) through the activity of the gut microbiota. The intestinal microbiota mediates the capacity to convert UA, and aging has been shown to reduce UA production. Nevertheless, direct supplementation in humans is now available. Figure extracted from D'Amico et al. (2021).

One of the most well-documented effects of UA in the scientific literature is its association with the mitophagy process in C2C12 models and in rodents, where increased gene expression of the proteins PINK1 and Parkin has been observed. This effect is also consistent with an improvement in muscle function in the animal model (Ryu et al., 2016). In humans, the administration of UA is safe; specifically, doses of 500 mg to 1,000 mg have resulted in detectable plasma levels of UA (Reaching a peak concentration at 6 hours, and returning to values similar to baseline at 12 hours.). Moreover, after four weeks of supplementation, an increase in the expression of genes associated with mitochondrial health in the skeletal muscle, as well as elevated plasma levels of acylcarnitines, has been observed (Andreux et al., 2019). As mentioned, mitochondria play a critical role

in skeletal muscle homeostasis, suggesting potential benefits of UA in conditions involving mitochondrial alterations.

In muscular diseases, one study reported reduced expression of mitophagy-related genes in myoblasts derived from DMD patients (Luan et al., 2021). In line with this, the effects of urolithin A were tested in an mdx mouse model, homologous to human DMD. They found that UA treatment enhanced the canonical mitophagy pathway in skeletal muscle. They also demonstrated that PINK1 protein levels were increased in human myoblasts derived from DMD donors (Figure 1.9).

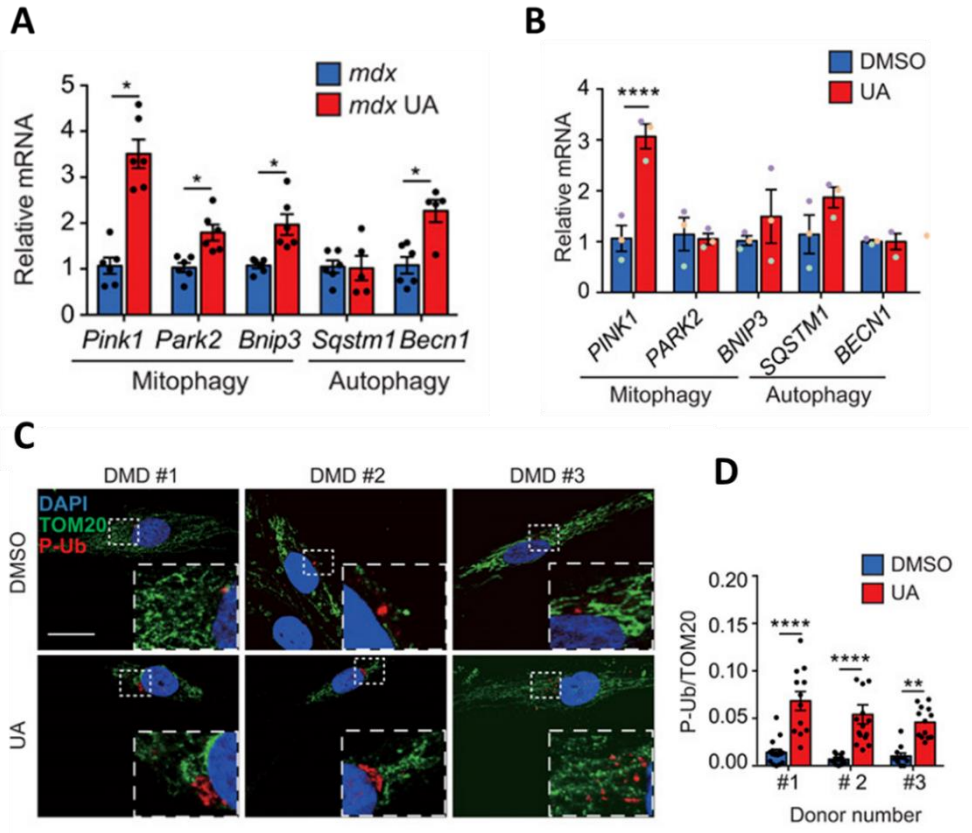


Figure 1.9 Effects of urolithin A on DMD, adapted from Luan et al. (2021). (A) mRNA levels of mitophagy (PINK1, PARK2, BNIP3) and autophagy (Sqstm1, Becn1) markers in forelimb muscles of mdx mice treated with or without UA (50 mg/kg/day, 10 weeks). UA significantly increased the expression of several genes compared to untreated mdx mice. (B) mRNA levels of mitophagy (PINK1, PARK2, BNIP3) and autophagy (SQSTM1, BECN1) markers in human myoblasts derived from DMD patients treated with DMSO or

UA (25 μM, 24 h). UA induced a marked upregulation of PINK1 expression. (C) Representative images of DMD patient–derived myoblasts treated as in (B), stained with phospho-ubiquitin (P-Ub, S65), TOM20 (mitochondria), and DAPI (nuclei). UA increased mitochondrial P-Ub signal, consistent with activation of the PINK1–Parkin pathway. (D) Quantification of the P-Ub/TOM20 signal in DMD myoblasts, showing a significant increase after UA treatment.

Regarding inflammation, it has been reported in a chondrocyte cell model that UA can reduce the levels of p38, JNK, and ERK proteins following stimulation in an IL-1β–induced inflammation model (Ding et al., 2020). Also, in the same model, UA increased protein expression related to the mitophagy process (D'Amico et al., 2022). Additionally, in an animal model of acute lung injury induced by LPS, UA was found to attenuate inflammation by downregulating MAPK protein expression after stimulation (Jiao et al., 2024). UA exerts anti-inflammatory effects by modulating NF-κB signaling, reducing pro-inflammatory cytokine production, and improving mitochondrial function, which secondarily dampens inflammation-associated cellular stress. Similarly, epicatechin has been shown to inhibit NF-κB activation, reduce oxidative stress, and improve endothelial and skeletal muscle function, thereby contributing to an anti-inflammatory response (Denk et al., 2025). This information may suggest that UA could represent a potential therapeutic tool in desminopathy. However, it is first essential to describe and understand the role of inflammation and its relationship with mitochondrial homeostasis in patients with desminopathy

UA supplementation in humans has been shown to be safe, including single doses up to 2,000 mg and repeated daily doses of 250–1,000 mg. In clinical trials of up to four months, UA has been associated with improvements in skeletal muscle mitochondrial health (Andreux et al., 2019). In older adults, 1,000 mg/day for four months improved muscular endurance (Liu et al., 2022). Additionally, 500 mg/day for four weeks increased muscle strength and functional performance markers in older adults (Singh et al., 2022). To date, there is no published evidence regarding UA supplementation specifically in desminopathy.

Epicatechin

The second compound is EPI, a natural flavonoid belonging to the flavan-3-ol subclass, which is found in various foods of plant origin, such as in cocoa (Borges et al., 2018). Its protective effects against inflammatory stimuli, particularly through modulation of the TLR4 signaling pathway, have been well documented. Specifically, in a rat model, EPI attenuated inflammation via TLR4 signaling after LPS-induced cortex renal inflammation (Prince et al., 2017). Additionally, in vitro findings showed that in macrophages exposed to LPS, EPI induced a reduction in the activation of TLR4 signaling (Chiou et al., 2016).

In the context of myopathies, it has been shown that a daily dose of 100 mg of EPI for a period of 8 weeks had positive effects in patients with Becker muscular dystrophy, specifically by increasing protein expression related to muscle regeneration processes such as myogenin, MyoD, Myf5, and MEF2a (McDonald et al., 2021).

Human supplementation with EPI has been associated with acute improvements in endothelial function at doses of 0.5–2 mg/kg in healthy men (Loke et al., 2008). In addition, chronic supplementation with 100 mg/day improved fasting insulin and HOMA-IR in adults aged 40–80 years (Dower et al., 2015). In skeletal muscle, a 7-day regimen of 1 mg/kg/day increased muscle strength and elevated the follistatin-to-myostatin ratio in young and adult participants (Gutierrez-Salmean et al., 2014). In sarcopenia, combining 1 mg/kg/day with resistance training enhanced strength gains and improved the follistatin/myostatin ratio (Mafi et al., 2019). In Becker muscular dystrophy, preliminary evidence from seven patients receiving 100 mg/day for eight weeks suggested improvements in markers of mitochondrial biogenesis and functional performance (McDonald et al., 2021).

Based on the available evidence regarding natural compounds such as UA and EPI, it is clear that both have been shown to regulate mechanisms associated with inflammation mediated by TLR4 signaling, while also exerting effects on

markers of mitochondrial homeostasis. On this premise, their role could represent an interesting area to explore in the context of muscular diseases.

General aims

To characterize the role of desmin in skeletal muscle function, with a particular focus on the interplay between TLR4 signaling, mitochondrial homeostasis, and systemic immune responses in desminopathy, and to evaluate the potential modulatory effects of natural compounds as exploratory therapeutic strategies

Specific aims

1. To characterize skeletal muscle function and inflammatory signatures skeletal muscle and PBMCs.

2. To investigate the effects of EPI on TLR4 signaling, and on mitochondrial dynamics and morphology, in cells derived from a patient carrying a DES mutation.

3. To determine the effects of UA on TLR4 signaling, and on mitochondrial dynamics and morphology in C2C12 myotubes exposed to LPS-induced inflammation.

Chapter II: Toll-Like Receptor 4 Signaling Dysregulation and Mitochondrial Morphology Alterations in Desminopathy

Manuscript in preparation

The chapter explained

Problem

Desminopathy is an uncommon myopathic disorder that usually emerges in adulthood, presenting with progressive muscle weakness and cardiomyopathy that collectively shorten life expectancy. No disease-specific therapy is currently available. Consequently, delineating its effects on skeletal muscle physiology and systemic homeostasis is essential for developing biomarkers and outcome measures to guide future therapeutic interventions.

Results

Desminopathy muscles displayed reduced ERK phosphorylation and normal fission–fusion protein levels, yet marked mitochondrial dysmorphology attributable to mutant, excess desmin. The consequent muscle damage and sarcopenia elicited systemic TLR4 dysregulation in PBMCs, whose mitochondrial morphology remained normal, consistent with their lack of desmin, despite balanced dynamics protein expressions. The resulting cytokine profile pointed to a shifted inflammatory set-point driven by altered TLR4 signalling.

Impact

These data support the hypothesis that inflammatory signaling pathways, including TLR4-associated mechanisms, may contribute to the molecular landscape of desminopathy. Further studies are required to determine whether modulation of these pathways can influence mitochondrial remodeling and muscle function in this condition.

Introduction

Desminopathy is an uncommon myopathy linked to mutations in the DES gene, encoding desmin, an essential intermediate filament protein that stabilizes striated muscle architecture (Kubanek et al., 2020). The disorder usually presents in adulthood, manifesting as steadily worsening muscle weakness that affects gait and overall movement. Cardiac involvement is frequent, with dilated cardiomyopathy and subsequent heart failure being the chief causes of premature death in desminopathy (Bermudez-Jimenez et al., 2024). While awareness of the condition has grown, treatment remains supportive and no disease-modifying therapies exist. Mechanistic details and the sequence of molecular maladaptation continue to elude complete clarification.

Historically, knowledge of desminopathy has expanded mainly through case reports documenting distinctive histopathological findings, particularly excessive desmin protein aggregation. However, such studies have seldom gone beyond descriptive pathology to explore underlying cellular and molecular pathways (Vajsar et al., 1993); (Yeow et al., 2022); (Maddison et al., 2012). Recently, experimental models have emerged, notably a CRISPR-Cas9-engineered rat harboring a mild DES mutation, recapitulating skeletal and cardiac phenotypes, thus facilitating mechanistic investigations (Langer et al., 2020). Complementary in vitro studies in immortalized myotubes expressing mutated desmin have highlighted a triad of pathological alterations: impaired mitochondrial respiration, dysregulated macroautophagy flux, and compromised proteasomal activity. Collectively, these findings implicate mitochondrial dysfunction as a central node in desminopathy pathophysiology (Bermudez-Jimenez et al., 2024); (Smolina et al., 2020).

Mitochondria are highly dynamic organelles undergoing continuous fusion and fission events. This ongoing remodeling, governed by the balance between these two processes, is essential to maintain mitochondrial and cellular homeostasis (Yapa et al., 2021). In skeletal muscle, precise regulation of mitochondrial dynamics is critical; imbalance frequently manifests as muscle wasting (Lei et

al., 2024). Central to the fission process is dynamin-related protein 1 (DRP1), particularly when phosphorylated at serine 616 (p-DRP1 S616). This phosphorylated form facilitates mitochondrial fragmentation and has been directly linked to muscle wasting (Nakano & Machida, 2022). Supporting this, increased p-DRP1 S616 levels have been reported in skeletal muscle of cancer patients experiencing cachexia, with in vitro studies further reinforcing its role in this severe wasting syndrome (Mao et al., 2021).

Alongside mitochondrial dynamics, inflammatory signalling pathways have emerged as critical modulators of skeletal muscle health, particularly via activation of key receptors such as Toll-like receptor 4 (TLR4). TLR4 plays a crucial role in inflammatory responses and its activation decreases myogenic processes (Ono & Sakamoto, 2017). Conversely, TLR4 inhibition during endotoxemia has demonstrated the potential to prevent muscle wasting, highlighting TLR4 as a therapeutic target in muscle disorders (Ono et al., 2020). Despite its recognized importance in various muscle pathologies, the involvement of TLR4 signalling in desminopathy remains largely unexplored.

Recent studies further underscore this relationship, demonstrating negative correlations between TLR4 signalling, mitochondrial fusion protein OPA1, and mitochondrial size in human skeletal muscle. Moreover, TLR4 activation by lipopolysaccharide (LPS) induces abnormal mitochondrial morphology in human myotubes (Castro-Sepulveda et al., 2023). Downstream signalling proteins related to TLR4, such as ERK, have also been linked to mitochondrial dynamics. Specifically, reduced ERK activity correlates with decreased DRP1 (S616) phosphorylation (Yuan et al., 2023). Thus, inflammatory signalling appears intimately linked with mitochondrial dynamics, both critical for maintaining skeletal muscle homeostasis.

Given these considerations, the interplay between inflammatory pathways and mitochondrial dynamics emerges as a potentially critical mechanism influencing muscle integrity and disease progression in desminopathy. Accordingly, the present study aimed to systematically analyse TLR4 signalling, mitochondrial

dynamics, and mitochondrial morphology in human skeletal muscle, the primary site affected in desminopathy, as well as in PBMCs to capture systemic responses.

Results

Skeletal muscle function and desmin protein aggregates in skeletal muscle from desminopathy patients

We assessed skeletal muscle functional capacity in patients with desminopathy using the Medical Research Council (MRC) scale (Ciesla et al., 2011). The left panel in Fig. 2.1A highlights the muscle groups most affected. Notably, patient DES5 maintained near-normal resistance across all movements. Consistent with this, DES5 did not rely on a walking stick in daily life, unlike the other four patients. Fig. 2.1B shows that skeletal muscle from desminopathy patients expresses more desmin than that of controls ($p=0.0483$), with corresponding immunoblot in Fig. 2.1C.

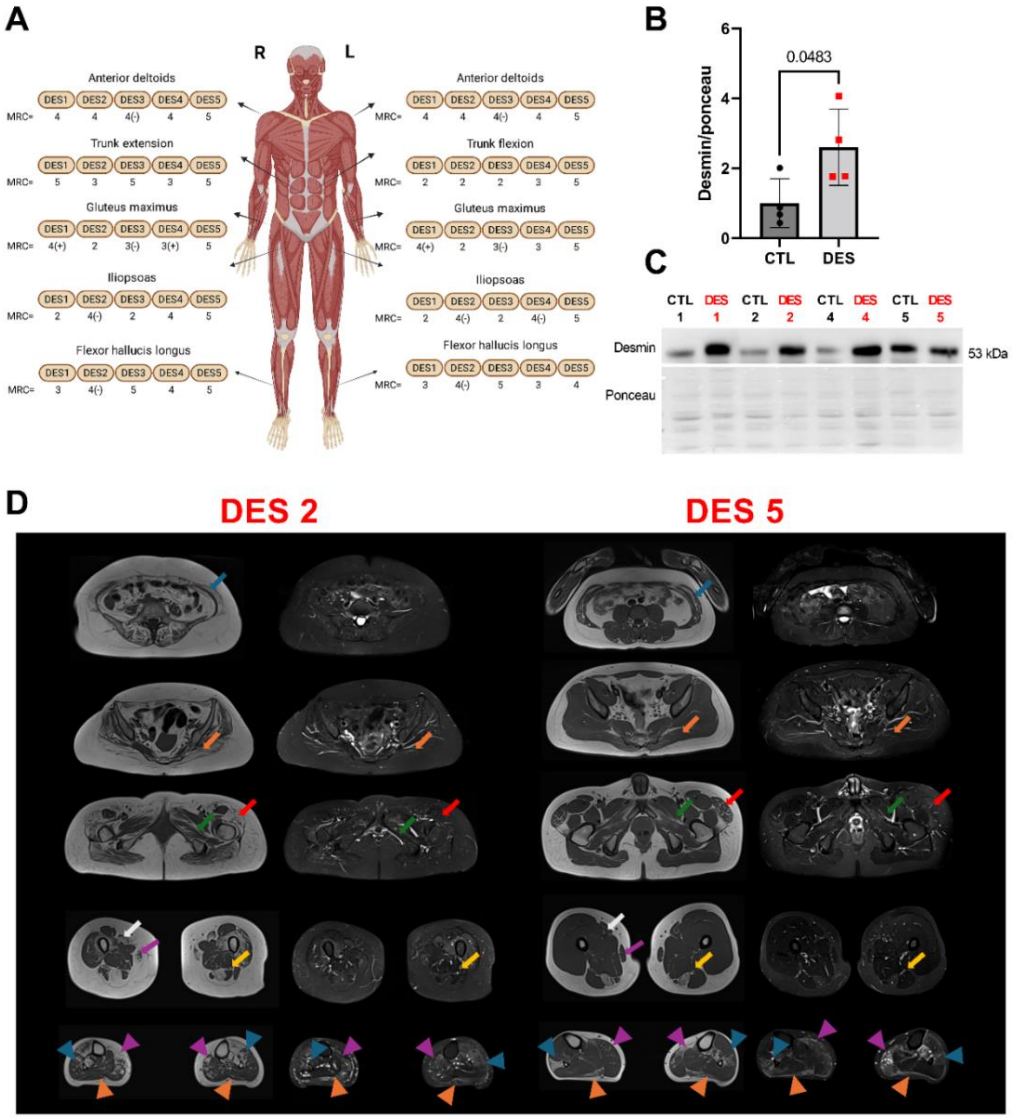


Figure 2.1 Clinical profile of desminopathy patients and skeletal muscle desmin expression. (A) Muscle groups most affected, scored with the Medical Research Council (MRC) scale (1 – 5). A plus sign (+) denotes full range of motion against gravity, whereas a minus sign (−) indicates that manual resistance is tolerated but the full range of motion is not achieved (n=5). (B) Quantitative comparison of desmin expression in skeletal muscle of patients versus controls (n=4/group). (C) Representative immunoblots for desmin in samples from desminopathy patients and matched controls. (D) Representative magnetic resonance image (MRI) axial T1-weighted images (left panels) from pelvis through legs depict fatty replacement, while matched STIR images (right panels) delineate edema. In the abdominal wall and pelvis, there is moderate fatty replacement of the external oblique muscles (blue arrows), mild proximal gluteal fatty infiltration with mild STIR hyperintensity (orange arrows), and moderate fatty replacement with proximal edema of the external obturators (green arrows).

In the thighs, there is mild fatty replacement with edema of the tensor fasciae latae (red arrows); mild fatty replacement of the sartorius (white arrow) and gracilis (purple arrow); and severe fatty replacement with diffuse, bilateral, symmetric edema of the semitendinosus (yellow arrows). In the legs, there is marked fatty replacement and edema of the peroneal (fibularis) compartment bilaterally—greater on the right (blue arrowheads); marked atrophy with fatty replacement and edema of the lateral gastrocnemius heads bilaterally (orange arrowheads); and mild-to-moderate fatty replacement with edema of the medial gastrocnemius heads (purple arrowheads). Student T-tests were used to test possible differences between groups. All data are reported as mean and standard deviation.

Skeletal muscle damage and insulin resistance markers

Age and anthropometric measures did not differ between the desminopathy patients and the matched controls (Table 2.1). Plasma total creatine kinase (CK-T) tended to be higher ($p=0.055$) and CK-MB, which is mainly found in cardiac muscle, was higher ($p=0.03$) in desminopathy patients compared to controls. Both values in desminopathy patients were higher than the upper clinical limit. Plasma creatinine concentrations were lower in desminopathy patients compared to controls ($p=0.03$) and fell below the clinical reference range. Despite similar and normal fasting insulin levels between the two groups, fasting glycaemia tended to be higher in desminopathy patients compared to controls ($p=0.06$), which remained within clinical limits. Consistently, HOMA-IR tended to be higher in desminopathy patients ($p=0.07$), approaching the upper limit of the clinical range. All other routine biochemical and lipid parameters were within normal limits in both groups, as detailed in Supplementary Table 2.1.

Table 2.1 Physical characteristics and biochemical profile.

	CTL (n=5)	**DES (n=5)**	**P value**		
Age (years)	39.2 ± 5.7	43.2 ± 7.8	0.38		
Height (cm)	179.5 ± 6.4	169.8 ± 6.9	0.80		
Weight (kg)	82.7 ± 18.5	75.5 ± 11.8	0.54		
BMI (kg/m^2)	28.3 ± 5.3	26.0 ± 2.3	0.47		
				Clinical range	
CK-T (IU/L)	142 ± 119	474 ± 342	0.055	30	135
Creatinine (mg/dL)	0.95 ± 0.3	0.49 ± 0.2	0.03	0.52	1.04
CK-MB	8 ± 7	39 ± 28	0.03	0	16
Insulin (uIU/mL)	6.1 ± 3.5	16.1 ± 8.5	0.15	2.6	24.9
Glycemia (mg/dL)	93 ± 7	101 ± 4	0.06	65	105
HOMA-IR	1.4 ± 0.9	4.2 ± 2.8	0.07		

BMI: body mass index; CK-T: total creatine kinase; CK-MB: creatine kinase muscle brain isoform; HOMA-IR: homeostatic model assessment for insulin resistance. Student T-tests were used to test possible differences between groups except for the following variables for which a Mann-Withney test was used as their distribution was not normal: CK-T, CK-MB and insulin. All data are reported as mean and standard deviation.

Supplementary Table 2.1 Biochemical and lipidic profile.

	CTL (n=5)	DES (n=5)	P value	Clinical range	
Uric nitrogen (mg/dL)	16 ± 4	13 ± 4	0.37	9	20
Uric acid (mg/dL)	4.6 ± 0.9	5.8 ± 1.3	0.12	3.5	8.5
Total bilirubin (mg/dL)	0.6 ± 0.1	0.4 ± 0.1	0.08	0.2	1.3
GOT (U/L)	28 ± 6	43 ± 19	0.16	17	59
Lactate dehydrogenase (U/L)	193 ± 36	261 ± 84	0.30	120	246
Phosphorous (mg/dL)	3.7 ± 0.3	4.2 ± 0.7	0.13	2.5	4.5
Total proteins (g/dL)	7.2 ± 0.5	7.8 ± 0.6	0.18	6.3	8.2
Albumin (g/dL)	4.2 ± 0.2	4.5 ± 0.4	0.57	3.5	5.0
Total cholesterol (mg/dL)	188 ± 15	176 ± 21	0.30		
HDL (mg/dL)	51 ± 17	42 ± 9	0.33	41	59
Triglycerides (mg/dL)	130 ± 33	129 ± 63	0.96		<200
VLDL (mg/dL)	26 ± 7	26 ± 13	0.96	0	40
LDL (mg/dL)	112 ± 19	108 ± 23	0.79	0	150
Cholesterol/HDL	4.0 ± 1.1	4.3 ± 1.0	0.63		

GOT: glutamic oxaloacetic transaminase; HDL: high-density lipoprotein; VLDL: very low-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol. Student T-tests were used to test possible differences between groups, except for the following variables for which a Mann-Withney test was used as their distribution was not normal: GOT, phosphorus, LDH and albumin. Data reported as mean and standard deviation.

TLR4 signalling and markers for mitochondrial dynamics in skeletal muscle

Markers for the TLR4 pathway were first analyzed in skeletal muscle (Fig. 2.2A). TLR4 (Fig. 2.2B) and MyD88 (Fig. 2.2C) protein expression did not differ between patients and controls. Looking at downstream effectors of the TLR4 pathway, the p-ERK/ERK ratio was found to be lower in desminopathy patients compared to controls (p=0.0175, Fig. 2.2D), whereas p-JNK/JNK (Fig.

2.2E) and p-p38/p38 (Fig. 2.2F) ratios were not different. Neither was it the case for p-IKKα/β and IκBα (Supplementary Fig. 2.1A, B and C). We then analyzed mitochondrial fusion-fission markers (Fig. 2.2G). OPA1 protein expression, a marker for mitochondrial fusion (Westermann, 2010) , tended to be higher in desminopathy patients compared to controls (p=0.0861, Fig. 2.2H). The fusion marker MFN2 (Fig. 2.2I) and the fission marker p-DRP1 (Pagliuso et al., 2018) (Fig. 2.2J) were not different between the 2 groups.

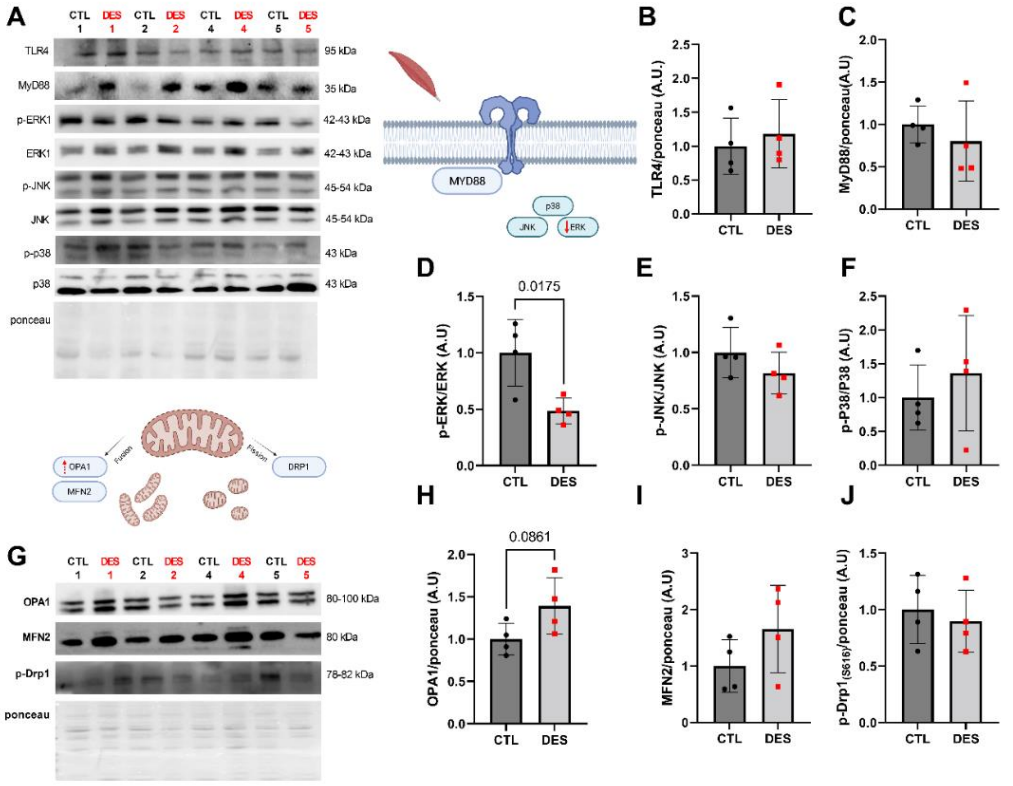
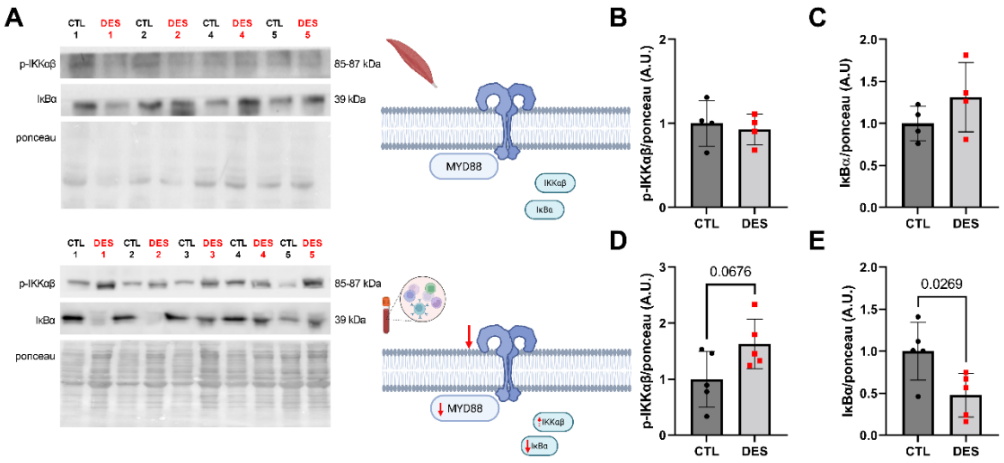


Figure 2.2 TLR4 signalling and mitochondrial dynamics in skeletal muscle of desminopathy patients and controls. A) Representative immunoblots for TLR4 signalling in skeletal muscle from desminopathy patients and matched controls. Protein expression of (B) TLR4, (C) MyD88, (D) p-ERK/ERK, (E) p-JNK/JNK and (F) p-p38/p38 (n=4/group). (G) Representative immunoblots for mitochondrial dynamics proteins in skeletal muscle from desminopathy patients and matched controls. Protein expression of (H) OPA1, (I) MFN2 and (J) p-DRP1 (n=4/group). Student T-tests were used to test possible differences between groups. All data are reported as mean and standard deviation.



Supplementary Figure 2.1 Dysregulated inflammatory signalling via the IKK complex in PBMCs, with normal protein levels of p-IKKα/β and IκBα in skeletal muscle. (A) Representative immunoblots for p-IKKαβ and IκBα in skeletal muscle and PBMCs from desminopathy patients and matched controls. (B) p-IKKαβ and (C) IκBα quantification from immunoblots in skeletal muscle of desminopathy patients and matched controls (n=4/group). (D) p-IKKαβ and (E) IκBα quantification from immunoblots in PBMCs of desminopathy patients and matched controls (n=5/group). Student T-tests were used to test possible differences between groups. All data are reported as mean and standard deviation.

Mitochondrial morphology alterations with desminopathy

As TLR4 signalling and ERK are known to regulate mitochondrial dynamics and morphology, we investigated whether structural changes took place in the skeletal muscle of desminopathy patients. Representative images of perinuclear and intermyofibrillar mitochondria are shown in Fig. 2.3A. Perinuclear mitochondria from desminopathy patients displayed larger area ($p<0.0001$, Fig. 2.3B) and perimeter ($p<0.0001$, Fig. 2.3C) than those from controls and tended to have a lower aspect ratio ($p=0.0637$, Fig. 2.3D), while circularity (Fig. 2.3E) and roundness (Fig. 2.3F) were unchanged. A comparable pattern emerged in the intermyofibrillar region: mitochondrial area (Fig. 2.3G) and perimeter (Fig. 2.3H) were larger in desminopathy patients than controls, and aspect ratio was reduced (Fig. 2.3I) (all $p<0.0001$). Consistent with these findings, circularity (Fig. 2.3J) and roundness (Fig. 2.3K) were both higher in desminopathy patients ($p<0.0001$).

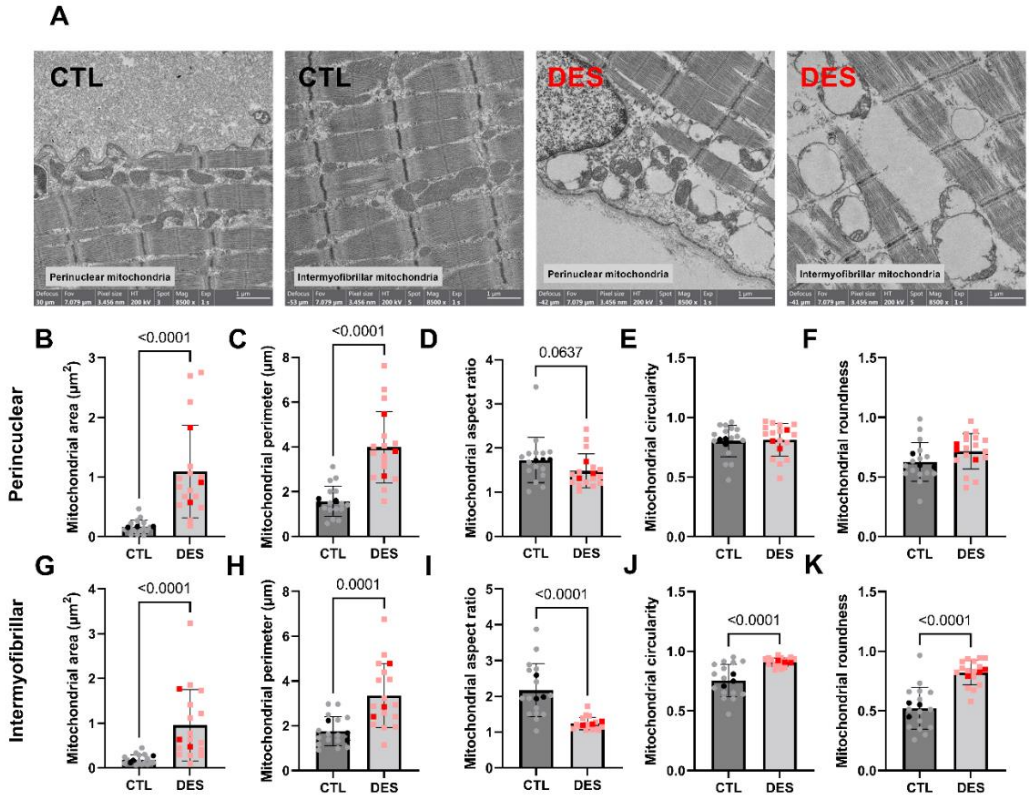


Figure 2.3 Mitochondrial morphology alterations in skeletal muscle with desminopathy. (A) Representative transmission-electron microscopy images (8500X) of perinuclear and intermyofibrillar mitochondria in skeletal muscle samples from desminopathy patients and matched controls. Area, perimeter, aspect ratio, circularity, roundness in perinuclear (B-F) and intermyofibrillar (G-K) mitochondria (n=3/group). A Mann-Withney test was used to test possible differences between groups, except for mitochondrial roundness, for which a Student T-test was used as it was the only normally distributed variable. All data are reported as mean and standard deviation.

TLR4 signalling and markers for mitochondrial dynamics in PBMCs

Systemic alterations in TLR4 signalling were assessed in PBMCs (Fig. 2.4A). TLR4 (p=0.002, Fig. 2.4B) and MyD88 (p=0.0266, Fig. 2.4C) protein expression was lower in desminopathy patients compared with controls. Conversely to skeletal muscle, the p-ERK/ERK ratio was not different between the 2 groups (Fig. 2.4D), whereas both p-JNK/JNK (p=0308, Fig. 2.4E) and p-p38/p38 (p=0.312, Fig. 2.4F) ratios were higher in desminopathy patients

compared to controls. We also noted a tendency toward higher p-IKKα/β (p=0.676, Supplementary Fig. 2.1D) and a lower protein expression of IκBα (p=0.269, Supplementary Fig. 2.1E). We then analyzed mitochondrial fusion-fission markers in PBMCs (Fig. 2.4G). Amongst the fusion markers, OPA1 protein expression did not differ between groups (Fig. 2.4H), while MFN2 protein expression tended to be lower in desminopathy patients compared to controls (p=0.0871, Fig. 2.4I). The fission marker p-DRP1 was not different between the 2 groups (Fig. 2.4J). Contrarily to skeletal muscle, mitochondrial morphology appeared normal in the PBMCs of desminopathy patients (Fig. 2.5).

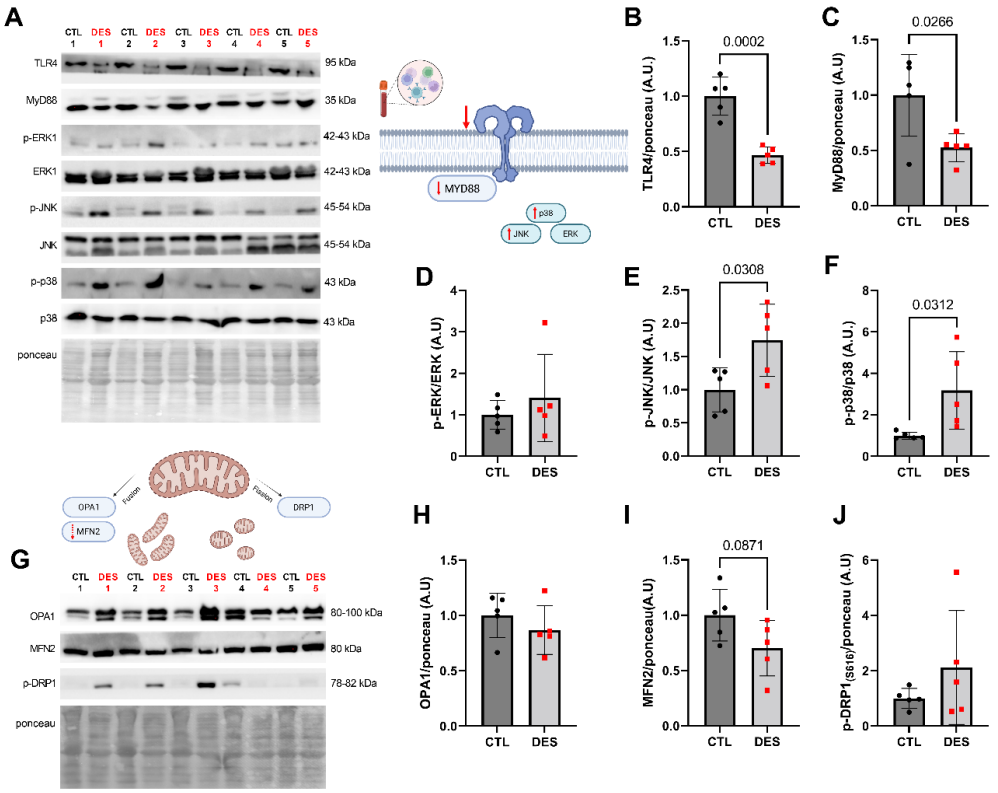


Figure 2.4 TLR4 signalling and mitochondrial dynamics in PBMCs of desminopathy patients and controls. A) Representative immunoblots for TLR4 signalling in PBMCs from desminopathy patients and matched controls. Protein expression of (B) TLR4, (C) MyD88, (D) p-ERK/ERK, (E) p-JNK/JNK and (F) p-p38/p38 (n=5/group). (G) Representative immunoblots for mitochondrial dynamics proteins in PBMCs from desminopathy patients and matched controls. Protein expression of (H) OPA1, (I) MFN2 and (J) p-DRP1

(n=5/group). Student T-tests were used to test possible differences between groups. All data are reported as mean and standard deviation.

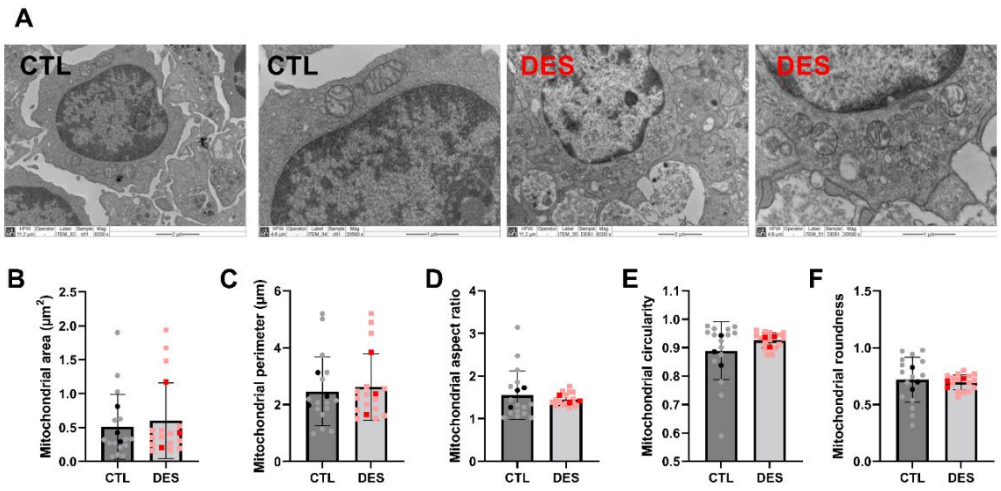
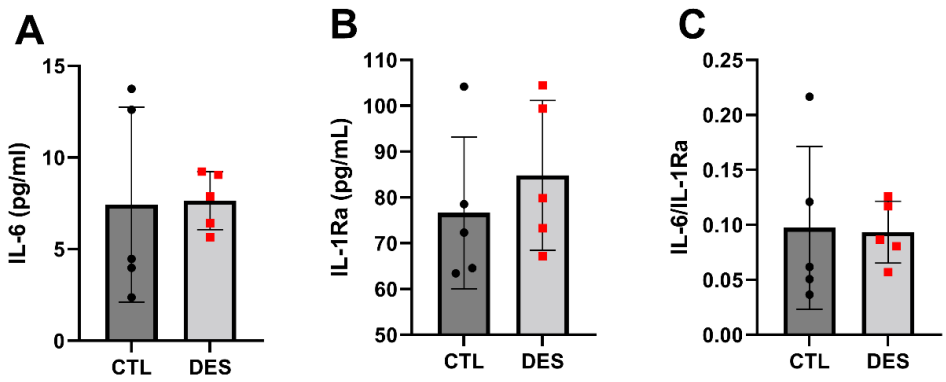


Figure 2.5 Mitochondrial morphology in PBMCs from desminopathy. (A) Representative transmission-electron microscopy images (8200X) of mitochondria in PBMC samples from desminopathy patients and matched controls. Mitochondria (B) area, (C) perimeter, (D) circularity, (E) aspect ratio and (F) roundness in PBMC samples of desminopathy patients (n=3/group) and matched controls (n=3/group). A Mann-Withney test was used to test possible differences between groups, except for mitochondrial roundness, for which a Student T-test was used as it was the only normally distributed variable. All data reported as mean and standard deviation.

Circulating inflammatory markers

Neither the plasma levels of the pro-inflammatory cytokine IL-6 (Supplementary Fig. 2.2A) nor the levels of the anti-inflammatory cytokine IL-1Ra (Supplementary Fig. 2.2B) differ between desminopathy patients and controls. Consequently, the IL-6/IL-1Ra ratio was not different between the 2 groups (Supplementary Fig. 2.2C).



Supplementary Figure 2.2 Circulating inflammatory markers in desminopathy patients and controls. Plasma (A) IL-6 levels, (B) IL-1Ra levels and (C) IL6/IL-1Ra ratio. Student T-tests were used to test possible differences between groups. All data are reported as mean and standard deviation.

Discussion

Desminopathy is a rare inherited myopathy, and recent research has intensified efforts to identify the cellular signaling pathways that drive its clinical progression. We observed increased desmin protein expression in skeletal muscle from desminopathy patients. Consistent with findings in murine DES-mutant muscle (Clemen et al., 2013; Liu et al., 2006), patients with desminopathy can show substantial fatty infiltration and edema. Frequently affected skeletal muscles include the gluteus maximus at the hip and the sartorius, gracilis, tensor fasciae latae and hamstrings in the thigh, and the process may extend further into the leg compartment (Wattjes et al., 2010). Our findings corroborate this distribution. Notably, patient DES5 exhibits the characteristic MRI pattern and associated fatty infiltration, yet remains clinically asymptomatic to date, whereas patient DES2 shows gait impairment with a visibly greater burden of fatty infiltration and associated edema. This divergence suggests mutation-dependent gradients of functional severity that align with the MRI phenotype. We further propose that the increased desmin abundance observed in skeletal muscle from desminopathy patients reflects ongoing synthesis coupled with impaired turnover and accumulation of a dysfunctional protein.

In the present context, the pro-inflammatory TLR4 signaling is of particular interest, as it is known to regulate skeletal muscle atrophy. Controlling the activity of that signaling could be a target to maintain skeletal muscle homeostasis (Fang et al., 2021; Hahn et al., 2020). In skeletal muscle from desminopathy patients, a reduced phosphorylated ERK, which is a downstream effector of the TLR4 pathway, coincided with pronounced alterations in mitochondrial morphology (Yuan et al., 2023). However, ERK fulfils several roles: it regulates mechanically-induced gene expression (Martineau & Gardiner, 2001), is essential for skeletal-muscle regeneration (Fu et al., 2015), modulates the IRS-1/PI3K–Akt axis during insulin resistance, thereby influencing glucose uptake (Hwang et al., 2013), contributes to the production

of pro-inflammatory cytokines (Frost et al., 2002), and regulates mitochondrial fission by phosphorylating DRP1 at Ser616 (Yuan et al., 2023). Based on those roles, we propose two, not mutually exclusive, explanations for the reduced p-ERK levels. First, desminopathy-associated sarcopenia may blunt mechanical load-dependent ERK activation in skeletal muscle, resulting in lower ERK phosphorylation (Williamson et al., 2003). Second, ERK down-regulation may be an adaptive response to keep mitochondrial fission under control. Dampening ERK could help maintain DRP1 activity within a physiological range (Favaro et al., 2019).

To obtain a complete view of mitochondrial dynamics, we also assessed fusion markers. MFN2 expression was unchanged, whereas OPA1 tended to increase in desminopathy. Because mitochondrial dynamics shape mitochondrial architecture, we extended our analysis and found clear morphological abnormalities in skeletal muscle of patients with desminopathy, i.e. larger mitochondria, with higher circularity and roundness. Those features reflect the structural role of desmin in stabilising mitochondrial networks. If desmin function is perturbed, protein accumulation and abnormal morphology can arise in both intermyofibrillar and perinuclear regions under stress (Capetanaki, 2002; Clemen et al., 2013). We therefore propose that OPA1 up-regulation, rather than MFN2 down-regulation, may partly offset the disturbed architecture, together with a relative restraint of DRP1 activity secondary to reduced p-ERK. Such adjustments might slow the progressive degeneration observed in desminopathy skeletal muscle. While it is not possible to test this in patients in vivo, this hypothetical mechanism would be worth to be further investigated in desminopathy models in rodents or in primary skeletal muscle cultures from patients.

Looking at systemic adaptations in PBMCs, TLR4 and MyD88 expression was reduced, while the phosphorylation of the downstream JNK and p38 MAPK proteins was increased. This pattern may reflect a compensatory down-regulation designed to restrain excessive activation of the pathway. It is

consistent with the idea that immune cells may respond to skeletal muscle injury, as reported in idiopathic inflammatory myopathies (Argyriou et al., 2023). To our knowledge, our results provide the first insights into this signaling axis in patients with desminopathy. Importantly, TLR4 can be internalized, ubiquitinated and degraded, together with its adaptor MyD88, during sustained stimulation, thereby preventing uncontrolled inflammation (Ciesielska et al., 2021; Tan et al., 2015). In dystrophic models, CK levels correlate with damage-associated molecular patterns such as HMGB1, which can contribute to muscle injury (Careccia et al., 2021). In line with this, our desminopathy cohort exhibited higher CK values, likely reflecting muscle damage due to altered desmin function. By contrast, lower serum creatinine levels were found in our patients and aligned with reduced MRC performance. In line with the latter, lower creatinine levels have been previously related to sarcopenia in a series of patients (Tang et al., 2020). Collectively, those observations support the usefulness of PBMCs as a minimally invasive tool to detect TLR4 dysregulation as well as other circulating markers, such as CK and creatinine, to screen for potential severe skeletal muscle atrophy with desminopathy.

Interestingly, TLR4 dysregulation in PBMCs did not result in abnormal PBMC morphology, as it was the case in skeletal muscle, highlighting the tissue specific regulation of mitochondria with desminopathy. Our results suggest that mitochondrial dynamics proteins in PBMCs succeed in maintaining an adequate balance under systemic cues. It remains to determine by which mechanisms the TLR4 pathway was modified in PBMCs of our patients as the main trigger, systemic inflammation, was not modified. Nor the plasma levels of the pro-inflammatory marker IL-6, nor those of the anti-inflammatory marker IL-1Ra, nor their ratio was different from healthy matched controls.

The strength of our study is the accessibility to skeletal muscle and blood samples from patients suffering from desminopathy to better characterize the pathology. This is not without limitations. First, the sample size was small (n = 5), reflecting the rarity of desminopathy and the practical constraints of

biopsy-based research. Blood was obtained from the five participants. Only four underwent muscle biopsy, and one specimen was scarce and therefore prioritized for Western blot analysis. Second, we did not perform in vivo assessments of mitochondrial function, which would have strengthened the interpretation of the morphological abnormalities. Finally, our work is relatively descriptive at this stage. As mentioned earlier in the discussion, future work should investigate nutritional or pharmacological compounds that modulate TLR4-mediated inflammatory signalling and mitochondrial dynamics in DES-mutant rodents or myotubes and/or PBMC culture models. Longitudinal studies using targeted interventions to track molecular adaptations and clinical trajectories are also warranted.

In summary, our findings bolster the evidence on structural abnormalities of skeletal muscle in desminopathy and, notably, highlight that those changes relate with patients' clinical manifestations and with a higher desmin expression in skeletal muscle. We propose that desminopathy features abnormal mitochondrial morphology in skeletal muscle driven by desmin mutation, while reduced ERK phosphorylation serves as a compensatory mechanism of mitochondrial fission under desminopathy-related stress in skeletal muscle. In parallel, muscle injury and sarcopenia are associated with systemic TLR4 dysregulation in PBMCs. Because PBMCs do not express desmin, their mitochondrial architecture is unlikely to be affected directly. Nonetheless, those cells may provide non-invasive biomarkers for therapeutic monitoring. Clarifying the interplay between inflammation and mitochondrial homeostasis in desminopathy therefore remains a priority for translational research.

Methods

Five patients diagnosed with desminopathy and five matched control patients (matched by physical characteristics, comorbidities, sex, and age) were included in this study. In the desminopathy cohort, two distinct families are represented: DES1, DES2, and DES3 are siblings (p.Arg350Pro mutation) (DES2 and DES3 are females), and DES4 and DES5 are siblings from another family (both males, p.Pro419Ser mutation). Controls were sex-matched; accordingly, CTL2 and CTL3 are females. All patients have a genetically confirmed autosomal-dominant DES mutation in exon 6.

The study consisted of three visits to the Exercise and Metabolism Laboratory at Finis Terrae University (Santiago, Chili). During the first visit, patients were recruited and informed about the study in detail. Those who agreed to participate then underwent clinical screening with the Medical Research Council (MRC) scale (Ciesla et al., 2011) and a comprehensive medical history interview. The second visit involved collecting skeletal muscle and blood samples in the fasted state at 9 am; and the final visit included medical monitoring of the incision site following the muscle biopsy. To assess skeletal muscle composition, axial T1-weighted (T1w) and T2-weighted short tau inversion recovery (T2-STIR) sequences were acquired on a 1.5-T Siemens MR system. The imaging volume extended from the pelvis to the ankles.

All of the subjects gave their written informed consent to participate in the study. The study was undertaken according to the Helsinki declaration, that was approved by the Finis Terrae University's scientific ethics committee (study ID: 23-030).

Blood samples

Blood samples were drawn and collected in 10-ml heparin EDTA tubes and subsequently centrifuged at 4000 rpm for 10 min at 4°C. Plasma was stored at -80°C. The clinical parameters of blood samples were measured by

chemiluminescence and dry chemistry in a clinical laboratory (Redlab, Chile). HOMA-IR was calculated using the following formula: (insulin (uIU/ml) x glycemia (mg/dL) / 405).

Skeletal muscle biopsy

Biopsies from the vastus lateralis of the dominant leg were obtained at the middle point between the anterior superior iliac spine and the patella. The modified Bergström needle technique with suction was used. After local anesthesia (2% lidocaine), a small incision was made into the skin and fascia, and the biopsy needle was inserted into the muscle. About 100 mg of tissue was withdrawn through manual suction. Excess blood, visible fat, and connective tissue was removed from the tissue. All biopsies were obtained at rest between 8 and 10 am. Half of the muscle tissue was immediately frozen in liquid nitrogen and stored at 80°C for Western blotting; the other half was used for transmission electronic microscopy.

PBMC isolation

PBMCs were isolated from approximately 10 mL of freshly drawn blood under sterile conditions by centrifugation on Ficoll-Histopaque 1077 gradients (Sigma, Milan, Italy) at 1500 rpm for 50 min at room temperature. After centrifugation, the intermediate phase containing PBMCs was carefully collected and resuspended in 8 mL of phosphate-buffered saline (PBS). Cells were washed three times by centrifugation at room temperature for 15 min each. A portion of the resulting cell pellet was fixed in 2% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7), while the remaining portion was frozen and stored at -80°C for subsequent western blot analysis.

Western blotting

Skeletal muscle and PBMC samples were homogenized in a lysis buffer containing: 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM EDTA, 20 mM NaF, 1 mM $Na_2P_2O_7$, 10% glycerol, 150 mM NaCl, 10 mM Na_3VO_4, 1 mM PMSF, and a protease inhibitor cocktail (Complete TM, Roche Applied Science). We used 15 µg proteins for each skeletal muscle sample and 40 µg proteins for each PBMC sample. Proteins were separated by SDS-PAGE and transferred to PVDF membranes with a 0.2 µm pore size (1620177, Bio-Rad). The following antibodies were used: TLR4 (293072, Santa Cruz Biotechnology), MyD88 (sc-74532, Santa Cruz Biotechnology), p-IKKα/β (Ser176/180) (2697S, Cell Signaling), IκBα (4814S, Cell Signaling), p44/42 Erk1/2 MAPK (4695S, Cell Signaling), p-p44/42 Erk1/2 MAPK (Thr202/Tyr204) (4377S, Cell Signaling), SAPK/JNK (9252S, Cell Signaling), p-SAPK/JNK (Thr183/Y185) (9251S, Cell Signaling), p38 MAPK (9212S, Cell Signaling), p-p38 MAPK (Thr180/Y182) (9215S, Cell Signaling), OPA1 (612606, BD Biosciences), MFN2 (ab56889, Abcam), p-DRP1 (Ser616) (3455, Cell Signaling). Protein expressions were normalised to Ponceau (BM-1492, Winkler) Red staining. The secondary antibodies were anti-rabbit IgG (7074P2, Cell Signalling) and anti-mouse IgG (31430, Invitrogen). ChemiDoc Imaging Systems was used to obtain images that were subsequently analyzed using the Image Lab software (Bio-Rad).

Interleukin analysis

Human IL-6 and IL-1Ra were measured by ELISA assays (R&D systems commercial kits; IL-6, D6050, and IL1Ra, DRA00B).

Transmission electron microscopy

Skeletal muscle and PBMC samples were processed in the Advanced Microscopy Facility, Faculty of Biological Sciences at Pontificia Universidad Católica de Chile. Sections of 80 nm were cut and mounted on a transmission electron microscope. Skeletal muscle tissue grids were examined with a Talos miscroscope (Thermo scientific) and PBMC grids with a Tecnai T12 microscope (Philips). Mitochondrial morphology was determined from 5 mitochondria per cell in each patient and all data were analyzed in Image-J.

Statistical analysis

Comparisons were performed using the parametric Student T-test or the non-parametric Mann-Whitney U-test, according to the normality or not of the data, respectively. The normality of the data sets was tested with the Shapiro-Wilk test. Protein analysis in PBMCs was conducted in all five desminopathy patients; however, skeletal muscle protein analysis included only four patients, as one patient did not undergo muscle biopsy for personal reasons. TEM analysis in muscle was performed with n=3, due to the difficulty in obtaining muscle samples from one patient with severe sarcopenia. Thus, morphological analysis in PBMCs was also conducted with the same three participants per group. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 9.3.0 software.

Chapter III: Effect of epicatechin consumption on the inflammatory pathway and mitochondria morphology in PBMC from a R350P desminopathy patient: A case report.

Germán Tapia-Curimil, Mauricio Castro-Sepulveda, Hermann Zbinden-Foncea

Physiological Reports: Volume 12, Issue 8

Keywords: Desminopathy, myopathy, epicatechin, PBMC, inflammation, TLR4 and mitochondrial dynamics

Abstract

Desminopathy R350P is a human myopathy that is characterized by the progressive loss of muscle fibre organization. This results in the loss of muscle size, mobility and strength. In desminopathy, inflammation affects muscle homeostasis and repair, and contributes to progressive muscle deterioration. Mitochondria morphology was also suggested to affect desminopathy progression. Epicatechin (Epi) - a natural compound found in cacao - has been proposed to regulate inflammatory signaling and mitochondria morphology in human and animal models. Hence, we hypothesise chronic Epi consumption to improve inflammatory pathway and mitochondria morphology in the peripheral blood mononuclear cells (PBMCs) of a desminopathy R350P patient. We found that 12 weeks of Epi consumption partially restored TLR4 signaling, indicative of inflammatory signalling and mitochondria morphology in the desminopathy patient. Moreover, Epi consumption improved blood health parameters, including reduced HOMA-IR and IL-6 levels in the desminopathy patient. This indicates that Epi consumption could be a useful tool to slow disease progression in desminopathy patients.

Keywords: Desminopathy, myopathy, epicatechin, PBMC, inflammation, TLR4 and mitochondrial dynamics.

Introduction

Desmin is a skeletal muscle structural protein that provides stability during muscle contraction. Mutations of the des gene can result in aberrant Desmin structures that may lead to desminopathy (DES), a type of myofibrillar myopathy (Kley et al., 2016). Myofibrillar myopathies are progressive skeletal muscle diseases characterized by progressive muscle dysfunction, muscle damage, the presence of protein aggregates, and inflammation. Acute muscle healing involves mounting an immune response that increase inflammation levels (Summan et al., 2006), which in turn contributes to progressive skeletal muscle atrophy (Mukund & Subramaniam, 2020) and affects the muscle's regenerative capacity.

Monocytes are a type of peripheral blood mononuclear cell (PBMC) that regulate the activity and expression of specific receptors that initiate inflammatory signaling pathways (Torres-Ruiz et al., 2020). The membrane protein Toll-like receptor 4 (TLR4) triggers an inflammatory response in cells. Upon activation, TLR4 first induces the myeloid differentiation gene 88 (Lu et al., 2008) and then initiates downstream signaling via either the IKK complex that induces IkBα activity, or via the mitogen protein kinases (MAPK) P38, JNK, and ERK. This culminates in NF-kB activation, interleukin transcription, and the generation of the systemic inflammatory response in cells (Li et al., 2019); (Rossol et al., 2011). The myokine IL-6, is produced in response to inflammation and other stressors, such as skeletal muscle damage (Welc & Clanton, 2013). Inflammatory responses are involved in myofibrillar myopathy repair (Rizzo et al., 2020), and affect mitochondria morphology in human skeletal muscle (Castro-Sepulveda et al., 2023). We showed that PBMCs from COVID-19 patients with acute inflammation present altered mitochondria morphology and mitochondria cristae architecture (Castro-Sepulveda et al., 2021; Castro-Sepulveda et al., 2022). Mitochondria are dynamic organelles that play an important role in highly active tissues, including skeletal muscle. Mitochondrial dynamics refers to mitochondria fusion/fission (Castro-Sepulveda et al., 2021),

which is essential for cell viability (Romanello & Sandri, 2015). Human PBMCs are responsive to metabolic states and substrate availability, and can be obtained less invasively than muscle biopsies (Castro-Sepulveda et al., 2021). PBMCs are thus well suited cell model to investigate the metabolic and inflammatory effects of potential pharmacological tools in desminopathy patients.

Epicatechin (Epi) is a flavonoid found in foods such as cacao. Epi administration affects oxidative stress, inflammatory pathways, mitochondria function, and muscle function in skeletal muscle (Zbinden-Foncea et al., 2022). Furthermore, Prince et al. (2017) demonstrated that Epi administration reduces TLR4 inflammatory signalling in the skeletal muscle of rats with systemic inflammation. Thus, we hypothesize that chronic Epi consumption by a desminopathy patient would reduce TLR4 inflammatory pathway signalling activity and thereby affect mitochondrial dynamics in the PBMCs.

Methods

Case presentation

The DES patient is a 56-year-old male with no family history of desminopathy. He was diagnosed in 2006 with desminopathy - R350P mutation of the des gene. His two main symptoms are: (a) severe muscular weakness in the lower extremities, the left leg is affected most severely; (b) muscular weakness in the upper left extremity (shoulder and shoulder girdle). The DES patient can only perform some of his basic of daily living activities (eating, sphincter control and some mobility for writing) independently and is bound to his electric wheelchair. His case has been previously clinically described by Monje et al. (2020). The control subject, the DES patient's 54-year-old brother, has no clinical desminopathy symptoms.

Epicatechin administration

The DES subject consumed cocoa powder rich in Epi (100 mg/day) mixed with yoghurt daily for 12 weeks. The control subject consumed just yoghurt for 12 weeks. The Epi content in the cocoa powder was determined by HPLC (634mg of Epi per 100g of cocoa powder).

Blood samples

Blood samples were drawn and collected in 10-ml heparin EDTA tubes and subsequently centrifuged at 4000rpm for 10min at 4°C. Plasma was stored at -80°C. The clinical parameters of blood samples were measured by chemiluminescence and dry chemistry in a clinical laboratory (Redlab, Chile).

PBMC isolation

PBMCs were separated under sterile conditions on Ficoll-Histopaque 1077 (Sigma, Milan, Italy) gradients from ~10ml of freshly drawn blood (see Castro-Sepulveda et al., 2022) to obtain ~80% lymphocytes. PBMCs were frozen and stored at -80°C for western blotting.

Western blotting

PBMCs were homogenized in a lysis buffer containing: 20mM Tris-HCl (pH 7.5), 1% Triton X-100, 2mM EDTA, 20mM NaF, 1mM Na2P2O7, 10% glycerol, 150mM NaCl, 10mM Na3VO4, 1mM PMSF, and a protease inhibitor cocktail (Complete TM, Roche Applied Science). Proteins were separated by SDS-PAGE and transferred to PVDF membranes with a 0.2 µm pore size (1620177, BIO-RAD). The following antibodies were used: TLR4 (293072, Santa-Cruz Biotechnology), MyD88 (sc-74532, Santa-Cruz Biotechnology), IKKα (2682S, Cell-Signaling), IKKβ (8943S, Cell-Signaling), p-IKKα/β (Ser176/180) (2697S, Cell-Signaling), IKBα (4814S, Cell-Signaling), SAPK/JNK (9252S, Cell-Signaling), p-SAPK/JNK (Thr183/Y185) (9251-S, Cell-Signaling), p38 MAPK (9212S, Cell-Signaling), p-p38 MAPK (Thr180/Y182) (9215S, Cell-Signaling), p44/42 Erk1/2 MAPK (137F5, Cell-Signaling), p-p44/42 Erk1/2 MAPK (Thr202/Tyr204) (197G2, Cell-Signaling), Optic atrophy 1 (Opa 1, 612,606, BD-Biosciences), p-DRP1 (Ser616) (3455, Cell-Signaling). Protein expressions were normalized to Ponceau (BM-1492, Winkler) Red staining and GAPDH (97166S, Cell-signaling). The secondary antibodies were anti-rabbit IgG (7074P2, Cell-signaling) and anti-Mouse IgG (31430, Invitrogen).

Interleukin analysis

Human IL-6 and IL-1Ra were measured by ELISA assays (Abcam commercial kit; IL-6, ab46042; IL1Ra, ab211650).

Transmission electron microscopy

PBMCs were prepared as previously described (Castro-Sepulveda et al., 2022). Sections of 80 nm were cut, mounted on electron microscopy grids, and examined using a transmission electron microscope (Philips, Tecnai 12 at 80 kV). Mitochondria density (%) and size (µm2) were assessed in 4 – 6 cells per subject, and 3– 10 mitochondria per cell. Mitochondria cristae density (cristae

number and length) was assessed as previously described (Castro-Sepulveda et al., 2021).

Data analysis

Western blot data are reported as arbitrary units (a.u). Protein content was normalized so that the control subject`s value obtained at the PRE measurement was set to 1.0 a.u. The colored area represents the random error, which corresponds to the changes over time (delta) between pre- and post-measurements of the control subject. This analysis was performed based on the high or low response to a stimulus previously reported by Dankel and Loenneke (2020).

Results

In the Control, TLR4 decreased from 1.0 a.u PRE to 0.8 a.u POST, whilst in the DES subject TLR4 increased from 0.3 a.u PRE to 1.1 a.u POST (Fig 3.1A). MyD88, decreased in the Control from 1.0 a.u PRE to 0.61 a.u POST, but in the DES subject increased from 0.77 a.u PRE to 1.52 a.u POST (Fig 3.1B).

In the Control p-IKKαβ decreased from 1.0 a.u PRE to 0.23 a.u POST, whilst the DES subject increased from 0.02 a.u PRE to 1.12 a.u POST (Fig 3.1C). IKBα decreased in the Control from 1.0 a.u to 0.83 a.u POST, whilst the DES subject increased from 0.35 a.u PRE to 1.60 a.u POST (Fig 3.1D).

In the Control, the p-P38/P38 ratio decreased from 1.0 a.u PRE to 0.21 a.u POST, whilst the DES subject showed a more pronounced decrease from 9.32 a.u PRE to 0.48 a.u POST (Fig 3.1E). The p-ERK/ERK ratio decreased in the Control from 1.0 PRE to 0.98 POST= 0.98 but increased in the DES subject from 1.13 a.u PRE to 1.18 a.u POST (Fig 3.1F). The p-JNK/JNK ratio increased in the Control from 1.0 a.u to 1.18 POST but decreased in the DES subject from 1.92 a.u PRE to 0.70 a.u POST (Fig 3.1G).

The inflammatory cytokine IL-6 returned to normal levels in the DES patient after Epi consumption (PRE= 11.1 pg/mL, POST= 18.7 pg/mL) with respect to the Control (PRE= 17.0 pg/mL, POST= 13.1 pg/mL; Fig 3.1H). Anti-inflammatory cytokine IL-1Ra increased in DES patient after Epi consumption (PRE= 394.1 pg/mL, POST= 593.1 pg/mL) with respect to the Control (PRE= 340.1 pg/mL, POST= 248.6 pg/mL; Fig 3.1I).

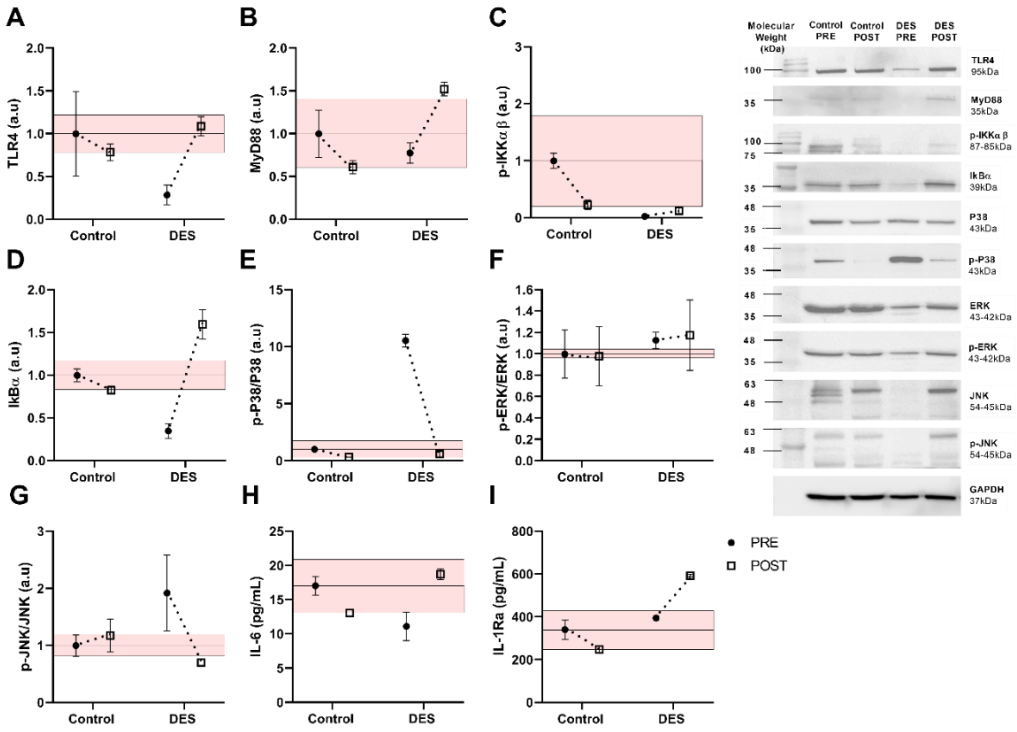


Figure 3.1 Inflammatory signaling in PBMCs and cytokine levels of the control subject and DES patient. A) TLR4, B) MyD88, C) p-IKKαβ, D) IkBα, E) p-P38/P38, F) p-ERK/ERK, G) p-JNK/JNK. Protein levels obtained by western-blot; H) IL-6 and I) IL-1Ra, plasma concentration obtained by ELISA. The colored area represents the random error.

Mitochondria remained larger in the DES subject after Epi consumption (PRE=0.18 µm, POST= 0.19 µm), with respect to the Control (PRE=0.11 µm, POST= 0.11 µm; Fig 3.2B). Cellular mitochondria number was unaffected in the DES subject (PRE= 6, POST= 6), but reduced in the control subject (PRE= 7, POST= 5; Fig 3.2C). Mitochondria density per total area remains in the random error range in DES (PRE=7.69 %, POST= 6.16 %) and control (PRE=5.85 %, POST= 3.69 %; Fig 3.2D). Mitochondria cristae number relative to mitochondria size increased in DES but (PRE=11.9 n/µm, POST= 16.8 n/µm) did not reach control levels (PRE=24.4 n/µm, POST= 27.7 n/µm; Fig 3.2E). Mitochondria cristae grew larger in DES (PRE=0.24 µm, POST= 0.27 µm) than the control (PRE=0.21 µm, POST= 0.25 µm; Fig 3.2F).

Mitochondrial dynamics was evaluated by determining p-DRP1 and Opa1 levels. p-DRP1 levels increased in both the DES (PRE= 0.99 a.u, POST= 4.95 a.u) and the Control subject (PRE= 1.0 a.u, POST= 5.66 a.u; Fig 3.2G). OPA1 levels decreased in the control subject (PRE=1.0 a.u, POST= 0.068 a.u), and the DES subject (PRE= 0.0 a.u, POST= 0.064 a.u; Fig 3.2H). In the control subject the OPA1/p-DRP1 ratio decreased (PRE= 1.0 a.u, POST= 0.004 a.u), whereas in the DES subject it increased to the normal values (PRE= 0.0 a.u, POST= 0.005 a.u; Fig 3.2I).

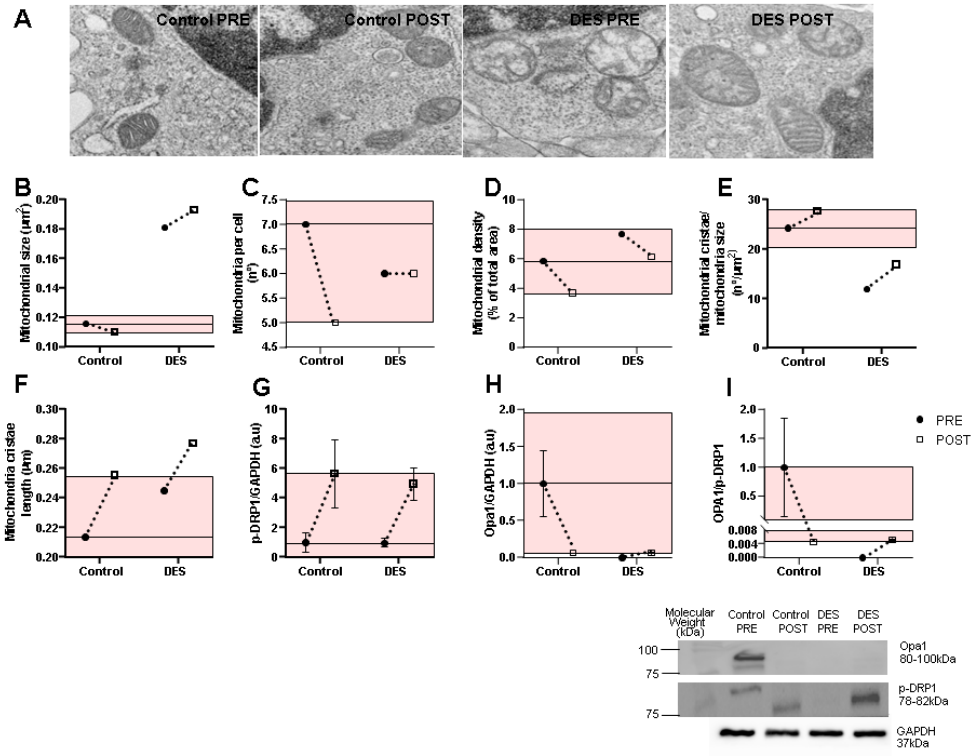


Figure 3.2 Mitochondrial morphology and mitochondrial dynamic protein levels. A) Representative image of mitochondria from PBMCs of the DES patient and the control subject, B) mitochondrial size, C) cellular mitochondrial number, and D) mitochondrial density, E) mitochondrial cristae relative to mitochondrial size, F) mitochondrial cristae length, G) p-DRP1, H) OPA1, and I) ratio OPA1/p-DRP1. The colored area represents the random error by change ratio of control between PRE and POST.

C reactive protein (CRP) levels increased in the DES patient from 8.6 mg/dL PRE to 12.7 mg/dL POST, but remained constant in the control (6 mg/dL PRE and 6.3 mg/dL POST). Creatinine levels were unchanged in control and DES. HOMA-IR scores decreased in the DES patient from 10.84 PRE to 8.88 POST, as in the Control subject from 2.0 PRE and 1.71 POST (Table 3.1).

Table 3.1 Clinical parameters of blood samples

	Control			DES			Clinical value	
	PRE	**POST**	**Change**	**PRE**	**POST**	**Change**	**Lower**	**Top**
CRP (mg/L)	6.0	6.3	0.3	8.6	12.7	4.1	<10	
Creatinine (mg/dL)	0.85	0.80	-0.05	0.25	0.20	-0.05	0.66	1.25
Insulin (uUI/ml)	7.3	6.5	-0.8	41.4	28.6	-12.9	2.6	24.9
Glycemia (mg/dL)	111	106	-5	106	126	20	75	110
HOMA-IR	2.00	1.71	-0.28	10.84	8.88	-1.95		
Uric nitrogen (mg/dL)	14	10	-4	22	14	-8	9	20
Uric acid (mg/dL)	4.8	5.5	0.7	6	7.3	1.3	3.5	8.5
Total bilirubin (mg/dl)	0.60	0.36	-0.24	0.34	0.42	0.08	0.20	1.30
GOT (U/L)	<13	19		16	16	0	17	59
Lactate dehydrogenase (U/L)	200	160	-40	168	155	-13	120	246
Phosphorous (mg/dL)	2.8	2.6	-0.2	2.8	3.6	0.8	2.5	4.5
Total proteins (g/dL)	7.6	7.2	-0.4	6.9	6.9	0	6.3	8.2
Albumin (g/dL)	>5.1	4.8		4.4	4.3	-0.1	3.5	5.0
Total cholesterol (mg/dL)	179	157	-22	160	164	4		
Col HDL (mg/dL)	54	49	-5	42	38	-4	41	59
Triglycerides (mg/dL)	119	96	-23	183	174	-9		
Cholesterol VLDL (mg/dL)	24	19	-5	37	35	-2	0	40
Cholesterol LDL (mg/dL)	101	89	-12	81	91	10	0	150
Cholesterol /HDL	3.3	3.2	-0.1	3.8	4.3	0.5		

CRP, C reactive protein; HOMA-IR, Homeostatic model assessment for insulin resistance; GOT, Glutamic oxaloacetic transaminase; Col HDL, cholesterol high-density lipoprotein; VLDL, very-low-density lipoprotein; LDL, Low-Density Lipoprotein; HDL, high-density lipoprotein.

Discussion

Our results indicate that 12 weeks of Epi consumption restored the level of certain inflammatory pathway proteins, such as TLR4 and p-P38/P38, in the DES patient's PBMCs. Similarly, IL-6 returned to normal levels, and the anti-inflammatory cytokine IL-1Ra levels became even higher in the DES patient than in the control. After the Epi intervention, the DES patient increased mitochondria cristae length and fusion/fission mitochondrial dynamic ratio proteins. Creatinine was unaffected in the DES patient, Furthermore, Epi consumption decreased HOMA-IR in the DES patient.

Inflammation is a crucial response in myopathies to promote the growth and repair of skeletal muscle (Mukund & Subramaniam, 2020). In mouse models of Duchenne muscular dystrophy, TRL4 was identified to be critical in muscle tissue repair (Petrof, 2022). In light sepsis in humans, an acute inflammatory response inducing the TLR4 pathway and inflammatory cytokine production is beneficial, but in severe sepsis these components are dysregulated, preventing inflammatory cytokine production by the PBMCs (Brunialti et al., 2006). Our study provides the first insights of Epi's effects on inflammatory signaling and cytokine production in a desminopathy patient's PBMCs, namely that certain inflammatory signal proteins return to their normal levels.

Accordingly, the grape polyphenol Malvidin diminished the inflammatory response in human PBMCs following LPS in vitro stimulation (Bastin et al., 2020). Barrera-Reyes et al. (2019) showed that healthy young adults consuming Epi acutely from cocoa modulated their PBMC's inflammatory response. Our data indicates the DES patient's IL-6 levels rose after Epi administration, which concurs with his increased TLR4 levels. This suggests that polyphenols, specifically Epi, may increase the protein levels of TLR4 pathway components in PBMCs, which is necessary to initiate tissue repair (Cooke, 2019).

Fix et al. (2019) showed that chronically elevated IL-6 levels in C2C12 myotubes induces DRP-1 and FIS-1 expression, which are both involved in mitochondria protein dynamics. Similarly, to Navarrete-Yanez et al. (2020) who

showed Epi to increase plasma IL-1ra in mice with inflammation, our data indicates that IL-1ra increased after Epi consumption in the DES subject compared to Control. Similarly, (Barrera-Reyes et al., 2019); Bartsakoulia et al. (2018) found an imbalance in mitochondria fission/fusion dynamics, and showed aberrant mitochondria cristae in fibroblasts from a human myopathy patient. Interestingly, our DES patient has aberrant PBMC mitochondria cristae prior to, but increased cristae length following Epi consumption. This suggests that Epi affects mitochondria fusion/fission dynamics, morphology and mitochondria cristae density in the desminopathy patient PBMCs.

A potential limitation is that we did not have a positive control or plasma marker to indicate Epi absorption. However, Actis-Goretta et al. (2013) showed that almost 46% of Epi was absorbed, so we speculate that a similar percentage was absorbed during the 12 weeks of Epi supplementation.

In conclusion, our results indicate that Epi consumption could regulate inflammatory signaling in desminopathy patients, and restore it to normal levels. This supports the idea that Epi affects inflammation to control systemic homeostasis of the immune response and be a potential trigger for repair. A future line of research would be to see if similar results can be obtained in the skeletal muscle of patients with desminopathy after Epi consumption, considering that these results correspond to a single patient.

Chapter IV: Urolithin A regulates TLR4-mediated inflammation induced by LPS by modulating the MAPK pathway and mitigating mitochondrial abnormalities in C2C12 myotubes

Germán Tapia-Curimil, Mayalen Valero-Breton, Ana-María Figueroa, Louise Deldicque, Hermann Zbinden-Foncea

Manuscript submitted

Abstract

Introduction: The Toll-like receptor 4 (TLR4) pathway plays a critical role in mediating inflammatory responses and regulates mitochondrial structural adaptations. By inducing mito- and autophagy, urolithin A (UA), a recent natural compound, may potentially enhance mitochondrial homeostasis and health.

Objective: To examine the effects of UA on key components and regulators of TLR4 signaling, mitochondrial dynamics and autophagy, and to analyze mitochondrial morphology after LPS incubation in C2C12 myotubes.

Methods: On day 6 of differentiation, C2C12 myotubes were incubated with LPS (1µg/ml) for 3h and/or UA (50µM) for 24h. Protein levels associated with TLR4 signaling, mitochondrial dynamics and autophagy were determined by Western blot, and mitochondrial morphology was assessed using electron microscopy.

Results: LPS-induced inflammation activated downstream TLR4 signaling, by increasing the expression of IκBα ($p=0.05$) and the phosphorylation of ERK ($p=0.003$), p38 ($p=0.04$) and JNK ($p=0.006$) and tended to increase the phosphorylation of TAK1 ($p=0.06$) and IKKαβ ($p=0.07$); 2) LPS reduced mitochondrial area ($p=0.05$) and circularity ($p=0.0004$) and tended to reduce mitochondrial perimeter ($p=0.07$); 3) LPS increased the phosphorylation of the fission marker DRP1 ($p=0.009$); 4) UA completely prevented, or at least attenuated, all previous effects induced by LPS.

Conclusion: Our findings suggest that UA partially mitigates LPS-induced inflammation by modulating the TLR4 signaling pathway and mitochondrial dynamics. Thus, UA may represent a promising therapeutic agent for conditions associated with mitochondrial dysfunction and inflammation, highlighting its potential role in promoting skeletal muscle health.

Keywords: TLR4, inflammation, Urolithin A, mitochondrial homeostasis and myotubes.

Introduction

Urolithin A (UA) is a gut microbiota-derived metabolite that is produced upon the breakdown of ellagitannins - polyphenols found in foods such as pomegranates and berries (Tomás-Barberán et al., 2017). This compound has gained significant attention in recent years due to its potential health benefits, particularly for muscle function and mitochondrial health (D'Amico et al., 2021). UA has been shown to stimulate mitophagy, a cellular process that removes dysfunctional mitochondria through mitophagolysosomes, mediated by proteins involved in both canonical and non-canonical pathways (Palikaras et al., 2018), thereby stimulating mitochondrial biogenesis to maintain cellular mitochondrial homeostasis and respiratory capacity (Faitg et al., 2024). Mitophagy is regulated by both canonical and non-canonical pathways, involving proteins such as PTEN induced putative kinase 1 (PINK1), Parkin, and Bcl-2 interacting protein 3 (BNIP3) (Palikaras et al., 2018). One recent study has reported that UA can restore mitochondrial and muscle function in murine models of muscular dystrophy, where mitochondrial dysfunction is a hallmark feature (Luan et al., 2021).

Mitochondria are highly dynamic organelles that undergo constant fusion and fission processes. These processes are essential for maintaining mitochondrial health and function. Fusion allows for the exchange of mitochondrial contents and can help mitigate damage, while fission is important for the segregation of damaged mitochondria for subsequent degradation through mitophagy (Picca et al., 2023). The balance between mitochondrial fusion and fission is essential for cellular homeostasis, and its disruption has been implicated in various pathological conditions, including myopathies (Tapia-Curimil et al., 2024). Dynamin-related protein 1 (DRP1) is a key regulator of mitochondrial fission. The phosphorylation of DRP1 (p-DRP1) plays a crucial role in its activation and recruitment to the mitochondrial outer membrane, where it facilitates fission (Chen et al., 2023). Interestingly, recent studies have shown that mitophagy can

be associated with p-DRP1 regardless of mitochondrial morphology (Han et al., 2020), suggesting a complex interaction between mitochondrial dynamics and quality control mechanisms.

Mitochondrial damage is sensed by the inflammasome, which triggers IL-1β production and the recruitment of the innate immune system to the affected tissues (Zhou et al., 2011). Also, a damage-associated molecular pattern (DAMP) can enter the circulation, where it binds to and activates pattern recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4) (Lu et al., 2008). Mitophagy inhibition elevates reactive oxygen species (ROS) production and inflammasome activity. TLR4 signalling triggers the activation of the NF-κB and MAPK pathways, leading to the production of pro-inflammatory cytokines such as tumoral nuclear factor alpha (TNFα), interleukin 1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and type-I interferons to mount a strong inflammatory response (Lu et al., 2008). Lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, is a potent activator of TLR4 signaling. Recent studies have shown that LPS-induced inflammation can affect mitochondrial morphology in human myotubes, highlighting the interconnection between inflammation and mitochondrial dysfunction in muscle cells (Castro-Sepulveda et al., 2023).

UA has been found to have anti-inflammatory effects in various disease models via, amongst others, the inhibition of the NF-κB pathway (Ding et al., 2020); (Tao et al., 2021). This suggests that UA may have a dual role in protecting muscle cells, namely by stimulating mitophagy and by mitigating inflammation. Given the complex interplay between inflammation, mitochondrial dysfunction, and muscle health, we hypothesized that UA could reduce the activation of components of the TLR4 inflammatory pathway following acute LPS stimulation. Furthermore, UA could restore optimal mitochondrial morphology in C2C12 myotubes, potentially through its effects on mitophagy. To investigate this, we examined the effects of UA on key components and regulators of TLR4

signaling, mitochondrial dynamics and autophagy, and analyzed mitochondrial morphology.

Materials and methods

Cell culture

C2C12 murine skeletal muscle myoblasts (ATCC) were maintained in complete DMEM (Dulbecco's modifed Eagle's medium) supplemented with 10% FBS and 1% antibiotic/antimycotic and incubated at 37°C and 5% CO2. Once at 80% confluence, the culture medium was changed to 1% horse serum to induce the differentiation process.

Experimental procedures

On the sixth day of differentiation, the cells were treated with 50µM UA (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one) for 24h, using DMSO as the vehicle, as previously described (Ryu et al., 2016). Cells were treated with LPS at 1µg/ml for 3h (PBS as the vehicle) from 21h with UA. The four experimental conditions were: 1) CTL, 2) LPS, 3) UA and 4) UA +LPS.

Western blotting

C2C12 myotubes were homogenized in a lysis buffer containing: 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM EDTA, 20 mM NaF, 1 mM Na2P2O7, 10% glycerol, 150 mM NaCl, 10 mM Na3VO4, 1 mM PMSF, and a protease inhibitor cocktail (Complete TM, Roche Applied Science). Fifteen µg of protein were loaded per sample, separated by SDS-PAGE and transfered to a PVDF membrane with a pore size of 0.2µm (1620177, BIO-RAD). The following antibodies were used: TLR4 (sc-293072, Santa-Cruz Biotechnology), MyD88 (sc-74532, Santa-Cruz Biotechnology), p-TAK1 (Ser412) (9339S, Cell Signaling), p-IKKα/β (Ser176/180) (2697S, Cell Signaling), IκBα (4814S, Cell Signaling), p65 NF-κB (8242S, Cell Signaling), p-SAPK/JNK (Thr183/Tyr185) (9251S, Cell Signaling), p-p38 MAPK (Thr180/Tyr182) (9215S, Cell Signaling), p-p44/42 ERK1/2 MAPK (Thr202/Tyr204) (4695S, Cell Signaling), p-DRP1 (Ser616) (4494S, Cell Signaling), OPA1 (612606, BD Biosciences), PINK1 (BC100-494SS, Novus Biologicals), p-AMPK (Thr172) (2531S, Cell Signaling), p62 (5114S, Cell Signaling), p-ULK (Ser757) (14202S, Cell

Signaling), LC3I-II (4108S, Cell Signaling). Subsequently, the membranes were incubated with the corresponding secondary antibody, goat anti-rabbit IgG-HRP (7074P2, Cell Signaling) and goat anti-mouse IgG-HRP (31430, Invitrogen). The ChemiDoc Imaging System was used to obtain images that were subsequently analyzed using the Image Lab software (Bio-Rad). Protein expressions were normalized to Ponceau S staining (BM-1492, Winkler).

Transmission electronic microscopy

C2C12 myotubes were dissociated with trypsin (Thermo Fisher Scientific), centrifuged, and fixed in 2% glutaraldehyde/0.1M cacodylate pH7.0. The cellular pellets were processed in the Advanced Microscopy Facility, Faculty of Biological Sciences at Pontificia Universidad Católica de Chile. Grids were visualized in Tecnai T12 microscopy (Philips). Mitochondrial morphology was analyzed by the ImageJ software

Data analysis

All results are expressed as mean and standard deviation. Statistical analyses were performed using two-way ANOVA to evaluate the effects of the LPS and UA factors, as well as their interaction. In case a main or interaction effect was found, a Sidak test was used as a post-hoc test. All statistical analyses were performed with GraphPad prism 9.3.0 analysis software. The p-values < 0.05 were considered statistically significant.

Results

Urolithin A reduces LPS-induced activation of the TLR4 pathway

TLR4 protein levels were unaffected by UA (p=0.69), LPS (p=0.53) or the interaction between LPS and UA (p=0.25) (Figure 4.1A). MyD88 protein levels were unaffected by UA (p=0.36) and LPS (p=0.15) alone but tended to interact (p=0.07) (Figure 4.1B). Downstream of TLR4, p-TAK was not regulated by UA (p=0.12), while a trend to upregulation was observed for LPS (p=0.06) (Figure 4.1C). An interaction between UA and LPS was observed as well (p=0.03). Post-hoc analysis revealed that p-TAK was lower in UA+LPS compared to LPS alone (p=0.0351). p-IKKαβ tended to be up-regulated by LPS (p=0.07) and down-regulated by UA (p=0.062), without any interaction between both factors (p=0.74) (Figure 4.1D). Similarly to p-TAK, IκBα protein levels were not modified by UA (p=0.19), while an upregulation by LPS was observed (p=0.050) (Figure 4.1E). No interaction between LPS and UA was present (p=0.24). p65 NF-κB protein levels were not modified by UA (p=0.51) or LPS (p=0.76) alone nor in combination (p=0.32) (Figure 4.1F). p-ERK was higher after LPS (p=0.003) but lower after UA incubation (p=0.02), with a significant interaction between LPS and UA (p=0.03) (Figure 4.1G). Post-hoc analysis showed that p-ERK was lower after UA+LPS compared to LPS alone (p=0.0215). p-p38 was higher after LPS (p=0.04) but was not modify by UA alone (p=0.11) (Figure 4.1H). Post-hoc analysis revealed that p-p38 was higher after LPS compared to CTL (p=0.05). When LPS and UA were combined, a tendency to an interaction effect was found (p=0.06). Finally, p-JNK was higher after LPS (p=0.006) but lower after UA incubation (p=0.01), with a significant interaction between both factors (p= 0.03) (Figure 4.1I). Post-hoc analysis showed that p-JNK was lower when UA and LPS were combined compared to LPS alone (p=0.0319).

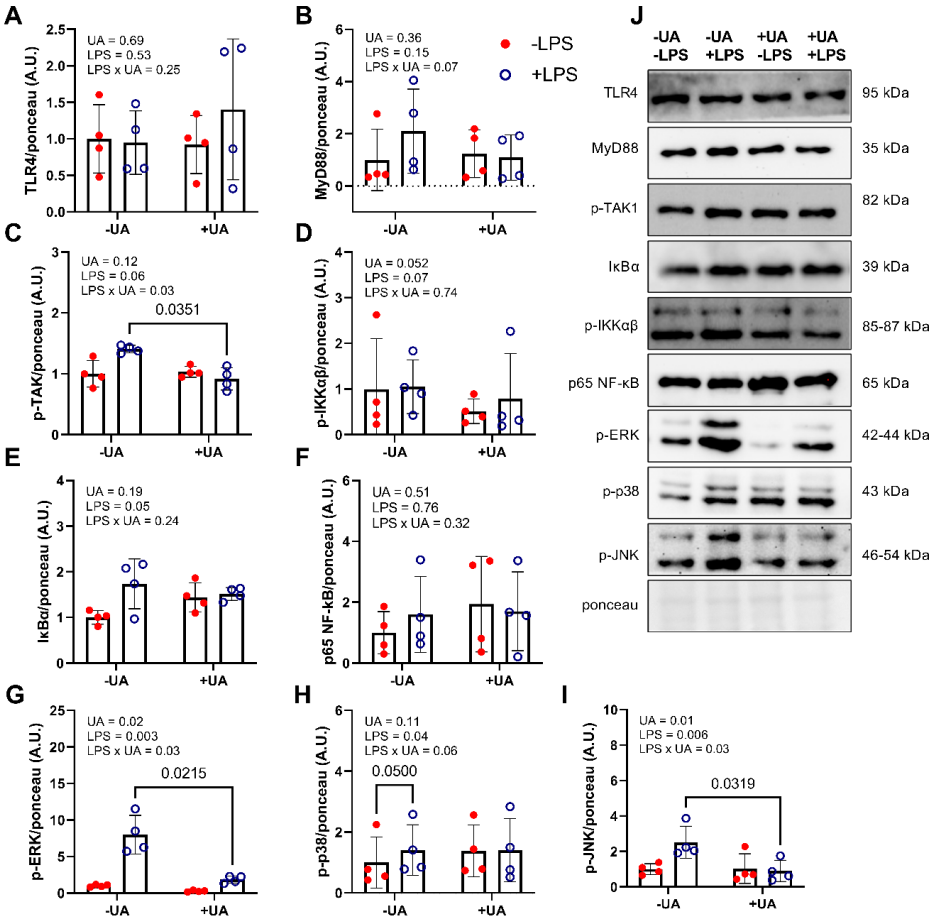


Figure 4.1 Regulation of the TLR4 pathway. A-I) Protein levels and phosphorylation status of main components of the TLR4 pathway after incubation with urolithin A (UA) and lipopolysaccharide (LPS) alone or in combination in C2C12 myotubes. J) Representative blots. All data are reported as mean and standard deviation. Main (UA and LPS) and interaction (UA x LPS) effects are reported at the top left of each graph. Statistical difference reported by the post-hoc test is given above the concerned conditions.

Urolithin A prevents some alterations in mitochondrial morphology induced by LPS

Cellular components associated with lysosomal processes were observed by electron microscopy after LPS and after UA incubation alone but not in the CTL conditions and when UA and LPS were combined (Figure 4.2A). LPS incubation decreased mitochondrial area (p=0.05) (Figure 4.2B) and tended to decrease the

perimeter (p=0.07) (Figure 4.2C). For the perimeter, an interaction between UA and LPS was observed (p=0.02). Post-hoc analysis revealed that mitochondrial perimeter was lower in LPS compared to CTL (p=0.0314). UA (p=0.04) and LPS (p=0.0004) decreased mitochondrial circularity, with an interaction between both factors (p=0.01) (Figure 4.2D). Finally, the mitochondrial aspect ratio was higher after UA (p=0.005) but was not modified by LPS alone (p=0.15) or when UA and LPS were combined (p=0.14) (Figure 4.2E).

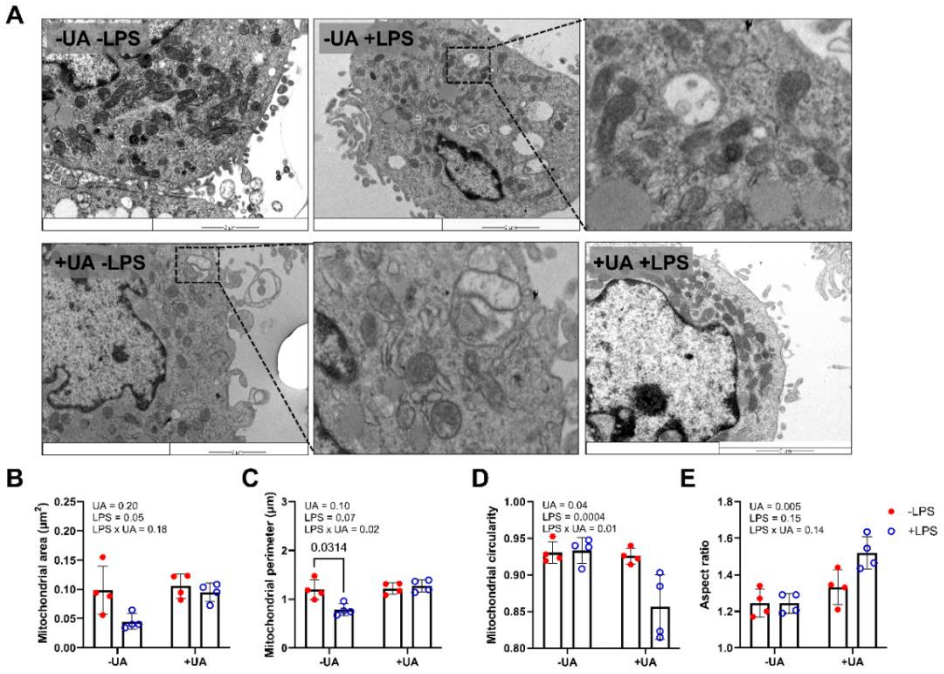


Figure 4.2 Mitochondrial morphology. A) Representative images of mitochondrial morphology after incubation with urolithin A (UA) and lipopolysaccharide (LPS) alone or in combination in C2C12 myotubes using electron microscopy. B-E) Quantitative analysis of mitochondrial morphology. All data are reported as mean and standard deviation. Main (UA and LPS) and interaction (UA x LPS) effects are reported at the top left of each graph. Statistical difference reported by the post-hoc test is given above the concerned conditions.

UA decreases mitochondrial fission induced by LPS

The mitochondrial fission marker p-DRP1 was higher with LPS (p=0.009) but lower with UA (p=0.04), with no interaction between UA and LPS (p=0.46) (Figure 4.3A). Post-hoc analysis indicated that p-DRP1 was lower when UA and LPS were combined compared to LPS alone (p=0.0363). The mitochondrial fusion marker Opa1 was not modified by any condition (Figure 4.3B).

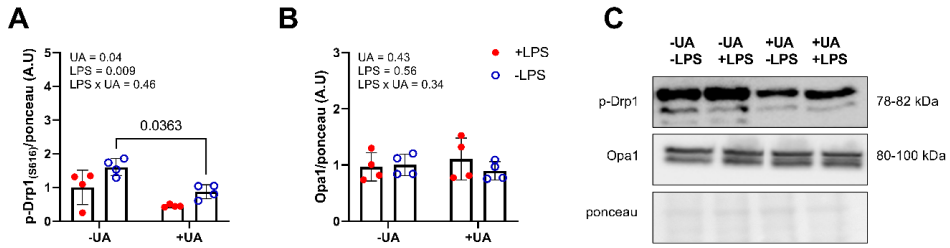


Figure 4.3 Markers for mitochondrial dynamics. A) p-DRP1 and B) Opa1 protein levels after incubation with urolithin A (UA) and lipopolysaccharide (LPS) alone or in combination in C2C12 myotubes. C) Representative blots. All data are reported as mean and standard deviation. Main (UA and LPS) and interaction (UA x LPS) effects are reported at the top left of each graph. Statistical difference reported by the post-hoc test is given above the concerned conditions.

UA stimulates autophagy initiation

PINK1 protein levels were unaffected by UA (p=0.53) and LPS (p=0.73) alone, but there was an interaction between both factors (p=0.04) (Figure 4.4A). Post-hoc analysis indicated a trend towards an upregulation of PINK1 protein levels between CTL and UA alone (p=0.08). A similar response was observed for p-AMPKα as no effect of UA (p=0.18) and LPS (p=0.43) alone was observed, while an interaction between both factors was present (p=0.03) (Figure 4.4B). Post-hoc analysis revealed that p-AMPKα was higher in UA compared to CTL (p=0.03). Analysis of autophagy-related proteins revealed no differences in p62 levels (Figure 4.4C) nor in ULK phosphorylation (Figure 4.4D) in any condition.

The LC3II/LC3I-II ratio was increased by UA (p=0.01) but remained unchanged after LPS incubation (p=0.27) (Figure 4.4E). A tendency towards significance was observed for the interaction between LPS and UA (p=0.08). Post-hoc analysis revealed that the ratio was higher in UA than in CTL conditions (p=0.03).

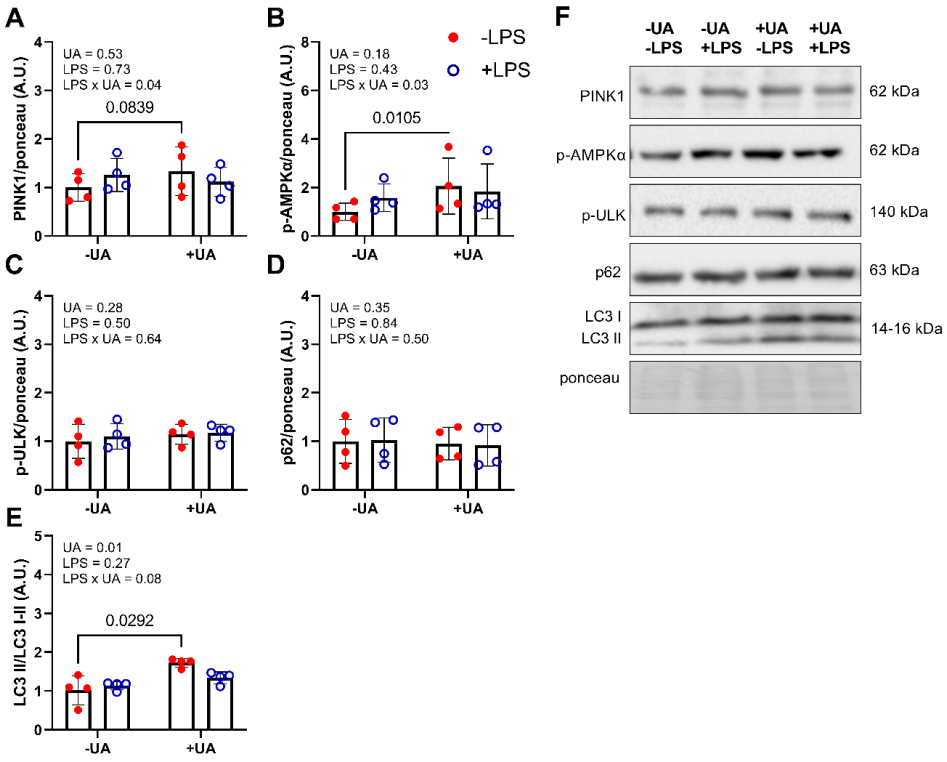


Figure 4.4 Markers for the autophagy pathway. A-E) Protein levels and phosphorylation status of main components of the autophagy pathway after incubation with urolithin A (UA) and lipopolysaccharide (LPS) alone or in combination in C2C12 myotubes. F) Representative blots. All data are reported as mean and standard deviation. Main (UA and LPS) and interaction (UA x LPS) effects are reported at the top left of each graph. Statistical difference reported by the post-hoc test is given above the concerned conditions.

Discussion

The aim of the present study was to examine the effects of UA on key components and regulators of TLR4 signaling, mitochondrial dynamics and mitophagy, and to analyze mitochondrial morphology after LPS incubation in C2C12 myotubes. Here we found that: 1) LPS-induced inflammation activated downstream TLR4 signaling, by increasing the expression of IκBα and the phosphorylation of ERK, p38 and JNK and tended to increase the phosphorylation of TAK1 and IKKαß; 2) LPS reduced mitochondrial area and circularity and tended to reduce mitochondrial perimeter; 3) LPS increased the phosphorylation of the fission marker DRP1; 4) UA completely prevented, or at least attenuated, all previous effects induced by LPS.

We first searched to confirm that LPS indeed activated the TLR4 pathway. We found that C2C12 incubation with 1µg LPS/ml for 3h increased the expression of IκBα and the phosphorylation of ERK, p38 and JNK and tended to increase the phosphorylation of TAK1 and IKKαß but no effect on p65 NF-κB expression was found. This partial activation of the TLR4 pathway is likely due to the dose of LPS used here. It was previously found that 1µg LPS/ml for 1h induced an increase in the DNA binding activity of NF-kB in C2C12 cells (Ono et al., 2020). Our mild activation of the TLR4 pathway by LPS could be prevented by pre-incubating cells for 21h with 50µM UA before adding LPS for an additional 3h. Our results are consistent with previous findings in chondrocytes. Specifically, UA was able to decrease the phosphorylation state of p38, ERK and JNK MAPK proteins following IL-1ß-induced inflammatory response (Ding et al., 2020). This could indicate that the anti-inflammatory effects of UA are mediated, at least partially, by MAPK proteins. Beyond its role in inflammation, ERK has been found to play a key role in skeletal muscle by promoting a transition from fast to slow fibre type, which may have a protective effect in skeletal muscle diseases such as muscular dystrophy (Boyer et al., 2019). Altogether, those results highlight the role of the MAPK family in pathological states.

Knowing that the TLR4 signalling pathway regulates mitochondrial dynamics, specifically through MyD88-dependent activation and downstream activation of ERK (Cook et al., 2017) (Weinberg et al., 2015), we were interested in further investigating fusion and fission. We found that the phosphorylation of Drp1, which promotes mitochondrial fission or fragmentation (Boyer et al., 2019); (Kapetanovic et al., 2020), was globally increased by LPS, but less so in the presence of UA. Thus, UA likely somewhat prevents LPS-induced Drp1 phosphorylation and thereby mitochondrial fission. The exact mechanisms by which UA might regulate Drp1 cannot be evidenced based on our results. However, knowing that ERK phosphorylation displays a similar regulation pattern by LPS and UA and that ERK has been previously shown to regulate Drp1 (Cook et al., 2017), it can be speculated that this MAPK might contribute to the higher phosphorylation state of Drp1 observed in the present study. Of note, we did not find any change in Opa protein levels, a marker of protein fusion (Quintana-Cabrera & Scorrano, 2023). Microscopy analyses revealed that the mitochondrial perimeter was reduced under LPS alone but not when pre-incubated with UA. Pre-treatment with UA thus prevented both the morphological alterations in the area and perimeter of mitochondria as well as the increase in phosphorylation of Drp1 following LPS stimulation.

Finally, as UA has been shown to induce some health, and more specifically muscle, benefits via an increase in mito- and autophagy (D'Amico et al., 2021); (Liu et al., 2022); (Singh et al., 2022), we investigated markers for autophagy under LPS stimulation in C2C12 cells. Additionally, it has been found that inflammation triggered by LPS might induce autophagy as a regulatory mechanism to control the inflammatory response (Shi et al., 2012). We were thus particularly interested in combining both UA and LPS and to investigate the autophagy pathway. However, our results showed that LPS-induced inflammation did not lead to changes in autophagy-related protein levels. As mentioned earlier, this could be due to the dose of LPS used in the present study and the absence of a frank activation of the TLR4/ NF-κB pathway. Though, we found that UA increased the ratio of LC3II/LC3I-II, which might be indicative

of a higher formation of autophagosomes. As the latter was not accompanied by any change in p62 expression, autophagic flux was probably not modified. Additionally, we found an interaction effect between UA and LPS for PINK1 protein expression and AMPKα phosphorylation. Our results are consistent with those reported by Ryu et al. (2016), showing that UA induced mitophagy in C2C12 cells, primarily by increasing PINK1 protein expression and the LC3II/LC3I-II ratio, concomitantly to an increase in the phosphorylation of AMPKα. However, in the present study, we did not perform mitochondrial isolation, which limits our ability to directly associate the observed increase in autophagy-related proteins with mitophagy. In summary, the relation between UA, autophagy, and inflammation is still highly speculative but altogether, it could contribute to a better preservation of mitochondrial morphology under LPS stimulation.

Conclusion

Our findings suggest that UA partially mitigates LPS-induced inflammation by modulating the TLR4 signaling pathway and mitochondrial dynamics. Thus, UA may represent a promising therapeutic agent for conditions associated with mitochondrial dysfunction and inflammation, highlighting its potential role in promoting skeletal muscle health.

CHAPTER V: General discussion

TLR4 signaling and mitochondrial dynamics and morphology in skeletal muscle and PBMCs in desminopathy

Overall, the findings of this thesis provide a multi-level characterization of desminopathy, integrating clinical, molecular, and cellular observations (table 4). In skeletal muscle, desminopathy was associated with structural alterations detected by imaging and functional assessments, accompanied by mitochondrial morphological abnormalities. Reduced ERK phosphorylation was observed, whereas pro-fission p-DRP1 levels remained unchanged, suggesting that mitochondrial remodeling in desminopathy may occur through mechanisms not exclusively dependent on DRP1 activation.

In PBMCs, we identified systemic inflammatory alterations characterized by reduced TLR4/MyD88 protein expression and changes in MAPK phosphorylation patterns. These tissue-specific findings indicate differential regulation of inflammatory and mitochondrial pathways between skeletal muscle and circulating immune cells. To our knowledge, this is the first study to examine both tissues in parallel in desminopathy.

Second, in our *in vitro* C2C12 myotube model inflamed by LPS, we observed increased expression of components of the MAPK pathway and a reduction in mitochondrial perimeter, alongside elevated pro-fission p-DRP1 (Ser616) expression. Interestingly, UA mitigated the aforementioned LPS-induced effects in C2C12 myotubes.

Finally, our case report provides the first scientific exploration of epicatechin supplementation in desminopathy, indicating that regularly dosed epicatechin may restore PBMC responsiveness. The functional impact remains to be determined and will require longitudinal follow-up in a larger cohort of patients with desminopathy.

Table 4. Western blot analysis comparing protein expression levels in patients with desminopathy versus controls and in the C2C12 cell model.

	TLR4	MyD88	p-TAK1	p-ERK	p-JNK	p-P38	p-IKKαβ	IκBα	OPA1	MFN2	p-Drp1
DES SKM	=	=	n/a	↓	=	=	=	=	↑*	=	=
DES PBMC	↓	↓	n/a	=	↑	↑	↑*	↓	=	↓*	=
C2C12 + LPS	=	=	↑*	↑	↑	↑	=	=	=	n/a	↑
C2C12 + UA	=	=	↓	↓	↓	=	=	=	=	n/a	↓

DES SKM: skeletal muscle from patients with desminopathy; DES PBMC: peripheral blood mononuclear cells from patients with desminopathy; C2C12+LPS: C2C12 myotubes stimulated with lipopolysaccharide; C2C12+UA: C2C12 myotubes treated with urolithin A. (=) no significant differences; (↓) decreased protein levels, statistically significant; (↑) increased protein levels, statistically significant; (↓*) decreased protein levels, trend toward statistical significance; (↑*) increased protein levels, trend toward statistical significance; (n/a) not available.

From clinical manifestation to molecular insights

We know that desmin mutation produces aggressive clinical manifestation characterized by sarcopenia, that finally results in an early limitation in activity daily routine and premature death in some cases. One of the current primary focus of the disease is on molecular mechanisms to identify therapeutic targets and to evaluate the effectiveness of future treatments. The hypothesis is that molecular signaling in skeletal muscle or blood could anticipate, or at least reflect, disease manifestations, specifically regarding the inflammatory signaling in PBMCs and mitochondrial morphology in skeletal muscle. The results of the present work are the first results in that direction in desminopathy.

Accordingly, TLR4 signaling regulates skeletal muscle homeostasis (Tu & Li, 2023) and evidence from cellular, animal and human models have shown that

inflammation through TLR4 can regulate skeletal muscle mass and function (Zong et al., 2013). Our results highlight that the clinical–biochemical profile of our patients is consistent with muscle injury and loss of muscle mass. In skeletal muscle, we observe pronounced mitochondrial dysmorphology despite no increase in canonical fission, suggesting that decreased ERK phosphorylation may act as a compensatory brake.

In PBMCs, we observed reduced TLR4 and MyD88 protein expression accompanied by altered MAPK phosphorylation patterns. Notably, phosphorylation of JNK and p38 was decreased, while downstream NF-κB signaling markers showed reduced activation. (Zbinden-Foncea et al., 2024), also the cytokine levels did not significantly differ between patients and controls, which may indicate a regulated or compensated inflammatory state rather than overt systemic inflammation. These findings suggest attenuation rather than activation of canonical TLR4 signaling in circulating immune cells from desminopathy patients. When the activity of the TLR4 pathway increases, a regulatory process—potentially mediated by the receptor—is triggered whereby TLR4 is internalized into the cytoplasm and degraded via autophagy, with the aim of reducing the production of pro-inflammatory cytokines (Husebye et al., 2006). This mechanism might be similar in our desminopathy patients: TLR4 and MyD88 protein expression was lower than in controls, whereas phosphorylation of MAPK proteins (JNK and p38), higher p-IKKαβ and IκBα was lower. Although receptor internalization and degradation via autophagy have been described as regulatory mechanisms controlling TLR4 signaling in other contexts, our study did not directly assess receptor trafficking or autophagic flux. Therefore, the involvement of autophagy in TLR4 downregulation in desminopathy remains speculative and warrants further mechanistic investigation.(Fujita et al., 2011; Wu et al., 2022) (Figure 5.1).

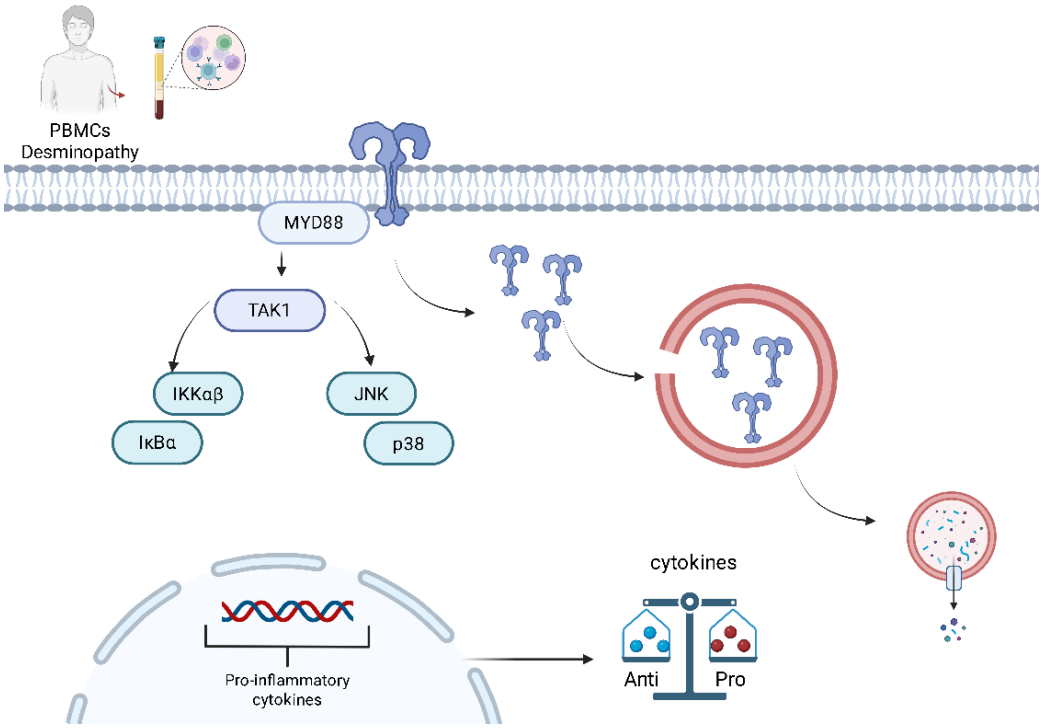


Figure 5.1 Downregulation of the activation of the TLR4 pathway as a compensatory response in PBMCs from desminopathy patients. Cytoplasmic internalization of TLR4 as a response to a higher JNK/p38 MAPK and a higher p-IKKαβ phosphorylation as well as a lower IκBα expression that results in a higher stimulation of cytokine production. Consequently, this downregulation of the activation of the TLR4 pathway might produce homeostasis in anti- and pro-inflammatory cytokine levels.

What is the role of PBMCs in desminopathy? Our results do not allow us to answer this question precisely; however, we can speculate that these cells respond to muscle damage (Careccia et al., 2021), with the aim of infiltrating skeletal muscle and promoting tissue repair (Argyriou et al., 2023). To investigate this, we propose that future studies include in vitro co-culture experiments using patient-derived myotubes from individuals with desminopathy together with PBMCs, in order to assess the role of these cells in the presence of the desmin protein mutation. Our findings constitute the first

evidence regarding an involvement of the TLR4 signaling pathway in skeletal muscle and PBMCs from patients with desminopathy.

From skeletal muscle to blood

The skeletal and heart muscle are the primary tissues affected in desminopathy, as introduced in chapter I. Skeletal muscle is a key tissue to maintain skeletal muscle function, which is a manifestation of strength, resistance and velocity that allow assuring daily activities (Mendoza et al., 2023).

Serum CK levels have been variably reported in desminopathy, with some patients exhibiting elevated levels consistent with muscle injury, while others remain within normal ranges. In our cohort, we observed a trend toward increased CK levels, although variability was high, likely reflecting heterogeneity in disease severity and activity status (Clemen et al., 2013). Consistent with these observations, our data show a trend toward higher total CK levels in the desminopathy cohort, albeit with a large standard deviation. We interpret this variability as reflecting heterogeneity in disease activity and stage—that is, greater clinical severity may be associated with higher CK values. In dystrophic models, increased CK has been linked to elevated levels of DAMPs (Careccia et al., 2021). Accordingly, we hypothesize that greater fibrosis and intramuscular desmin accumulation may be associated with increased muscle damage in patients with desminopathy. An exploratory analysis revealed a correlational trend supporting this hypothesis; however, the limited sample size in our desminopathy group precludes definitive conclusions (Figure 5.2).

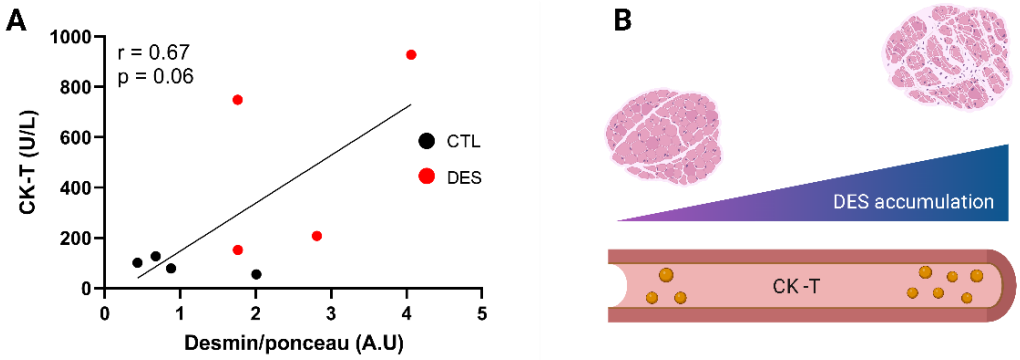


Figure 5.2. Muscle damage across desminopathy progression. A) Unpublished data: correlation between skeletal muscle desmin expression and serum total creatine kinase (CK-T) in control subjects (CTL) and patients with desminopathy (DES), showing a positive trend ($R^2 = 0.42$; $r = 0.67$; $p = 0.06$). B) Theoretical model: higher skeletal muscle desmin levels—potentially reflecting greater fibrosis in desminopathy—are hypothesized to be associated with higher circulating CK-T concentrations.

The injury response described above may underlie the phenotype observed in PBMCs. Specifically, muscle damage can activate TLR4 signaling (Zong et al., 2013). TLR4 expressed in PBMCs would therefore sense this damage and trigger the downstream MyD88→NF-κB/p38–JNK cascade, promoting the sterile inflammatory response required for tissue repair (Tu & Li, 2023). Within this context, we propose that muscle injury in desminopathy drives a compensatory downregulation of TLR4 signaling in PBMCs (Figure 5.3).

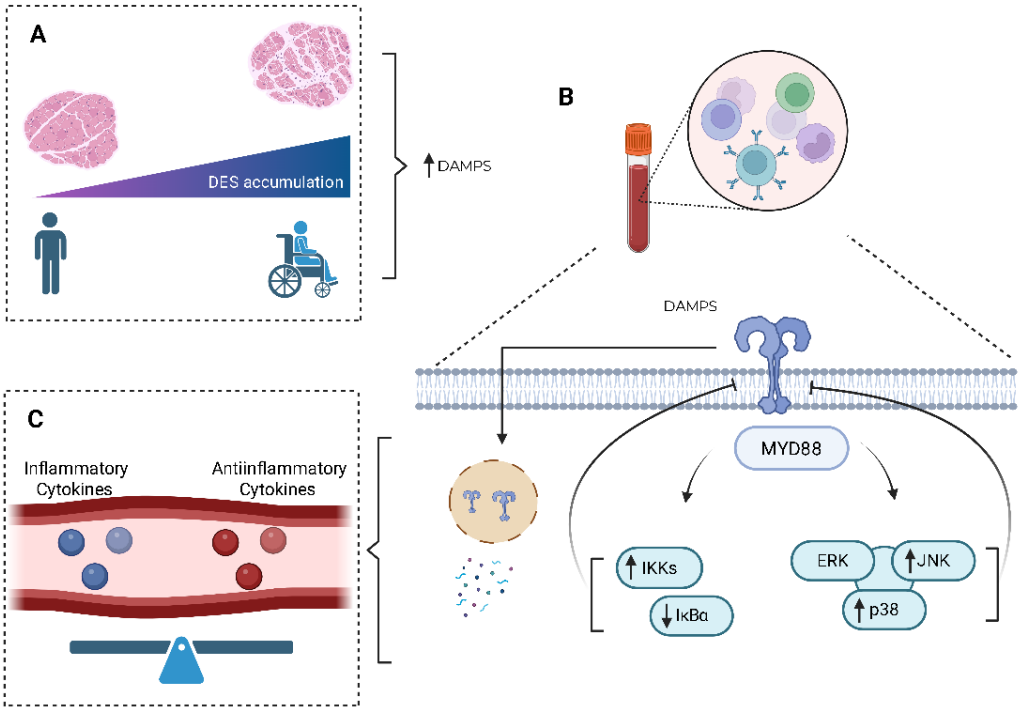


Figure 5.3. Proposed model: impact of desminopathy in PBMCs. A) Desmin accumulation is associated with disease progression and increases damage-associated molecular patterns (DAMPs), which are recognized by Toll-like receptor 4 (TLR4). B) TLR4 activation upregulates MAPK signaling (notably JNK and p38) and the IKK pathway, promoting IκBα degradation. This downstream response may establish a TLR4-dependent feedback loop that drives receptor internalization and degradation. C) Ultimately, this downregulation promotes the restoration of pro- and anti-inflammatory cytokine homeostasis.

Conceptual model of molecular signaling in desminopathy

We propose that the TLR4 pathway, together with mitochondrial dynamics and morphology, may play a role in desminopathy in both skeletal muscle and PBMCs. Specifically, in skeletal muscle we observe structural alterations of mitochondria, as desmin mutations can impose stress on this organelle due to loss of muscle architecture (Capetanaki, 2002). Moreover, changes in p-ERK may be explained by its stimulation through mechanotransduction during muscle contraction, mechanotransduction refers to the conversion of mechanical stress at the sarcolemma into intracellular signaling cascades in skeletal muscle after contraction. (Crozet & Levayer, 2023). Accordingly, the reduction in p-ERK in desminopathy could reflect the underlying disruption of muscle structure. In PBMCs, we observed dysregulation of the TLR4/MAPK pathway with no alteration of mitochondrial ultrastructure, likely because desmin is not expressed in those cells. It is therefore possible that the level of stress is lower in PBMCs. Based on our results, it seems that the skeletal muscle and PBMCs are regulated differently at a molecular level in desminopathy (Figure 5.4). Nonetheless, we currently lack data to delineate how these mechanisms may be related, or not, to each other in desminopathy.

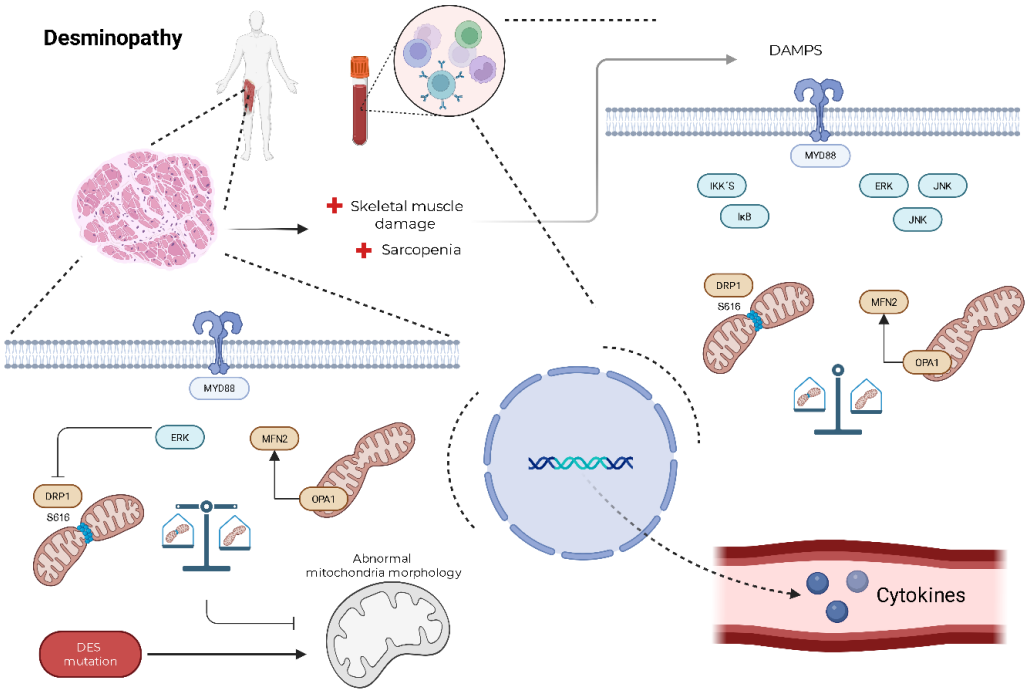


Figure 5.4. Initial evidence of TLR4 pathway dysregulation in PBMCs and altered mitochondrial morphology in skeletal muscle in desminopathy, suggesting a pivotal role for those mechanisms in disease pathogenesis. Specifically, we observed: (i) lower ERK phosphorylation with normal DRP1 Ser616 levels, consistent with tempered mitochondrial fission in skeletal muscle; (ii) DES mutations per se underlying markedly abnormal mitochondrial morphology despite otherwise intact fission–fusion dynamics in skeletal muscle; and (iii) alterations in TLR4 signaling in PBMCs associated with muscle damage and sarcopenia, while PBMC mitochondria remain morphologically normal, possibly due to the absence of desmin.

From mechanisms to intervention

This thesis supports the hypothesis that some proteins related to the TLR4 pathway may play a role in PBMCs in desminopathy. Therefore, investigating the specific role of this pathway in the context of inflammation associated with sarcopenia, muscle damage, and disease progression could represent a potential therapeutic target. Furthermore, studying the regulation of this pathway and how it may modulate disease progression—together with the mitochondrial morphological alterations in skeletal muscle described in the scientific literature—could provide new mechanistic insights.

One natural way to modulate the activity of the TLR4 pathway is to ingest polyphenols, which are known to have anti-oxidant and anti-inflammatory effects. Evidence supports that UA is safe in humans; specifically, daily doses of 500 mg or 1000 mg for four weeks have been shown to modulate plasma acylcarnitines and increase the expression of mitochondrial genes in skeletal muscle in older adults (Andreux et al., 2019). The main effects of UA are associated with biological responses such as mitochondrial function, gut microbiome modulation, microbial metabolites, and inflammation. Moreover, UA has been linked to clinical improvements including enhanced cardiovascular markers, aerobic capacity, and muscle strength (Kuerec et al., 2024). Consistent with these findings, our results from the in vitro C2C12 myotube model suggest that UA can prevent the effects induced by LPS-triggered inflammation on the regulation of the TLR4 pathway, as well as the inflammation-induced mitochondrial morphological alterations. However, no evidence is currently available regarding supplementation in patients suffering from desminopathy. Therefore, we propose that UA could represent a therapeutic approach to be explored in desminopathy. We therefore investigated the effects of UA on TLR4

signaling in PBMCs, mitochondrial structure in skeletal muscle, and ERK protein expression.

Additionally, evidence also supports EPI supplementation in humans to reduce oxidative stress and inflammation. Specifically, a daily dose of 100 mg for three months has shown positive effects on oxidative stress in patients with type 2 diabetes mellitus and heart failure (Ramirez-Sanchez et al., 2013). Improvements in mitochondrial biogenesis and cristae structure in skeletal muscle have also been reported with the same dose and treatment duration (Taub et al., 2012). Moreover, EPI has been proposed to mitigate skeletal muscle alterations in the context of muscular dystrophy due to its antioxidant and anti-inflammatory properties (Zbinden-Foncea et al., 2022). In agreement with this, our results in Chapter III represent the first scientific evidence regarding the TLR4 pathway and mitochondrial morphology in PBMCs under EPI treatment in a patient with desminopathy. These findings suggest that EPI in PBMCs could regulate both TLR4 signaling and mitochondrial morphology in desminopathy.

However, a relevant question arises, i.e. what is better for patients with desminopathy—UA or EPI, or a combination of both? It is currently impossible to answer that question due to the lack of data in the literature. Nevertheless, based on our results, we can speculate that each compound might regulate signaling pathways associated with inflammation and mitochondrial morphology, aiming to control disease progression. This, however, must be correlated with clinical and functional outcomes in patients. Any synergistic effect sill needs to be tested as well.

Limitations and perspectives

As discussed throughout this thesis, desminopathy is a rare disease, and we were able to recruit five patients and present the first results concerning TLR4 signaling pathways, mitochondrial morphology, and dynamics across two biological systems—the skeletal muscle and PBMCs—which were associated with the patients' clinical features. Furthermore, we reported the first study examining the effect of EPI supplementation in a patient with desminopathy and its potential modulation of the TLR4 pathway and mitochondrial morphology in PBMCs. Finally, we tested another natural compound, UA, in an in vitro C2C12 model to evaluate its effects on TLR4-mediated inflammation, as well as the regulation of mitochondrial dynamics and morphology.

Among the limitations of our study, we acknowledge the absence of mitochondrial functional assays and their association with the inflammatory response in Chapter II, as well as the lack of a mutant desmin cellular model in human myotubes to investigate the underlying mechanisms of the pathway. Additionally, co-culture experiments involving PBMCs could further elucidate cellular interactions and signaling behavior.

We believe that future research should prioritize well-designed longitudinal studies that evaluate supplementation with natural compounds such as UA or EPI while prospectively tracking disease trajectory. Ideally, randomized, placebo-controlled, dose-response trials, preceded by short safety/run-in phases, would determine whether these interventions can meaningfully modulate or slow desminopathy progression. Beyond standard clinical endpoints (muscle strength, functional capacity, and activities of daily living), protocols should incorporate mechanistic readouts aligned with our observations: PBMC TLR4/MyD88 abundance and MAPK phosphorylation (JNK, p38, and IκBα), skeletal muscle mitochondrial morphology and ultrastructural measurements as well as signaling nodes such as ERK and DRP1 Ser616. Multimodal assessment (serum

biomarkers including CK and DAMPs, imaging for muscle quality and fibrosis, and patient reported outcomes) would enable an integrated view of efficacy.

Given potential heterogeneity across genotypes and disease stage, stratified analyses (e.g., by DES variant class, baseline fibrosis, or CK levels) and longitudinal modeling (mixed-effects trajectories) are recommended. For UA, ancillary measures of gut microbiome composition and metabolite profiling could clarify exposure–response relationships. For EPI, assays of oxidative stress and mitochondrial biogenesis may be informative. Adherence monitoring, pharmacokinetic sampling, and adverse event surveillance should be embedded throughout. Collectively, this framework would test whether UA or EPI can re-equilibrate inflammatory signaling and mitochondrial architecture toward a more physiological state, and—critically—whether such remodeling translates into clinically meaningful preservation of muscle function over time.

Conclusion

Taken together, the present work investigated the hypothesis that re-equilibrating inflammatory signaling and mitochondrial architecture could be translated into clinically meaningful preservation of muscle function over time. Our results suggest that PBMCs may constitute both an accessible compartment for monitoring disease progression and a potential therapeutic target, as those cells likely respond to ongoing muscle damage in affected patients. In parallel, p-ERK may play a key role within skeletal muscle: its reduction plausibly reflects disrupted mechanotransduction secondary to desmin-related architectural loss, and may also be influenced by an insulin-resistant state. These findings position TLR4/MAPK signaling in PBMCs and altered mitochondrial architecture in skeletal muscle as complementary mechanisms relevant to disease pathogenesis.

Beyond these mechanistic insights, our data demonstrate that UA mitigates LPS-induced inflammatory signaling and restores mitochondrial morphology in vitro,

while EPI-rich cocoa modulates the TLR4 pathway in human PBMCs. These findings establish a proof-of-concept for the use of natural compounds to target the inflammation-mitochondrial axis in desminopathy.

Consequently, this work provides a specific molecular framework for future clinical applications. We propose that therapeutic success in desminopathy should be evaluated through an integrated approach: monitoring systemic biomarkers in PBMCs (TLR4/MyD88 and MAPK signaling) alongside skeletal muscle ultrastructure (DRP1 Ser616 and ERK expression). By linking these mechanistic endpoints to clinical markers such as serum CK and muscle quality imaging, this research moves beyond hypothesis-generation to provide actionable targets for slowing disease progression. Ultimately, this translational model identifies the TLR4/MAPK-mitochondrial signaling pathway as a primary, measurable target for pharmacological intervention, offering a novel path toward preserving muscle function in patients with desmin mutations.

References

Actis-Goretta, L., Leveques, A., Rein, M., Teml, A., Schafer, C., Hofmann, U., Li, H., Schwab, M., Eichelbaum, M., & Williamson, G. (2013). Intestinal absorption, metabolism, and excretion of (-)-epicatechin in healthy humans assessed by using an intestinal perfusion technique. *Am J Clin Nutr*, *98*(4), 924-933. https://doi.org/10.3945/ajcn.113.065789

Alam, S., Abdullah, C. S., Aishwarya, R., Miriyala, S., Panchatcharam, M., Peretik, J. M., Orr, A. W., James, J., Robbins, J., & Bhuiyan, M. S. (2018). Aberrant Mitochondrial Fission Is Maladaptive in Desmin Mutation-Induced Cardiac Proteotoxicity. *J Am Heart Assoc*, *7*(14). https://doi.org/10.1161/JAHA.118.009289

Alexovic, M., Lindner, J. R., Bober, P., Longuespee, R., Sabo, J., & Davalieva, K. (2022). Human peripheral blood mononuclear cells: A review of recent proteomic applications. *Proteomics*, *22*(15-16), e2200026. https://doi.org/10.1002/pmic.202200026

Andreux, P. A., Blanco-Bose, W., Ryu, D., Burdet, F., Ibberson, M., Aebischer, P., Auwerx, J., Singh, A., & Rinsch, C. (2019). The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. *Nat Metab*, *1*(6), 595-603. https://doi.org/10.1038/s42255-019-0073-4

Argyriou, A., Horuluoglu, B., Galindo-Feria, A. S., Diaz-Boada, J. S., Sijbranda, M., Notarnicola, A., Dani, L., van Vollenhoven, A., Ramskold, D., Nennesmo, I., Dastmalchi, M., Lundberg, I. E., Diaz-Gallo, L. M., & Chemin, K. (2023). Single-cell profiling of muscle-infiltrating T cells in idiopathic inflammatory myopathies. *EMBO Mol Med*, *15*(10), e17240. https://doi.org/10.15252/emmm.202217240

Barrera-Reyes, P. K., Hernandez-Ramirez, N., Cortes, J., Poquet, L., Redeuil, K., Rangel-Escareno, C., Kussmann, M., Silva-Zolezzi, I., & Tejero, M. E. (2019). Gene expression changes by high-polyphenols cocoa powder intake: a randomized crossover clinical study. *Eur J Nutr*, *58*(5), 1887-1898. https://doi.org/10.1007/s00394-018-1736-8

Bartsakoulia, M., Pyle, A., Troncoso-Chandia, D., Vial-Brizzi, J., Paz-Fiblas, M. V., Duff, J., Griffin, H., Boczonadi, V., Lochmuller, H., Kleinle, S., Chinnery, P. F., Grunert, S., Kirschner, J., Eisner, V., & Horvath, R. (2018). A novel mechanism causing imbalance of mitochondrial fusion and fission in human myopathies. *Hum Mol Genet*, *27*(7), 1186-1195. https://doi.org/10.1093/hmg/ddy033

Bastin, A. R., Sadeghi, A., Abolhassani, M., Doustimotlagh, A. H., & Mohammadi, A. (2020). Malvidin prevents lipopolysaccharide-induced oxidative stress and inflammation in human peripheral blood mononuclear cells. *IUBMB Life*, *72*(7), 1504-1514. https://doi.org/10.1002/iub.2286

Bermudez-Jimenez, F. J., Protonotarios, A., Garcia-Hernandez, S., Perez Asensio, A., Rampazzo, A., Zorio, E., Brodehl, A., Arias, M. A., Macias-Ruiz, R., Fernandez-Armenta, J., Remior Perez, P., Munoz-Esparza, C., Pilichou, K., Bauce, B., Merino, J. L., Moliner-Abos, C., Ochoa, J. P., Barriales-Villa, R., Garcia-Pavia, P., . . . Jimenez-Jaimez, J. (2024). Phenotype and Clinical Outcomes in Desmin-Related Arrhythmogenic Cardiomyopathy. *JACC Clin Electrophysiol*, *10*(6), 1178-1190. https://doi.org/10.1016/j.jacep.2024.02.031

Borges, G., Ottaviani, J. I., van der Hooft, J. J. J., Schroeter, H., & Crozier, A. (2018). Absorption, metabolism, distribution and excretion of (-)-epicatechin: A review of recent findings. *Mol Aspects Med*, *61*, 18-30. https://doi.org/10.1016/j.mam.2017.11.002

Boyer, J. G., Prasad, V., Song, T., Lee, D., Fu, X., Grimes, K. M., Sargent, M. A., Sadayappan, S., & Molkentin, J. D. (2019). ERK1/2 signaling induces skeletal muscle slow fiber-type switching and reduces muscular dystrophy disease severity. *JCI Insight*, *5*(10). https://doi.org/10.1172/jci.insight.127356

Brunialti, M. K., Martins, P. S., Barbosa de Carvalho, H., Machado, F. R., Barbosa, L. M., & Salomao, R. (2006). TLR2, TLR4, CD14, CD11B, and CD11C expressions on monocytes surface and cytokine production in patients with sepsis, severe sepsis, and septic shock. *Shock*, *25*(4), 351-357. https://doi.org/10.1097/01.shk.0000217815.57727.29

Capetanaki, Y. (2002). Desmin cytoskeleton: a potential regulator of muscle mitochondrial behavior and function. *Trends Cardiovasc Med*, *12*(8), 339-348. https://doi.org/10.1016/s1050-1738(02)00184-6

Capetanaki, Y., Papathanasiou, S., Diokmetzidou, A., Vatsellas, G., & Tsikitis, M. (2015). Desmin related disease: a matter of cell survival failure. *Curr Opin Cell Biol*, *32*, 113-120. https://doi.org/10.1016/j.ceb.2015.01.004

Careccia, G., Saclier, M., Tirone, M., Ruggieri, E., Principi, E., Raffaghello, L., Torchio, S., Recchia, D., Canepari, M., Gorzanelli, A., Ferrara, M., Castellani, P., Rubartelli, A., Rovere-Querini, P., Casalgrandi, M., Preti, A., Lorenzetti, I., Bruno, C., Bottinelli, R., . . . Venereau, E. (2021). Rebalancing expression of HMGB1 redox isoforms to counteract muscular dystrophy. *Sci Transl Med*, *13*(596). https://doi.org/10.1126/scitranslmed.aay8416

Castro-Sepulveda, M., Morio, B., Tunon-Suarez, M., Jannas-Vela, S., Diaz-Castro, F., Rieusset, J., & Zbinden-Foncea, H. (2021). The fasting-feeding metabolic transition regulates mitochondrial dynamics. *FASEB J*, *35*(10), e21891. https://doi.org/10.1096/fj.202100929R

Castro-Sepulveda, M., Tapia, G., Tunon-Suarez, M., Diaz, A., Marambio, H., Valero-Breton, M., Fernandez-Verdejo, R., & Zbinden-Foncea, H. (2022). Severe COVID-19 correlates with lower mitochondrial cristae density in PBMCs and greater sitting time in humans. *Physiol Rep*, *10*(14), e15369. https://doi.org/10.14814/phy2.15369

Castro-Sepulveda, M., Tunon-Suarez, M., Rosales-Soto, G., Vargas-Foitzick, R., Deldicque, L., & Zbinden-Foncea, H. (2023). Regulation of mitochondrial morphology and cristae architecture by the TLR4 pathway in human skeletal muscle. *Front Cell Dev Biol*, *11*, 1212779. https://doi.org/10.3389/fcell.2023.1212779

Chen, W., Zhao, H., & Li, Y. (2023). Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduct Target Ther*, *8*(1), 333. https://doi.org/10.1038/s41392-023-01547-9

Chiou, Y. S., Huang, Q., Ho, C. T., Wang, Y. J., & Pan, M. H. (2016). Directly interact with Keap1 and LPS is involved in the anti-inflammatory mechanisms of (-)-epicatechin-3-gallate in LPS-induced macrophages and endotoxemia. *Free Radic Biol Med*, *94*, 1-16. https://doi.org/10.1016/j.freeradbiomed.2016.02.010

Ciesielska, A., Matyjek, M., & Kwiatkowska, K. (2021). TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci*, *78*(4), 1233-1261. https://doi.org/10.1007/s00018-020-03656-y

Ciesla, N., Dinglas, V., Fan, E., Kho, M., Kuramoto, J., & Needham, D. (2011). Manual muscle testing: a method of measuring extremity muscle strength applied to critically ill patients. *J Vis Exp*(50). https://doi.org/10.3791/2632

Clemen, C. S., Herrmann, H., Strelkov, S. V., & Schroder, R. (2013). Desminopathies: pathology and mechanisms. *Acta Neuropathol*, *125*(1), 47-75. https://doi.org/10.1007/s00401-012-1057-6

Cook, S. J., Stuart, K., Gilley, R., & Sale, M. J. (2017). Control of cell death and mitochondrial fission by ERK1/2 MAP kinase signalling. *FEBS J*, *284*(24), 4177-4195. https://doi.org/10.1111/febs.14122

Cooke, J. P. (2019). Inflammation and Its Role in Regeneration and Repair. *Circ Res*, *124*(8), 1166-1168. https://doi.org/10.1161/CIRCRESAHA.118.314669

Crozet, F., & Levayer, R. (2023). Emerging roles and mechanisms of ERK pathway mechanosensing. *Cell Mol Life Sci*, *80*(12), 355. https://doi.org/10.1007/s00018-023-05007-z

D'Amico, D., Andreux, P. A., Valdes, P., Singh, A., Rinsch, C., & Auwerx, J. (2021). Impact of the Natural Compound Urolithin A on Health, Disease, and Aging. *Trends Mol Med*, *27*(7), 687-699. https://doi.org/10.1016/j.molmed.2021.04.009

D'Amico, D., Olmer, M., Fouassier, A. M., Valdes, P., Andreux, P. A., Rinsch, C., & Lotz, M. (2022). Urolithin A improves mitochondrial health, reduces cartilage degeneration, and alleviates pain in osteoarthritis. *Aging Cell*, *21*(8), e13662. https://doi.org/10.1111/acel.13662

Dankel, S. J., & Loenneke, J. P. (2020). A Method to Stop Analyzing Random Error and Start Analyzing Differential Responders to Exercise. *Sports Med*, *50*(2), 231-238. https://doi.org/10.1007/s40279-019-01147-0

de Sousa, A. R. S., Ottestad, I., Gjevestad, G. O., Holven, K. B., Ulven, S. M., & Christensen, J. J. (2023). Associations between PBMC whole genome transcriptome, muscle strength, muscle mass, and physical performance in healthy home-dwelling older women. *Geroscience*, *45*(6), 3175-3186. https://doi.org/10.1007/s11357-023-00819-0

Denk, D., Singh, A., Kasler, H. G., D'Amico, D., Rey, J., Alcober-Boquet, L., Gorol, J. M., Steup, C., Tiwari, R., Kwok, R., Arguello, R. J., Faitg, J., Sprinzl, K., Zeuzem, S., Nekljudova, V., Loibl, S., Verdin, E., Rinsch, C., & Greten, F. R. (2025). Effect of the mitophagy inducer urolithin A on age-related immune decline: a randomized, placebo-controlled trial. *Nat Aging*, *5*(11), 2309-2322. https://doi.org/10.1038/s43587-025-00996-x

Ding, S. L., Pang, Z. Y., Chen, X. M., Li, Z., Liu, X. X., Zhai, Q. L., Huang, J. M., & Ruan, Z. Y. (2020). Urolithin a attenuates IL-1beta-induced inflammatory responses and cartilage degradation via inhibiting the MAPK/NF-kappaB signaling pathways in rat articular chondrocytes. *J Inflamm (Lond)*, *17*, 13. https://doi.org/10.1186/s12950-020-00242-8

Dower, J. I., Geleijnse, J. M., Gijsbers, L., Zock, P. L., Kromhout, D., & Hollman, P. C. (2015). Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: a randomized, double-blind, placebo-controlled, crossover trial. *Am J Clin Nutr*, *101*(5), 914-921. https://doi.org/10.3945/ajcn.114.098590

Drummond, M. J., Timmerman, K. L., Markofski, M. M., Walker, D. K., Dickinson, J. M., Jamaluddin, M., Brasier, A. R., Rasmussen, B. B., & Volpi, E. (2013). Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults. *Am J Physiol Regul Integr Comp Physiol*, *305*(3), R216-223. https://doi.org/10.1152/ajpregu.00072.2013

Engel, A. G., & Arahata, K. (1986). Mononuclear cells in myopathies: quantitation of functionally distinct subsets, recognition of antigen-specific cell-mediated cytotoxicity in some diseases, and implications for the pathogenesis of the different inflammatory myopathies. *Hum Pathol*, *17*(7), 704-721. https://doi.org/10.1016/s0046-8177(86)80180-0

Faitg, J., D'Amico, D., Rinsch, C., & Singh, A. (2024). Mitophagy Activation by Urolithin A to Target Muscle Aging. *Calcif Tissue Int*, *114*(1), 53-59. https://doi.org/10.1007/s00223-023-01145-5

Fang, W. Y., Tseng, Y. T., Lee, T. Y., Fu, Y. C., Chang, W. H., Lo, W. W., Lin, C. L., & Lo, Y. C. (2021). Triptolide prevents LPS-induced skeletal muscle atrophy via inhibiting NF-kappaB/TNF-alpha and regulating protein synthesis/degradation

pathway. *Br J Pharmacol*, *178*(15), 2998-3016. https://doi.org/10.1111/bph.15472

Favaro, G., Romanello, V., Varanita, T., Andrea Desbats, M., Morbidoni, V., Tezze, C., Albiero, M., Canato, M., Gherardi, G., De Stefani, D., Mammucari, C., Blaauw, B., Boncompagni, S., Protasi, F., Reggiani, C., Scorrano, L., Salviati, L., & Sandri, M. (2019). DRP1-mediated mitochondrial shape controls calcium homeostasis and muscle mass. *Nat Commun*, *10*(1), 2576. https://doi.org/10.1038/s41467-019-10226-9

Fix, D. K., VanderVeen, B. N., Counts, B. R., & Carson, J. A. (2019). Regulation of Skeletal Muscle DRP-1 and FIS-1 Protein Expression by IL-6 Signaling. *Oxid Med Cell Longev*, *2019*, 8908457. https://doi.org/10.1155/2019/8908457

Frost, R. A., Nystrom, G. J., & Lang, C. H. (2002). Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. *Am J Physiol Regul Integr Comp Physiol*, *283*(3), R698-709. https://doi.org/10.1152/ajpregu.00039.2002

Fu, X., Wang, H., & Hu, P. (2015). Stem cell activation in skeletal muscle regeneration. *Cell Mol Life Sci*, *72*(9), 1663-1677. https://doi.org/10.1007/s00018-014-1819-5

Fujita, K., Maeda, D., Xiao, Q., & Srinivasula, S. M. (2011). Nrf2-mediated induction of p62 controls Toll-like receptor-4-driven aggresome-like induced structure formation and autophagic degradation. *Proc Natl Acad Sci U S A*, *108*(4), 1427-1432. https://doi.org/10.1073/pnas.1014156108

Giacomello, M., Pyakurel, A., Glytsou, C., & Scorrano, L. (2020). The cell biology of mitochondrial membrane dynamics. *Nat Rev Mol Cell Biol*, *21*(4), 204-224. https://doi.org/10.1038/s41580-020-0210-7

Goldfarb, L. G., & Dalakas, M. C. (2009). Tragedy in a heartbeat: malfunctioning desmin causes skeletal and cardiac muscle disease. *J Clin Invest*, *119*(7), 1806-1813. https://doi.org/10.1172/JCI38027

Gutierrez-Salmean, G., Ciaraldi, T. P., Nogueira, L., Barboza, J., Taub, P. R., Hogan, M. C., Henry, R. R., Meaney, E., Villarreal, F., Ceballos, G., & Ramirez-Sanchez, I. (2014). Effects of (-)-epicatechin on molecular modulators of skeletal muscle

growth and differentiation. *J Nutr Biochem*, *25*(1), 91-94. https://doi.org/10.1016/j.jnutbio.2013.09.007

Hahn, A., Kny, M., Pablo-Tortola, C., Todiras, M., Willenbrock, M., Schmidt, S., Schmoeckel, K., Jorde, I., Nowak, M., Jarosch, E., Sommer, T., Broker, B. M., Felix, S. B., Scheidereit, C., Weber-Carstens, S., Butter, C., Luft, F. C., & Fielitz, J. (2020). Serum amyloid A1 mediates myotube atrophy via Toll-like receptors. *J Cachexia Sarcopenia Muscle*, *11*(1), 103-119. https://doi.org/10.1002/jcsm.12491

Han, H., Tan, J., Wang, R., Wan, H., He, Y., Yan, X., Guo, J., Gao, Q., Li, J., Shang, S., Chen, F., Tian, R., Liu, W., Liao, L., Tang, B., & Zhang, Z. (2020). PINK1 phosphorylates Drp1(S616) to regulate mitophagy-independent mitochondrial dynamics. *EMBO Rep*, *21*(8), e48686. https://doi.org/10.15252/embr.201948686

Hughes, D. C., Wallace, M. A., & Baar, K. (2015). Effects of aging, exercise, and disease on force transfer in skeletal muscle. *Am J Physiol Endocrinol Metab*, *309*(1), E1-E10. https://doi.org/10.1152/ajpendo.00095.2015

Husebye, H., Halaas, O., Stenmark, H., Tunheim, G., Sandanger, O., Bogen, B., Brech, A., Latz, E., & Espevik, T. (2006). Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity. *EMBO J*, *25*(4), 683-692. https://doi.org/10.1038/sj.emboj.7600991

Hwang, S. L., Jeong, Y. T., Li, X., Kim, Y. D., Lu, Y., Chang, Y. C., Lee, I. K., & Chang, H. W. (2013). Inhibitory cross-talk between the AMPK and ERK pathways mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. *Br J Pharmacol*, *169*(1), 69-81. https://doi.org/10.1111/bph.12124

Jiao, P., Wang, Y., Ren, G., Chu, D., Li, Y., Yang, Y., & Sang, T. (2024). Urolithin A exerts a protective effect on lipopolysaccharide-induced acute lung injury by regulating HMGB1-mediated MAPK and NF-kappaB signaling pathways. *Naunyn Schmiedebergs Arch Pharmacol*, *397*(8), 5765-5777. https://doi.org/10.1007/s00210-024-02977-0

Kalovidouris, A. E., Pourmand, R., Passo, M. H., & Plotkin, Z. (1989). Proliferative response of peripheral blood mononuclear cells to autologous and allogeneic

muscle in patients with polymyositis/dermatomyositis. *Arthritis Rheum*, *32*(4), 446-453. https://doi.org/10.1002/anr.1780320414

Kapetanovic, R., Afroz, S. F., Ramnath, D., Lawrence, G. M., Okada, T., Curson, J. E., de Bruin, J., Fairlie, D. P., Schroder, K., St John, J. C., Blumenthal, A., & Sweet, M. J. (2020). Lipopolysaccharide promotes Drp1-dependent mitochondrial fission and associated inflammatory responses in macrophages. *Immunol Cell Biol*, *98*(7), 528-539. https://doi.org/10.1111/imcb.12363

Kley, R. A., Olive, M., & Schroder, R. (2016). New aspects of myofibrillar myopathies. *Curr Opin Neurol*, *29*(5), 628-634. https://doi.org/10.1097/WCO.0000000000000357

Kubanek, M., Schimerova, T., Piherova, L., Brodehl, A., Krebsova, A., Ratnavadivel, S., Stanasiuk, C., Hansikova, H., Zeman, J., Palecek, T., Houstek, J., Drahota, Z., Nuskova, H., Mikesova, J., Zamecnik, J., Macek, M., Jr., Ridzon, P., Maluskova, J., Stranecky, V., . . . Kmoch, S. (2020). Desminopathy: Novel Desmin Variants, a New Cardiac Phenotype, and Further Evidence for Secondary Mitochondrial Dysfunction. *J Clin Med*, *9*(4). https://doi.org/10.3390/jcm9040937

Kuerec, A. H., Lim, X. K., Khoo, A. L., Sandalova, E., Guan, L., Feng, L., & Maier, A. B. (2024). Targeting aging with urolithin A in humans: A systematic review. *Ageing Res Rev*, *100*, 102406. https://doi.org/10.1016/j.arr.2024.102406

Langer, H. T., Mossakowski, A. A., Willis, B. J., Grimsrud, K. N., Wood, J. A., Lloyd, K. C. K., Zbinden-Foncea, H., & Baar, K. (2020). Generation of desminopathy in rats using CRISPR-Cas9. *J Cachexia Sarcopenia Muscle*, *11*(5), 1364-1376. https://doi.org/10.1002/jcsm.12619

Lei, Y., Gan, M., Qiu, Y., Chen, Q., Wang, X., Liao, T., Zhao, M., Chen, L., Zhang, S., Zhao, Y., Niu, L., Wang, Y., Zhu, L., & Shen, L. (2024). The role of mitochondrial dynamics and mitophagy in skeletal muscle atrophy: from molecular mechanisms to therapeutic insights. *Cell Mol Biol Lett*, *29*(1), 59. https://doi.org/10.1186/s11658-024-00572-y

Li, P., Wu, Y. H., Zhu, Y. T., Li, M. X., & Pei, H. H. (2019). Requirement of Rab21 in LPS-induced TLR4 signaling and pro-inflammatory responses in macrophages and monocytes. *Biochem Biophys Res Commun*, *508*(1), 169-176. https://doi.org/10.1016/j.bbrc.2018.11.074

Liu, J., Chen, Q., Huang, W., Horak, K. M., Zheng, H., Mestril, R., & Wang, X. (2006). Impairment of the ubiquitin-proteasome system in desminopathy mouse hearts. *FASEB J*, *20*(2), 362-364. https://doi.org/10.1096/fj.05-4869fje

Liu, S., D'Amico, D., Shankland, E., Bhayana, S., Garcia, J. M., Aebischer, P., Rinsch, C., Singh, A., & 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. https://doi.org/10.1001/jamanetworkopen.2021.44279

Lobrinus, J. A., Janzer, R. C., Kuntzer, T., Matthieu, J. M., Pfend, G., Goy, J. J., & Bogousslavsky, J. (1998). Familial cardiomyopathy and distal myopathy with abnormal desmin accumulation and migration. *Neuromuscul Disord*, *8*(2), 77-86. https://doi.org/10.1016/s0960-8966(98)00005-4

Loke, W. M., Hodgson, J. M., Proudfoot, J. M., McKinley, A. J., Puddey, I. B., & Croft, K. D. (2008). Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *Am J Clin Nutr*, *88*(4), 1018-1025. https://doi.org/10.1093/ajcn/88.4.1018

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, *42*(2), 145-151. https://doi.org/10.1016/j.cyto.2008.01.006

Luan, P., D'Amico, D., Andreux, P. A., Laurila, P. P., Wohlwend, M., Li, H., Imamura de Lima, T., Place, N., Rinsch, C., Zanou, N., & Auwerx, J. (2021). Urolithin A improves muscle function by inducing mitophagy in muscular dystrophy. *Sci Transl Med*, *13*(588). https://doi.org/10.1126/scitranslmed.abb0319

Maddison, P., Damian, M. S., Sewry, C., McGorrian, C., Winer, J. B., Odgerel, Z., Shatunov, A., Lee, H. S., & Goldfarb, L. G. (2012). Clinical and myopathological characteristics of desminopathy caused by a mutation in desmin tail domain. *Eur Neurol*, *68*(5), 279-286. https://doi.org/10.1159/000341617

Mafi, F., Biglari, S., Ghardashi Afousi, A., & Gaeini, A. A. (2019). Improvement in Skeletal Muscle Strength and Plasma Levels of Follistatin and Myostatin Induced by an 8-Week Resistance Training and Epicatechin Supplementation in Sarcopenic Older Adults. *J Aging Phys Act*, *27*(3), 384-391. https://doi.org/10.1123/japa.2017-0389

Mao, X., Gu, Y., Sui, X., Shen, L., Han, J., Wang, H., Xi, Q., Zhuang, Q., Meng, Q., & Wu, G. (2021). Phosphorylation of Dynamin-Related Protein 1 (DRP1) Regulates Mitochondrial Dynamics and Skeletal Muscle Wasting in Cancer Cachexia. *Front Cell Dev Biol*, *9*, 673618. https://doi.org/10.3389/fcell.2021.673618

Martineau, L. C., & Gardiner, P. F. (2001). Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. *J Appl Physiol (1985)*, *91*(2), 693-702. https://doi.org/10.1152/jappl.2001.91.2.693

McDonald, C. M., Ramirez-Sanchez, I., Oskarsson, B., Joyce, N., Aguilar, C., Nicorici, A., Dayan, J., Goude, E., Abresch, R. T., Villarreal, F., Ceballos, G., Perkins, G., Dugar, S., Schreiner, G., & Henricson, E. K. (2021). (-)-Epicatechin induces mitochondrial biogenesis and markers of muscle regeneration in adults with Becker muscular dystrophy. *Muscle Nerve*, *63*(2), 239-249. https://doi.org/10.1002/mus.27108

Mendoza, E., Moen, D. S., & Holt, N. C. (2023). The importance of comparative physiology: mechanisms, diversity and adaptation in skeletal muscle physiology and mechanics. *J Exp Biol*, *226*(Suppl_1). https://doi.org/10.1242/jeb.245158

Monje, C., Jannas-Vela, S., Baar, K., & Zbinden-Foncea, H. (2020). Case report of an exercise training and nutritional intervention plan in a patient with A350P mutation in DES gene. *Clin Case Rep*, *8*(2), 283-288. https://doi.org/10.1002/ccr3.2607

Mukund, K., & Subramaniam, S. (2020). Skeletal muscle: A review of molecular structure and function, in health and disease. *Wiley Interdiscip Rev Syst Biol Med*, *12*(1), e1462. https://doi.org/10.1002/wsbm.1462

Nakano, D., & Machida, S. (2022). Mitochondrial fusion- and fission-related protein expression in the regulation of skeletal muscle mass. *Physiol Rep*, *10*(8), e15281. https://doi.org/10.14814/phy2.15281

Navarrete-Yanez, V., Garate-Carrillo, A., Rodriguez, A., Mendoza-Lorenzo, P., Ceballos, G., Calzada-Mendoza, C., Hogan, M. C., Villarreal, F., & Ramirez-Sanchez, I. (2020). Effects of (-)-epicatechin on neuroinflammation and

hyperphosphorylation of tau in the hippocampus of aged mice. *Food Funct*, *11*(12), 10351-10361. https://doi.org/10.1039/d0fo02438d

Ono, Y., Maejima, Y., Saito, M., Sakamoto, K., Horita, S., Shimomura, K., Inoue, S., & Kotani, J. (2020). TAK-242, a specific inhibitor of Toll-like receptor 4 signalling, prevents endotoxemia-induced skeletal muscle wasting in mice. *Sci Rep*, *10*(1), 694. https://doi.org/10.1038/s41598-020-57714-3

Ono, Y., & Sakamoto, K. (2017). Lipopolysaccharide inhibits myogenic differentiation of C2C12 myoblasts through the Toll-like receptor 4-nuclear factor-kappaB signaling pathway and myoblast-derived tumor necrosis factor-alpha. *PLoS One*, *12*(7), e0182040. https://doi.org/10.1371/journal.pone.0182040

Pagliuso, A., Cossart, P., & Stavru, F. (2018). The ever-growing complexity of the mitochondrial fission machinery. *Cell Mol Life Sci*, *75*(3), 355-374. https://doi.org/10.1007/s00018-017-2603-0

Palikaras, K., Lionaki, E., & Tavernarakis, N. (2018). Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. *Nat Cell Biol*, *20*(9), 1013-1022. https://doi.org/10.1038/s41556-018-0176-2

Petrof, B. J. (2022). Macrophage plasticity in Duchenne muscular dystrophy: a nexus of pathological remodelling with therapeutic implications. *J Physiol*, *600*(15), 3455-3464. https://doi.org/10.1113/JP281673

Picca, A., Faitg, J., Auwerx, J., Ferrucci, L., & D'Amico, D. (2023). Mitophagy in human health, ageing and disease. *Nat Metab*, *5*(12), 2047-2061. https://doi.org/10.1038/s42255-023-00930-8

Prince, P. D., Fischerman, L., Toblli, J. E., Fraga, C. G., & Galleano, M. (2017). LPS-induced renal inflammation is prevented by (-)-epicatechin in rats. *Redox Biol*, *11*, 342-349. https://doi.org/10.1016/j.redox.2016.12.023

Quintana-Cabrera, R., & Scorrano, L. (2023). Determinants and outcomes of mitochondrial dynamics. *Mol Cell*, *83*(6), 857-876. https://doi.org/10.1016/j.molcel.2023.02.012

Ramirez-Sanchez, I., Taub, P. R., Ciaraldi, T. P., Nogueira, L., Coe, T., Perkins, G., Hogan, M., Maisel, A. S., Henry, R. R., Ceballos, G., & Villarreal, F. (2013). (-)-Epicatechin rich cocoa mediated modulation of oxidative stress regulators in

skeletal muscle of heart failure and type 2 diabetes patients. *Int J Cardiol*, *168*(4), 3982-3990. https://doi.org/10.1016/j.ijcard.2013.06.089

Rizzo, G., Di Maggio, R., Benedetti, A., Morroni, J., Bouche, M., & Lozanoska-Ochser, B. (2020). Splenic Ly6Chi monocytes are critical players in dystrophic muscle injury and repair. *JCI Insight*, *5*(2). https://doi.org/10.1172/jci.insight.130807

Roberson, E. D. O., Mesa, R. A., Morgan, G. A., Cao, L., Marin, W., & Pachman, L. M. (2022). Transcriptomes of peripheral blood mononuclear cells from juvenile dermatomyositis patients show elevated inflammation even when clinically inactive. *Sci Rep*, *12*(1), 275. https://doi.org/10.1038/s41598-021-04302-8

Romanello, V., & Sandri, M. (2015). Mitochondrial Quality Control and Muscle Mass Maintenance. *Front Physiol*, *6*, 422. https://doi.org/10.3389/fphys.2015.00422

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., & Hauschildt, S. (2011). LPS-induced cytokine production in human monocytes and macrophages. *Crit Rev Immunol*, *31*(5), 379-446. https://doi.org/10.1615/critrevimmunol.v31.i5.20

Ryu, D., Mouchiroud, L., Andreux, P. A., Katsyuba, E., Moullan, N., Nicolet-Dit-Felix, A. A., Williams, E. G., Jha, P., Lo Sasso, G., Huzard, D., Aebischer, P., Sandi, C., Rinsch, C., & 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. https://doi.org/10.1038/nm.4132

Scala, P., Manzo, P., Lamparelli, E. P., Lovecchio, J., Ciardulli, M. C., Giudice, V., Selleri, C., Giordano, E., Rehak, L., Maffulli, N., & Della Porta, G. (2022). Peripheral blood mononuclear cells contribute to myogenesis in a 3D bioengineered system of bone marrow mesenchymal stem cells and myoblasts. *Front Bioeng Biotechnol*, *10*, 1075715. https://doi.org/10.3389/fbioe.2022.1075715

Shi, C. S., Shenderov, K., Huang, N. N., Kabat, J., Abu-Asab, M., Fitzgerald, K. A., Sher, A., & Kehrl, J. H. (2012). Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol*, *13*(3), 255-263. https://doi.org/10.1038/ni.2215

Singh, A., D'Amico, D., Andreux, P. A., Fouassier, A. M., Blanco-Bose, W., Evans, M., Aebischer, P., Auwerx, J., & Rinsch, C. (2022). Urolithin A improves muscle

strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. *Cell Rep Med*, *3*(5), 100633. https://doi.org/10.1016/j.xcrm.2022.100633

Smolina, N., Khudiakov, A., Knyazeva, A., Zlotina, A., Sukhareva, K., Kondratov, K., Gogvadze, V., Zhivotovsky, B., Sejersen, T., & Kostareva, A. (2020). Desmin mutations result in mitochondrial dysfunction regardless of their aggregation properties. *Biochim Biophys Acta Mol Basis Dis*, *1866*(6), 165745. https://doi.org/10.1016/j.bbadis.2020.165745

Summan, M., Warren, G. L., Mercer, R. R., Chapman, R., Hulderman, T., Van Rooijen, N., & Simeonova, P. P. (2006). Macrophages and skeletal muscle regeneration: a clodronate-containing liposome depletion study. *Am J Physiol Regul Integr Comp Physiol*, *290*(6), R1488-1495. https://doi.org/10.1152/ajpregu.00465.2005

Tabara, L. C., Segawa, M., & Prudent, J. (2025). Molecular mechanisms of mitochondrial dynamics. *Nat Rev Mol Cell Biol*, *26*(2), 123-146. https://doi.org/10.1038/s41580-024-00785-1

Tan, Y., Zanoni, I., Cullen, T. W., Goodman, A. L., & Kagan, J. C. (2015). Mechanisms of Toll-like Receptor 4 Endocytosis Reveal a Common Immune-Evasion Strategy Used by Pathogenic and Commensal Bacteria. *Immunity*, *43*(5), 909-922. https://doi.org/10.1016/j.immuni.2015.10.008

Tang, T., Zhuo, Y., Xie, L., Wang, H., & Yang, M. (2020). Sarcopenia index based on serum creatinine and cystatin C is associated with 3-year mortality in hospitalized older patients. *Sci Rep*, *10*(1), 1260. https://doi.org/10.1038/s41598-020-58304-z

Tao, H., Li, W., Zhang, W., Yang, C., Zhang, C., Liang, X., Yin, J., Bai, J., Ge, G., Zhang, H., Yang, X., Li, H., Xu, Y., Hao, Y., Liu, Y., & Geng, D. (2021). Urolithin A suppresses RANKL-induced osteoclastogenesis and postmenopausal osteoporosis by, suppresses inflammation and downstream NF-κB activated pyroptosis pathways. *Pharmacol Res*, *174*, 105967. https://doi.org/10.1016/j.phrs.2021.105967

Tao, H., Tang, X., & Tao, H. (2022). TLR4 activation inhibits the proliferation and osteogenic differentiation of skeletal muscle stem cells by downregulating

LGI1. *J Physiol Biochem*, *78*(3), 667-678. https://doi.org/10.1007/s13105-022-00888-3

Tapia-Curimil, G., Castro-Sepulveda, M., & Zbinden-Foncea, H. (2024). Effect of epicatechin consumption on the inflammatory pathway and mitochondria morphology in PBMC from a R350P desminopathy patient: A case report. *Physiol Rep*, *12*(8), e16020. https://doi.org/10.14814/phy2.16020

Taub, P. R., Ramirez-Sanchez, I., Ciaraldi, T. P., Perkins, G., Murphy, A. N., Naviaux, R., Hogan, M., Maisel, A. S., Henry, R. R., Ceballos, G., & Villarreal, F. (2012). Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clin Transl Sci*, *5*(1), 43-47. https://doi.org/10.1111/j.1752-8062.2011.00357.x

Theret, M., Saclier, M., Messina, G., & Rossi, F. M. V. (2022). Macrophages in Skeletal Muscle Dystrophies, An Entangled Partner. *J Neuromuscul Dis*, *9*(1), 1-23. https://doi.org/10.3233/JND-210737

Tomás-Barberán, F. A., González-Sarrías, A., García-Villalba, R., Núñez-Sánchez, M. A., Selma, M. V., García-Conesa, M. T., & Espín, J. C. (2017). Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. *Mol Nutr Food Res*, *61*(1). https://doi.org/10.1002/mnfr.201500901

Torres-Ruiz, J., Carrillo-Vazquez, D. A., Padilla-Ortiz, D. M., Vazquez-Rodriguez, R., Nunez-Alvarez, C., Juarez-Vega, G., & Gomez-Martin, D. (2020). TLR expression in peripheral monocyte subsets of patients with idiopathic inflammatory myopathies: association with clinical and immunological features. *J Transl Med*, *18*(1), 125. https://doi.org/10.1186/s12967-020-02290-3

Tu, H., & Li, Y. L. (2023). Inflammation balance in skeletal muscle damage and repair. *Front Immunol*, *14*, 1133355. https://doi.org/10.3389/fimmu.2023.1133355

Vajsar, J., Becker, L. E., Freedom, R. M., & Murphy, E. G. (1993). Familial desminopathy: myopathy with accumulation of desmin-type intermediate filaments. *J Neurol Neurosurg Psychiatry*, *56*(6), 644-648. https://doi.org/10.1136/jnnp.56.6.644

Verhoeckx, K., Cotter, P., Lopez-Exposito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., & Wichers, H. (2015). The Impact of Food Bioactives on

Health: in vitro and ex vivo models. In K. Verhoeckx, P. Cotter, I. Lopez-Exposito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models*. https://doi.org/10.1007/978-3-319-16104-4

Verzola, D., Bonanni, A., Sofia, A., Montecucco, F., D'Amato, E., Cademartori, V., Parodi, E. L., Viazzi, F., Venturelli, C., Brunori, G., & Garibotto, G. (2017). Toll-like receptor 4 signalling mediates inflammation in skeletal muscle of patients with chronic kidney disease. *J Cachexia Sarcopenia Muscle*, *8*(1), 131-144. https://doi.org/10.1002/jcsm.12129

Wai, T. (2024). Is mitochondrial morphology important for cellular physiology? *Trends Endocrinol Metab*, *35*(10), 854-871. https://doi.org/10.1016/j.tem.2024.05.005

Wattjes, M. P., Kley, R. A., & Fischer, D. (2010). Neuromuscular imaging in inherited muscle diseases. *Eur Radiol*, *20*(10), 2447-2460. https://doi.org/10.1007/s00330-010-1799-2

Weinberg, S. E., Sena, L. A., & Chandel, N. S. (2015). Mitochondria in the regulation of innate and adaptive immunity. *Immunity*, *42*(3), 406-417. https://doi.org/10.1016/j.immuni.2015.02.002

Welc, S. S., & Clanton, T. L. (2013). The regulation of interleukin-6 implicates skeletal muscle as an integrative stress sensor and endocrine organ. *Exp Physiol*, *98*(2), 359-371. https://doi.org/10.1113/expphysiol.2012.068189

Westermann, B. (2010). Mitochondrial fusion and fission in cell life and death. *Nat Rev Mol Cell Biol*, *11*(12), 872-884. https://doi.org/10.1038/nrm3013

Williamson, D., Gallagher, P., Harber, M., Hollon, C., & Trappe, S. (2003). Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. *J Physiol*, *547*(Pt 3), 977-987. https://doi.org/10.1113/jphysiol.2002.036673

Winter, L., Wittig, I., Peeva, V., Eggers, B., Heidler, J., Chevessier, F., Kley, R. A., Barkovits, K., Strecker, V., Berwanger, C., Herrmann, H., Marcus, K., Kornblum, C., Kunz, W. S., Schroder, R., & Clemen, C. S. (2016). Mutant desmin substantially perturbs mitochondrial morphology, function and maintenance

in skeletal muscle tissue. *Acta Neuropathol*, *132*(3), 453-473. https://doi.org/10.1007/s00401-016-1592-7

Wu, Z., Xu, Z., Zhou, X., Li, H., Zhao, L., Lv, Y., Guo, Y., Shen, G., He, Y., & Lei, P. (2022). sGRP78 enhances selective autophagy of monomeric TLR4 to regulate myeloid cell death. *Cell Death Dis*, *13*(7), 587. https://doi.org/10.1038/s41419-022-05048-5

Yapa, N. M. B., Lisnyak, V., Reljic, B., & Ryan, M. T. (2021). Mitochondrial dynamics in health and disease. *FEBS Lett*, *595*(8), 1184-1204. https://doi.org/10.1002/1873-3468.14077

Yeow, D., Katz, M., Henderson, R., Prasad, S., Denman, R., Blum, S., Davis, M., Robertson, T., & McCombe, P. (2022). Phenotypic variability within the desminopathies: A case series of three patients. *Front Neurol*, *13*, 1110934. https://doi.org/10.3389/fneur.2022.1110934

Youle, R. J., & van der Bliek, A. M. (2012). Mitochondrial fission, fusion, and stress. *Science*, *337*(6098), 1062-1065. https://doi.org/10.1126/science.1219855

Yuan, Z. L., Mo, Y. Z., Li, D. L., Xie, L., & Chen, M. H. (2023). Inhibition of ERK downregulates autophagy via mitigating mitochondrial fragmentation to protect SH-SY5Y cells from OGD/R injury. *Cell Commun Signal*, *21*(1), 204. https://doi.org/10.1186/s12964-023-01211-3

Zbinden-Foncea, H., Castro-Sepulveda, M., Fuentes, J., & Speisky, H. (2022). Effect of Epicatechin on Skeletal Muscle. *Curr Med Chem*, *29*(6), 1110-1123. https://doi.org/10.2174/0929867329666211217100020

Zbinden-Foncea, H., Tapia-Curimil, G., b, A. D., Tuñón-Suárez, M., a, M. C.-S., a, J. F., b, H. M., d, M. F., & d, L. D. (2024). Physical activity levels, gut permeability, circulating inflammatory cytokines and TLR4 signalling in PBMCs of COVID-19 patients. *Advanced Exercise and Health Science*, *1*(1), 8. https://doi.org/10.1016/j.aehs.2024.01.001

Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature*, *469*(7329), 221-225. https://doi.org/10.1038/nature09663

Zong, M., Bruton, J. D., Grundtman, C., Yang, H., Li, J. H., Alexanderson, H., Palmblad, K., Andersson, U., Harris, H. E., Lundberg, I. E., & Westerblad, H. (2013). TLR4

as receptor for HMGB1 induced muscle dysfunction in myositis. *Ann Rheum Dis*, *72*(8), 1390-1399. https://doi.org/10.1136/annrheumdis-2012-202207