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**Characterization of a newly generated knock-in
mouse model carrying a human mutation associated
to Tubular Aggregate Myopathy**

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ATP: adenosine triphosphate; Ca^{2+} : Calcium; CASQ1: calsequestrin type-1; CEUs: calcium release units; CRUs: calcium release units; CEU, calcium entry unit; DHPR: dihydropyridine receptors; EC coupling: excitation-contraction coupling; EM, electron microscopy; ER: endoplasmic reticulum; RyR1: ryanodine receptor type-1; SOCE: store operated calcium entry; SR: sarcoplasmic reticulum; STIM1: stromal interaction molecule-1; TAM: tubular aggregates myopathy; TAs: tubular aggregates; TTs: transverse tubules; WT, wild type.

OVERVIEW OF THE THESIS

Background:

Store Operated Calcium (Ca^{2+}) Entry (SOCE) is a mechanism, first described in non-excitable cells, that is triggered by depletion of intracellular Ca^{2+} stores (endoplasmic or sarcoplasmic reticulum, ER and SR) (Parekh & Penner, 1997; Putney, 1986). SOCE mediates recovery of extracellular Ca^{2+} thanks to the interaction between stromal interaction molecule 1 (STIM1) and ORAI1 (Liou, Kim, Won, et al., 2005; Vig et al., 2006). It seems that, in skeletal muscle fibers, the mechanism is modulated by Calsequestrin 1 (CASQ1) (Shin et al., 2003). Mutations in the genes encoding for STIM1, ORAI1, and CASQ1 have been linked to tubular aggregate (TA) myopathy (TAM)(Barone et al., 2017; Böhm et al., 2014; Y. Endo et al., 2015), a rare myopathy characterized by muscle pain, cramping, weakness, dysfunction of SOCE, and presence of tubular aggregates (TAs)(Jain et al., 2008; Pierobon-Bormioli et al., 1985), dysfunctional accumulation of SR membranes that are also found in fast twitch muscle of ageing-sedentary male mice (Boncompagni et al., 2012). The mechanisms linking human mutations to SOCE dysfunction, and to development of TAM are still unknown. The lack of a robust animal model that accurately mirrors the human pathology has been a significant barrier to elucidating the pathophysiological mechanisms underlying TAM and the development of effective treatments.

After Introduction dealing with a) mechanisms controlling intracellular Ca^{2+} homeostasis (EC coupling and SOCE), and b) TA and TAM (Chapter 1), my PhD thesis contains a detailed description of the project developed in these three years as PhD student (Chapter 2).

Scientific Hypotheses:

Both a) inactivity and b) mutations in proteins involved in SOCE (STIM1, ORAI1, and CASQ1) result in dysfunctional SOCE, and accumulation of TAs. On the other hand, c) muscle activity inducing fatigue, result in activation-recruitment of SOCE and reduction in TA formation.

Aim of the project:

Understand the pathophysiology of TAM caused by a mutation in CASQ1, a protein involved in modulation of SOCE.

In the first part of the project, we studied the formation of TAs at different ages in male and female animals in a newly generated mouse model of TAM carrying the human D44N mutation and coupled these advancements to the understanding of how their accrual is linked to dysfunctional SOCE and muscle weakness. In the second part of my work we investigated if voluntary training in wheel cages was able to counteract TAs formation and ameliorate signs of pathology in affected muscles.

As a side aspect, we also investigated mitochondrial damage in affected fibers.

Significance:

Our project may improve the current understanding of the mechanisms leading to generate possible mechanistic insights that could provide the basis for the development of safe/effective interventions to limit muscle fatigue, weakness, and dysfunction in TAM with a potential application to other human myopathies/conditions associated with the altered Ca^{2+} homeostasis.

Chapter 1: Ultrastructure of Skeletal Muscle Fibers

Skeletal muscle is a highly organized tissue composed of bundles of muscle fibers which contain several myofibrils. The typical striation of skeletal muscle tissue is due to the regular arrangement of contractile proteins (actin and myosin) constituting the light and dark zones. The light bands, i.e. I bands, are less dense protein, stain weakly and are formed by thin filaments; the dark bands, i.e. A band, have dense protein and stain deeply with basic dyes and are formed by thick filaments (Bagshaw, 1982). In the middle of the I band there is a zone called the Z-line or Z-disk, more dense of proteins that appears darker; in the middle of the A band there is a dense protein zone called the H-zone where thick filaments do not overlapped with thin filaments (Loeb, 1999); (Bagshaw, 1982). In the center of the H-zone there is a region called the M-line (for middle) and it is composed of a connective tissue network binding the thick filaments and maintaining them in a hexagonal pattern when viewed in a transverse plane. The functional unit of a skeletal muscle fiber is the sarcomere, a highly organized arrangement of the contractile myofilaments - actin (thin filament) and myosin (thick filament) - delimited by two Z-lines (Figure 1). The thin filament consists of two F-actin chains wrapped in a double helix, while the thick filament is formed by a few hundred molecules of myosin. In the thick filament, the myosin molecules aggregate together at the level of the tails, while the heads are projected to the outside. Each head has two sites: one capable of binding actin and the other endowed with ATPase action which, through hydrolysis of ATP, frees a considerable amount of energy necessary for contraction. As muscle shortens the sarcomere I band and H zone decrease in length while the A band length remains constant. These observations lead to development of the sliding filament theory (Huxley & Niedergerke, 1954). In addition to the contractile proteins, which are the main actors in filament sliding during the muscle contraction-relaxation cycle, there are also regulatory proteins in the myofibrils: troponin (Mizuno & Honda, 2006) and tropomyosin. These proteins are involved in controlling muscle contraction by preventing

actin from binding to myosin, thus preventing contraction. At low concentrations of Ca^{2+} , tropomyosin sterically blocks the myosin binding site on actin, while at high concentrations, its binding to the troponin complex induces a conformational change that unmask the actin-myosin binding site, making muscle contraction possible. Finally, there are accessory and structural proteins: nebulin and titin. Nebulin is an inelastic protein located in the thin filaments; it anchors to the Z line, aligns the actin filaments, and stabilizes the two F-actin filaments. Titin, unlike nebulin, is an elastic protein: it stabilizes the contractile filaments, ensuring their alignment even without tension, and allows the muscle to return to its initial length once the contraction is completed.

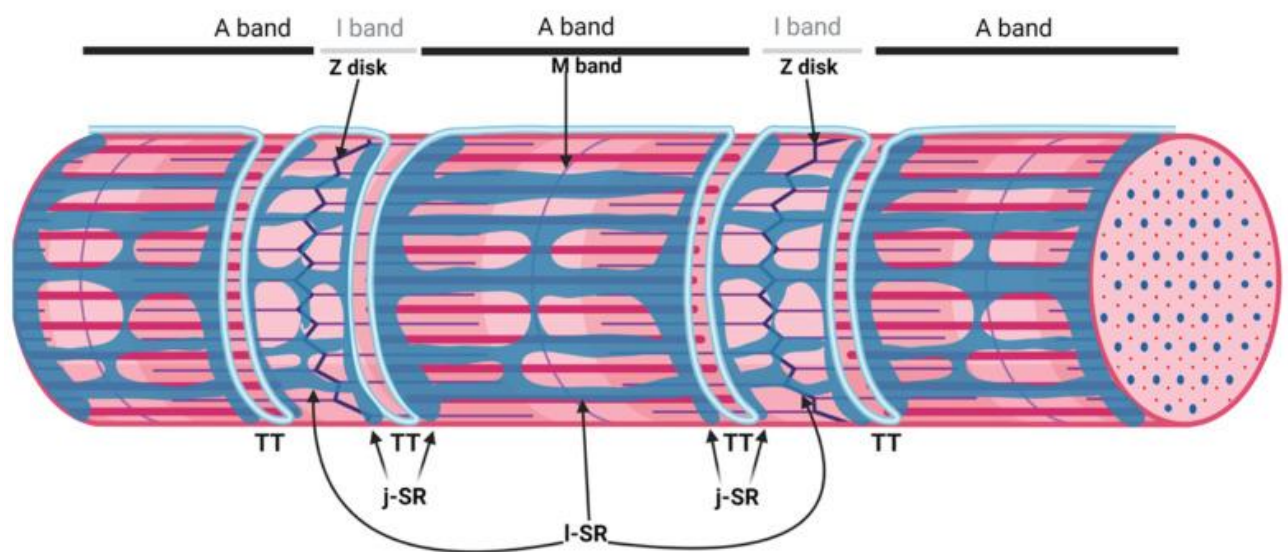


Figure 1. Organization of the sarcoplasmic reticulum in skeletal muscle cells. The regular alternation of anisotropic (A) and isotropic (I) bands along a single myofibril is depicted in the upper part of the image. A- and Ibands are bisected by the Mband and the Z-disk, respectively. The portion of the myofibril between two Z-disks is the sarcomere. The sarcoplasmic reticulum (SR) is composed of tubules and cisternae surrounding each myofibril. The elongated tubules are known as longitudinal SR (l-SR); they are dedicated to the removal of Ca^{2+} from the cytosol and are localized around the A- and Iband of each sarcomere. At the borders between the A- and Iband, the l-SR merges to form the terminal cisternae. These are positioned at the opposite sides of a transverse tubule (TT); the structure formed by two terminal cisternae and one TT is called a "triad". The region of the terminal cisternae that opposes the TT membrane is called junctional SR (j-SR). Adapted from "Myofibril Structure" using BioRender.com (2022).

1. Organization of organelles and intracellular membranes involved in aerobic ATP production and Ca^{2+} handling.

Calcium (Ca^{2+}) ions are extremely versatile intracellular messengers that control many physiological functions including mitochondrial activity (Clapham, 2007; Dolmetsch, 2003; Endo, 2006; Gerke et al., 2005; Marchi et al., 2018; Rasmussen et al., 1976). Entry of Ca^{2+} in the mitochondrial matrix (defined *Excitation-Metabolism Coupling*) stimulates the respiratory chain (Bolaños & Calderón, 2022; Gherardi et al., 2020; Rudolf et al., 2004; Shkryl & Shirokova, 2006) and relies on a bi-directional communication between the sarcoplasmic reticulum (SR) and mitochondria that involves Ca^{2+} ions and reactive oxygen species (ROS) signalling (Bolaños et al., 2007; Csordás & Hajnóczky, 2009; Eisner et al., 2013; Gherardi et al., 2020; A. E. Rossi et al., 2009; Rudolf et al., 2004; Shkryl & Shirokova, 2006).

Calcium handling in muscle fibers depends on several mechanisms, the most important being: a) release of Ca^{2+} from SR in response to action potentials delivered by motor neurons which controls the contraction and relaxation of the contractile elements, a mechanism known as *Excitation-Contraction (EC) Coupling* (Calderón et al., 2014; Franzini-Armstrong & Protasi, 1997; Hernández-Ochoa et al., 2015; Sandow, 1952; Sandow et al., 1965; Schneider et al., 2004; Stokes & Wagenknecht, 2000); and b) entry of Ca^{2+} from the extracellular space following repetitive stimulation that causes SR depletion, defined as *Store Operated Ca^{2+} Entry* (SOCE) (Dirksen, 2009; Kurebayashi & Ogawa, 2001; Launikonis et al., 2003; Launikonis & Ríos, 2007; Lilliu et al., 2021; Michelucci et al., 2018; Parekh & Penner, 1997; Protasi et al., 2022; Putney, 1986; Sztretye et al., 2017; Wei-Lapierre et al., 2013).

Each of the different mechanisms described above uses different intracellular membrane systems and organelles (Figure 2): a) mitochondrial uptake of Ca^{2+} during *Excitation-Metabolism Coupling* likely relies on the position of mitochondria next to sites of SR (Bolaños et al., 2007; Boncompagni et al., 2009a; Franzini-Armstrong & Boncompagni, 2011; Hatano et

al., 2013; Marchioretti et al., 2023; Rossi et al., 2009, 2011a; Santo-Domingo & Demareux, 2010), and possibly also to sites of SOCE (see below); b) EC coupling is mediated by *triads*, also known as *Ca²⁺ Release Units* (CRUs), specialized intracellular junctions formed by transverse tubules (TTs, invaginations of the surface membrane that carry the action potential into the fiber interior) and specialized domains of the SR (i.e. the terminal cisternae or junctional SR, jSR) dedicated to accumulate and release the Ca²⁺ needed to activate contraction (Flucher et al., 1994; Flucher & Franzini-Armstrong, 1996; Franzini-Armstrong, 1970; Franzini-Armstrong & Jorgensen, 1994; Takekura et al., 1994); finally c) SOCE occurs in *Ca²⁺ Entry Units* (CEUs), also junctions between SR and TT, that are few and small in rest condition, but that grow in number and size during exercise (Boncompagni et al., 2017, 2018; Michelucci et al., 2019, 2020, 2022; Protasi et al., 2021, 2023). Mitochondria, triads, and CEUs in adult/healthy fibers retain a specific intracellular position, which is dictated by the dark-pale cross striation, which in turn is generated by the lateral alignment of contractile elements, i.e. the *myofibrils*.

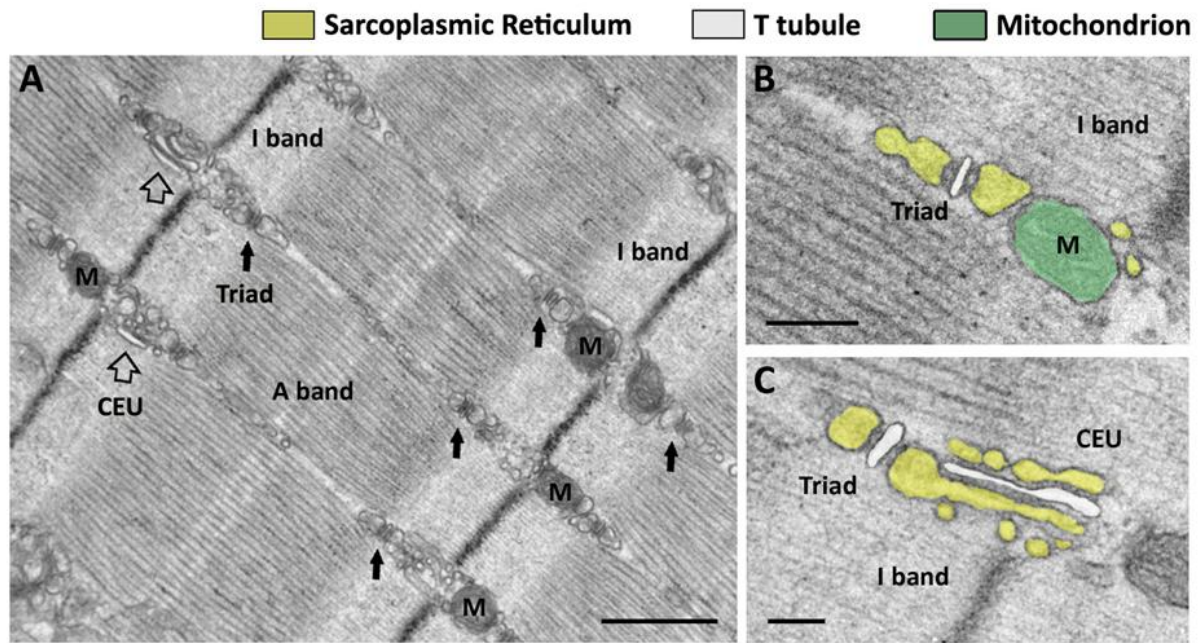


Figure 2. Disposition (panel A) and morphology (panels B and C) of Mitochondria, Triads, and Ca^{2+} Entry Units in adult skeletal muscle fibers. (A) Skeletal muscle fibers are striated cells (i.e. characterized by alternating I and A bands) in which mitochondria (M), triads (small arrows), and Ca^{2+} entry units (CEUs, empty arrows) occupy a specific position: mitochondria and CEUs at the I band, while triads are placed in proximity of the transition between I- and A bands. (B and C) In a higher magnification electron microscopy image, we have false-colored a triad (formed by a central TT flanked by two SR terminal cisternae) with a closely associated mitochondrion (panel B) and a representative CEU, the site of SOCE in skeletal muscle, next to a triad (panel C). Scale bars: A = 0.5 μm ; B and C = 0.1 μm .

1.1 The organelles deputing aerobic ATP production: Mitochondria.

Adenosine triphosphate (ATP) is the source of energy for many cellular functions. In muscle fibers, ATP: i) allows dissociation of the myosin heads from actin; ii) provides the energy needed for the power stroke, hence force generation; iii) allows relaxation of fibers thanks to Ca^{2+} re-uptake operated by SERCA pumps (sarco-endoplasmic reticulum Ca^{2+} ATP-ase)(Ebashi & Endo, 1968; Lymn & Taylor, 1971); iv) re-establishes the proper balance of Na^+ and K^+ following the propagation of action potentials thanks to Na^+/K^+ ATPases in the plasma membrane (Castillo et al., 2015). As ATP is continuously consumed, the cells must be capable of producing new ATP in a fast and efficient manner. Consumed ATP can be replenished either anaerobically or aerobically: the anaerobic alactic (meaning that it does not produce lactic acid) and lactic systems replenish ATP without the need of oxygen (Sweeney, 1994), while the aerobic system replenishes ATP in presence of oxygen.

The aerobic metabolism occurs within the mitochondrial matrix and represents the major source of cellular ATP production in muscle providing about 90% of all cellular metabolism (Bonora et al., 2012). Aerobic metabolism is the process by which carbohydrates and fatty acids are converted into energy in presence of oxygen (Farrell et al., 2012).

It has been proposed that mitochondria have a bi-directional interaction with the SR in adult skeletal muscle fibers, an orthograde and a retrograde communication: a) SR-to-mitochondrion signalling enhances aerobic ATP production through Ca^{2+} -influx in the mitochondrial matrix (Hansford, 1987; McMillin & Madden, 1989; Territo et al., 2000); whereas b) mitochondrion-to-SR signalling supposedly uses reactive oxygen species (ROS) to suppress SR Ca^{2+} release (Isaeva et al., 2005; Isaeva and & Shirokova, 2003). This crosstalk has been also defined excitation-metabolism coupling (A. E. Rossi et al., 2009, 2011).

As ATP production during mitochondrial respiration uses mechanisms stimulated by elevations in concentration of Ca^{2+} ($[\text{Ca}^{2+}]$) in the mitochondrial matrix (Hansford, 1994; McMillin & Madden, 1989; Territo et al., 2000), activation of muscle contraction induced by transient

elevation in intracellular $[Ca^{2+}]$ ($i[Ca^{2+}]$) also serves as a trigger to boost aerobic ATP production needed to keep pace with the increased crossbridge cycle activity and SERCA-mediated Ca^{2+} removal from the cytoplasm. While it was reported that contractile relaxation was slower in more glycolytic fibers (poor in mitochondria) than in slow twitch fibers (rich in mitochondria)(Gillis, 1997), suggesting that under physiological conditions mitochondria may influence the temporal developments of Ca^{2+} transients, the fact that mitochondria accumulate large amount of Ca^{2+} (suggested by some authors) remains controversial. This is because, on one side, the $[Ca^{2+}]$ required for mitochondrial Ca^{2+} transport is considerably higher than the one achieved during Ca^{2+} transients elicited by EC coupling (only $\sim 1-2\mu M$) while, on the other, the relative percentage of fiber volume occupied by mitochondria is relatively small, ranging from about 3-4% to approximately double, respectively in fast vs. slow twitch fibers (Boncompagni et al., 2009; Pietrangelo et al., 2015, 2019). Abnormalities in Ca^{2+} handling and inefficient aerobic ATP production by mitochondria underline several physio-pathological conditions, such as dysfunction in myopathies, compromised metabolic balance, skeletal muscle fatigue, and reduced endurance in ageing. While different mechanisms were hypothesized for entry of Ca^{2+} into the mitochondria (Beutner et al., 2001; Buntinas et al., 2001; Gunter & Gunter, 1994), the identification of a mitochondrial Ca^{2+} uniporter (MCU) may have revealed the main pathways for mitochondrial Ca^{2+} uptake (Baughman et al., 2011; De Stefani et al., 2011; Pan et al., 2013). While the $[Ca^{2+}]$ needed for Ca^{2+} uptake in isolated mitochondria is about $10\mu M$ (Sembrowich et al., 1985), studies that directly measured mitochondrial Ca^{2+} in intact skeletal fibers (Bolaños et al., 2007; Shkryl & Shirokova, 2006) proposed that this uptake occurs also during single twitches and tetanic stimulation (Rudolf et al., 2004), conditions in which $[Ca^{2+}]$ do not rise as high. Physiologists tried to reconcile this apparent discrepancy postulating the concept of *local Ca^{2+} microdomains*, i.e. narrow spaces inside muscle fibers in which $[Ca^{2+}]$ may rise high enough in proximity of mitochondria before Ca^{2+} will diffuse away to myofibrils. For this to happen mitochondria would have to be

strategically positioned in proximity to sites of Ca^{2+} release. Indeed, an intimate structural interaction between mitochondria and endoplasmic/sarcoplasmic reticulum (ER/SR) has been identified in non-muscle cells (Csordás et al., 2006; Mannella et al., 1998), in cardiomyocytes (Sharma et al., 2000), and finally also in skeletal muscle fibers (Boncompagni et al., 2009).

In mammalian skeletal fibers, most mitochondria (M in Figure 2, panel A) seem to occupy a preferential position in proximity to Z-lines, in correspondence of the pale band generated by the lateral alignment of myofibrils. Already in 1985, Ogata and Yamasaki in a elegant study performed by EM described a population of intermyofibrillar mitochondria surrounding myofibrils as incomplete rings on both sides of the Z-line (Ogata & Yamasaki, 1985). These I-band mitochondria were present in all types of fibers, i.e. fast, intermediate, and slow in adult rat leg muscles. It should be acknowledged that in slow-twitch fibers there are also other populations of mitochondria: some under the sarcolemma in proximity of capillaries and others forming longitudinal columns between myofibrils next to the A band (Ogata & Yamasaki, 1985). In 2009, Boncompagni and colleagues described how I band mitochondria in skeletal muscle fibers are placed in proximity of sites of Ca^{2+} release, the triads (Bolaños et al., 2007; Boncompagni et al., 2009), and detected the presence of small electron-dense strands (generically named *tethers*) between the outer mitochondrial membrane and the SR. Tethers must provide a strong link between the two organelles, as treatment with hypotonic solution causing swelling of the cytoplasmic space was unable to disrupt the SR-mitochondria association (Boncompagni et al., 2009). The molecular identity of tethers remains to be determined, even if a possible contribution of Mitofusin-2 was proposed (Ainbinder et al., 2015). Interestingly, before reaching their final position at the I band in adult fibers, most mitochondria in the first weeks after birth in mice are disposed longitudinally between myofibrils. The shift of the mitochondrial network from longitudinal to transversal during post-natal maturation of skeletal fibers mimics (but with a temporal delay) the maturation of the EC

coupling apparatus, in which TTs are initially longitudinal before becoming completely transversal (see Chapter 2, Section 2.2 for additional detail).

1.2 The sites of Excitation-Contraction (EC) Coupling: Triads (also named Ca^{2+} Release Units, CRUs).

EC coupling is a physiological event that converts the propagation of an action potential in the sarcolemma into a mechanical response of muscle fibers (named single twitch) due to release of Ca^{2+} from the SR, which activates contractile elements (Calderón et al., 2014; Ebashi, 1991; Protasi, 2002; Schneider, 1994; Wray et al., 2004), i.e. the myofibrils. The fact that contraction and relaxation of muscle fibers was the result of release and reuptake of Ca^{2+} ions from the SR is known long before the molecular machinery controlling EC coupling was discovered (Hasselbach, 1964; Hasselbach & Makinose, 1961; Hasselbach & Oetliker, 1983; Heilbrunn & Wiercinski, 1947; Niedergerke, 1955). In skeletal muscle fibers, EC coupling is defined as mechanical, to describe a direct communication between TTs that carries the electrical signal with the SR containing Ca^{2+} (Ríos et al., 1991, 1993; Ríos & Pizarro, 1991; Schneider, 1994; Schneider & Chandler, 1973). *Mechanical EC coupling* is the evolution of *Ca^{2+} -induced Ca^{2+} release (CICR)*, a more primitive EC coupling mechanism utilized by cardiac and smooth muscle cells, where the entry of Ca^{2+} from the extracellular space activates Ca^{2+} release from SR stores (Bers, 2002; Cannell et al., 1995; Collier et al., 1999, 2000; Fabiato, 1983, 1985; Näbauer et al., 1989; Orkand & Niedergerke, 1964). Mechanical EC coupling relies on the direct communication between two proteins: a) skeletal dihydropyridine receptors (DHPRs), voltage-gated L-type Ca^{2+} channels of external membranes that in skeletal muscle mainly acts as voltage sensors (Mikami et al., 1989; Rios & Brum, 1987; Ríos & Pizarro, 1991; Schneider & Chandler, 1973; Tanabe et al., 1987, 1988, 1990); and b) ryanodine receptors type-1 (RYR1), the main isoform of SR Ca^{2+} release channel expressed in adult skeletal muscle fibers (Franzini-Armstrong & Protasi, 1997; Giannini & Sorrentino, 1995; Nakai et al., 1998;

Sorrentino, 1995; Sorrentino & Volpe, 1993; Takeshima et al., 1989; Zorzato et al., 1990). The direct association between one RYR1 in the SR terminal cisternae (formed by four identical subunits) and four DHPRs in the TT membrane was demonstrated by the discovery of tetrads, intramembrane particles visualized in freeze fracture replicas of TTs (Block et al., 1988; Nakai et al., 1997, 1998; Protasi, 2002; Protasi et al., 1997, 1998, 2000, 2002; Takekura et al., 2004). The fact that a tetrad is formed by four DHPRs mechanically coupled to one RYR1 was proved in experiments showing how: i) tetrads are missing in dysgenic muscle (a natural mutation causing ablation of the $\alpha 1$ subunit of the DHPR)(Takekura et al., 1994); and ii) DHPRs do form tetrads in dyspedic muscle (a genetically engineered knockout of RYR1), but restored by transfection of dyspedic cells with RYR1 (Protasi et al., 1998). In mechanical coupling RYR1 Ca^{2+} release channels are directly opened by 4 voltage sensors contained in a DHPR-tetrad. While DHPR-tetrads and RYR1 are unquestionably the two main players in the skeletal EC coupling, many other proteins modulate and coordinate their interaction. Among them, calsequestrin, triadin, junctin, junctophilins, FKBP12, and STAC3 are the proteins more studied in the field (Caswell et al., 1991; Guo & Campbell, 1995; K. C. Kim et al., 1990; MacLennan & de Leon, 1983; MacLennan & Wong, 1971; Rebbeck et al., 2014; Ríos & Györke, 2009; Takeshima et al., 1998, 2000; L. Zhang et al., 1997; Zorzato et al., 2000). All those proteins, together with RYR1 and DHPR, constitute a macromolecular complex that regulates the activation of Ca^{2+} release from the SR terminal cisternae, i.e. EC coupling.

The molecular assembly of all those proteins requires the association of membranes carrying the action potential to those that store Ca^{2+} : this association occurs in specialized intracellular junctions, known CRUs, formed by the association of: a) invaginations of TTs(Franzini-Armstrong & Porter, 1964), which carries the depolarization of the sarcolemma (triggered by the motor-neuron at the neuromuscular junction, NMJ) and that contain DHPRs; and b) terminal cisternae, specialized SR domains that are decorated by feet, the cytoplasmic domain of RYRs, and that contain Calsequestrin (CASQ). CASQ is the main SR buffer that allows

terminal cisternae to accumulate the large amounts of Ca^{2+} needed to generate tetanic contractions, required for skeletal muscle function (Franzini-Armstrong, 1999). CRUs in adult skeletal muscle fibers are named triads because formed by 3 elements: a central TT - showing a narrow and flat profile – associated to two lateral terminal cisternae (Franzini-Armstrong, 1970) (Figure 2, panels B and C). Triads are the final result of differentiation and maturation that occurs while myotubes become adult fibers: a) at first CRUs are formed by only two elements, in which the interaction DHPR-RYR occurs in peripheral couplings formed between the SR and the sarcolemma; b) then, once the sarcolemma invaginates to form a primitive TT network, the junctional SR associates with TTs that are initially longitudinally oriented, i.e. parallel to the long axis of myofibrils (Flucher et al., 1994; Franzini-Armstrong & Jorgensen, 1994; Takekura et al., 1994); finally c) when the TT network becomes transversal and forms two networks that are positioned approximately at the transition between A and I bands of relaxed sarcomeres (approximately 2.2 – 2.4 μm long), CRUs becomes transversal triads. This position of triads is common to all mammalian muscles and is retained both in fast and slow twitch fibers (Protasi et al., 1998) (small arrows in Figure 2, panel A).

Looking at triads at higher magnifications by electron microscopy (EM) two structural details are visible: a) the presence dark electron-densities in the junctional gap between TT and SR, representing feet, the cytoplasmic domain of RYRs. RYR-feet in triads of mammals usually form two rows in which individuals RYRs touch each other corner-to-corner (Paolini et al., 2004)(feet are visible in Figure 2, panel B as densities between TT and SR, respectively labelled in white and yellow); b) the lumen of SR terminal cisternae filled dark matrix that reveals the presence of CASQ, the main SR Ca^{2+} buffer accumulating Ca^{2+} in proximity of the sites of release (Guo & Campbell, 1995; Ikemoto et al., 1989; MacLennan & de Leon, 1983; MacLennan & Wong, 1971). C. Franzini Armstrong, using deep etch replicas demonstrated that CASQ matrix is anchored at the SR terminal cisterna via thin-long strands, supposedly triadin or junctin molecules (Boncompagni et al., 2012; Franzini-Armstrong et al., 1987).

Contrary to RYR-feet, DHPRs voltage sensors could not be visualized in standard EM because almost entirely embedded within the TT lipid bilayer. C. Franzini-Armstrong used a different technique, freeze fracture replicas, to discover that in skeletal muscle DHPRs were grouped in 4 particles (Block et al., 1988; Paolini et al., 2004; Protasi, 2002) and demonstrated that DHPRs need association with RYR1 subunits to form tetrads (Protasi et al., 1997, 1998). This assembly is peculiar of skeletal muscle cells, because in smooth and cardiac muscle, which express different RYR isoforms (RYR2 and RYR3), tetrads are missing. Presence or absence of DHPR-tetrads has been related to EC coupling being either mechanical or CICR instead. Indeed, re-expression of RYR1, but not RYR2 or RYR3, in dyspedic myotubes (i.e. RYR1-knockout) restored tetrads arrangements of DHPRs, that otherwise were randomly disposed (Protasi et al., 2000, 2002).

1.3 The sites of Store-Operated Ca^{2+} Entry (SOCE): the Ca^{2+} Entry Units (CEUs).

The importance of external Ca^{2+} for the proper function of skeletal muscle fibers has been overlooked for many years because of EC coupling being mechanical (see above), i.e. it does not rely on CICR as cardiac or smooth muscle. Nonetheless, two forms of Ca^{2+} entry have been characterized in skeletal fibers:

- *Excitation-Coupled Ca^{2+} Entry (ECCE)*, a pathway first described in early 2000, that was later associated to the opening of the alpha-1 subunit of the DHPR, the voltage sensor in mechanical EC coupling that also forms an L-type Ca^{2+} channel that (in skeletal fibers) is slow and has little ion conductance (Bannister et al., 2009; Hurne et al., 2005). As ECCE is altered in malignant hyperthermia susceptibility (MHS), it was argued that it may contribute to the dysfunctional Ca^{2+} signaling found in muscle fibers of MHS patients (Cherednichenko et al., 2008).

- *Store-Operated Ca^{2+} Entry* (SOCE), a pathway that allows extracellular Ca^{2+} to enter the cytosol and refill SR stores during repetitive muscle activity (Dirksen, 2009; Hogan & Rao, 2015; Michelucci et al., 2018; Parekh & Penner, 1997; Putney, 1986). SOCE is activated by a phenomenon known as *SR depletion*, i.e. a reduction of the total amount of Ca^{2+} stored in the SR caused by loss of intracellular Ca^{2+} across the sarcolemma during prolonged muscle activity (Balnave & Allen, 1998; Brini & Carafoli, 2011; Cheng et al., 2018; Hidalgo et al., 1986). SOCE was first characterized in non-excitable cells (Parekh & Penner, 1997; Putney, 1986), but detected in skeletal muscle myotubes and fibers only several years later (Kurebayashi & Ogawa, 2001; Launikonis et al., 2003; Launikonis & Ríos, 2007). Initially, the molecular players of SOCE remained elusive for years after the first identification of the mechanism, until in 2005-2007 the two main proteins involved were discovered in patients affected by a severe immunodeficiency (Feske et al., 2006; R. S. Lewis, 2007; Liou, Kim, Do Heo, et al., 2005; Roos et al., 2005; Vig et al., 2006): a) STIM1 (stromal-interacting molecule-1), an ER/SR protein that acts as Ca^{2+} sensors due to the presence of an intraluminal N-terminal EF-hand domain; b) ORAI1, a Ca^{2+} release activated channels (CRAC) of the plasma membrane. Also, CASQ1, a protein involved in EC coupling with the dual role of being the main SR buffer that accumulates Ca^{2+} in proximity of the sites of release, and also a direct modulator of RYR1 (Ikemoto et al., 1989; MacLennan & de Leon, 1983; MacLennan & Wong, 1971), has been proposed to modulate SOCE in skeletal muscle (Shin et al., 2003; Wang et al., 2015; L. Zhang et al., 2016).

In non-muscle cells, the proposed mechanism for activation of SOCE involves depletion of intracellular stores (i.e. the ER), which would induce Ca^{2+} dissociation from the luminal N-terminal EF-hand domain of STIM1 followed by conformational changes, dimerization and relocation of STIM1 to sites of contact between ER and plasmalemma. The association of ER containing STIM1 with the surface membrane (junctions defined as *puncta*) would allow aggregated STIM1 dimers to recruit, trap, and open ORAI1 and permit entry of Ca^{2+} from the

extracellular space and replenishment of intracellular ER stores (Feske et al., 2006; R. S. Lewis, 2007; Liou et al., 2007; Liou, Kim, Do Heo, et al., 2005; Luik et al., 2006, 2008; Roos et al., 2005; Vig et al., 2006; Wu et al., 2006, 2014; S. L. Zhang et al., 2006).

The importance of SOCE for skeletal muscle function and adaptation to exercise (i.e. contribute the Ca^{2+} needed to modulate muscle-specific gene expression and sustain internal SR Ca^{2+} stores, hence prevent muscle fatigue/weakness) started to become evident when the two main players in SOCE were also identified in striated skeletal fibers (Lyfenko & Dirksen, 2008; Stiber et al., 2008). Lyfenko and Dirksen demonstrated that SOCE function in skeletal muscle fibers (but not ECCE) was dependant on STIM1 and ORAI1. Besides being important to counteract fatigue, it seems that SOCE may be important also for differentiation and development (Avila-Medina et al., 2018; Carrell et al., 2016; Kiviluoto et al., 2011; Seth et al., 2012).

In skeletal muscle, during prolonged-repetitive muscle activity, a small fraction of Ca^{2+} ions cycled by the SR during EC coupling is lost across the sarcolemma due to either simple leak or active extrusion by $\text{Na}^+/\text{Ca}^{2+}$ exchangers or plasma membrane Ca^{2+} ATP-ases (PMCA) (Balnave & Allen, 1998; Brini & Carafoli, 2011; Cheng et al., 2018; Hidalgo et al., 1986). As reduction of the amount of Ca^{2+} stored in the SR, i.e. SR depletion, is one of the factors that contributes to the onset of premature fatigue, also muscle (that often needs to work for extended periods of time) has changed during evolution to adopt a system that helps SR function: i.e. SOCE, the pathway that allows extracellular Ca^{2+} to enter the cytosol and refill SR stores during muscle fatigue (Dirksen, 2009; Wei-Lapierre et al., 2013). Though, SOCE in skeletal muscles developed peculiar features compared to other tissues. First, its activation following SR depletion seems to be much faster. Indeed, while in non-muscle cell activation of ORAI1 channels needs tens of seconds (Wu et al., 2014), experiments in skeletal fibers suggested that Ca^{2+} influx can be activated very quickly (<1 second) (Edwards et al., 2010; Launikonis et al., 2009; Launikonis & Ríos, 2007). To explain this rapid SOCE activation it

was hypothesized the presence of pre-formed SR-TT junctions in which STIM1 and ORAI1 could be already clustered before depletion (Dirksen, 2009). A STIM1 splice variant highly expressed in skeletal muscle, STIM1-long, was proposed to mediate rapid SOCE activation, referred to as *phasic-SOCE* (Darbellay et al., 2009, 2011).

For more than a decade after detection of SOCE in skeletal muscle fibers, it was speculated that SOCE would occur at the triad junction (i.e. the same sites of EC coupling; see above). The reasons underlying this speculation was the fact that, in triads, SR (presumably containing STIM1) and TT (invagination of the surface membrane that should contain ORAI1) are already preassembled, providing a convenient and suitable site for the fast-mode activation of skeletal SOCE (Dirksen, 2009). Although, this hypothesis was logical, this assumption ignored the fact that there was no direct evidence for the presence of STIM1 and ORAI1 in triads. Furthermore, the junctional space in triads already contains many proteins (junctophilins, FKBP12, triadin, junctin, mitsugumin, STAC3, etc.; see Chapter 1, Section 2.3), which are tightly assembled around regular arrays of RYR-feet in the SR mechanically coupled to DHPR-tetrads in TTs. This macromolecular complex could in principle interfere with the migrations of STIM1 oligomers in the SR membrane to form puncta, needed to recruit ORAI1 channels in TTs.

To determine the most likely sites for STIM1-ORAI1 interaction during SOCE in adult skeletal muscle fibers, Boncompagni and colleagues employed EM combined with immunogold, immunofluorescence, and functional fatigue protocols (Boncompagni et al., 2017, 2018). The experiments were performed in adult mice (4 months of age), before (control group) and after the animals were subjected to a fatigue running protocol on a treadmill (exercised group). The idea underlying these experiments was to induce fatigue and SR depletion, stimuli that would trigger SOCE activation. Unexpectedly, immunofluorescence and immunogold labelling revealed that colocalization of STIM1 with ORAI1 was minimal in control conditions, with most of STIM1 placed in the SR at the I band, while ORAI1 was retained in TTs at the triad junctions, colocalized with EC coupling protein (i.e. RYR1)(Boncompagni et al., 2017, 2018).

Parallel functional experiments showed that in muscles from control mice the contribution of external Ca^{2+} to contractility was indeed limited. EM then provided the evidence that explained how STIM-ORAI1 localization could increase during exercise: a single bout of incremental exercise on treadmill triggered a significant remodeling of both SR and TT at the I band, involving formation of SR-stacks and elongation of TTs from triads into the I band (Figure 2, panel C). This remodeling allowed the formation of new SR-TT junctions structurally and molecularly different from triads. These new SR-TT junctions did not contain RYR1, but colocalized STIM1 and ORAI1, promoted by the elongation of TT bearing ORAI1 into the I band (shown by a specific staining obtained with ferrocyanide) (Boncompagni et al., 2017) where SR-stacks contained aggregated STIM1. The authors proposed that these new SR-TT junctions would function as *Ca²⁺ entry units* (CEUs) during SOCE, because functional experiments showed that their presence in muscles isolated from pre-exercised mice resulted in increased resistance to fatigue in presence of external Ca^{2+} . This gain-of-function promoted by exercise-induced assembly of CEUs could be blocked by removal of external Ca^{2+} , or using SOCE inhibitors (Bootman et al., 2002; Zitt et al., 2004).

The final evidence that the new SR-TT junctions that assemble at the I band during exercise were indeed the sites of SOCE came from measurements of the rate of Mn^{2+} quenching in isolated single FDB fibers, the gold standard technique used to measure the entry of divalent cations from the extracellular space following SR depletion. In these experiments, the rate of Mn^{2+} quench was significantly increased in FDB fibers isolated from mice after they were subjected to the fatigue protocol (i.e. with CEUs formed), while Mn^{2+} quench was abolished in fibers from ORAI1 knockout mice. In the same study were also collected evidence that CEUs are dynamic junctions that promptly assemble during exercise, but that also disassemble during the following hours of recovery (Michelucci et al., 2019). The parallel between a) structural evidence showing elongation of TTs toward SR-stacks during exercise followed by retraction during recovery and b) Mn^{2+} quench being either up or down regulated by these changes

allowed the Authors to conclude that the formation of functional CEUs is dependent on the elongation of TTs and on the formation of contacts with SR-stacks at the I band, hence making TT remodelling the key event in the process of formation of functional CEUs (Michelucci et al., 2019).

The mechanisms leading to the exercise-induced remodelling of SR and TT are still quite obscure. In Girolami et al. 2023 it was demonstrated that: a) CEUs can assemble in isolated muscles, in complete absence of innervation and blood supply; b) CEUs assembly is pH and temperature sensitive: assembly is boosted by higher temperature and lower pH, two parameters that do change accordingly during exercise (Girolami et al., 2023). The fact that STIM1 has been proposed to function as a multipurpose stress transducer sensitive to changes like oxidation, temperature, hypoxia, etc (Mancarella et al., 2011; Soboloff et al., 2012) may explain how changes in pH and temperature may boost CEU assembly during repetitive electrical stimulation ex-vivo (Girolami et al., 2023).

Why CEUs were never identified and characterized before is a puzzling question with an easy explanation. CEUs are few and quite small in muscles isolated from control mice (empty arrows in Figure 2, panel A), hence never identified as an independent functional unit. Accordingly, muscle from control mice (not exercised) can use only little external Ca^{2+} during repetitive stimulation. In addition, immunolabeling for confocal or immunogold for EM to detect colocalization between STIM1 and ORAI1 was never performed with the level of resolution performed by Boncompagni and colleagues in 2017 and 2018 (Boncompagni et al., 2017, 2018). Additionally, the identification of CEUs was allowed by an experiment that was not performed before: fixation for EM and immunolabeling of EDL muscles from mice after they were subjected to an *in vivo* fatigue protocol designed to induce fatigue, and possibly SR depletion. As the *in vivo* fatigue protocol on treadmill induced a striking increase in size and number of SR-stacks, identification of the new structures in EM came from the simple comparison between muscles isolated from exercised mice with those of control mice, which

only had few and small SR-stacks. Complementary experiments then allowed verification that these stacks also i) contained elongated TTs, ii) allowed increased colocalization of STIM1 and ORAI1, and iii) boosted Ca^{2+} entry (Protasi et al., 2021a). A commentary published in *J. Gen. Physiol.* proposed CEUs as *the backdoor for Ca^{2+} ions in muscle cells*, bringing new attention to the importance of external Ca^{2+} in the function skeletal fibers (Melzer, 2020).

The discovery of CEUs between 2017 and 2019 also led to other significant findings and hypotheses:

a) Since we generated CASQ1 knockout mice about 20 years ago (Paolini et al., 2007), we have been always puzzled by the fact that these mice could live quite normally without CASQ1, hence with reduced Ca^{2+} stored in the SR (not considering the fact they were oversensitive to heat (Dainese et al., 2009; Protasi et al., 2009)). In 2010, we reported that CASQ1-knockout fibers undergo SR depletion under high frequency stimulation (Canato et al., 2010). Only in 2020 we came to the final conclusion that CASQ1-knockout mice could live a quite normal life because, during post-natal development, skeletal fibers progressively adapted to downregulation of CASQ2 expression (that is not accompanied by increased expression of CASQ1, because this gene is knocked out (Murzilli et al., 2023)) assembling constitutive CEUs (Michelucci et al., 2020). Constitutive presence of CEUs would allow muscle fibers lacking CASQ1 to deal with the insufficient storage of Ca^{2+} in the SR, supplementing constantly internal function with external Ca^{2+} coming into the fibers via the constitutively present CEUs.

b) It has been suggested by different authors that SOCE is compromised in ageing (Brotto, 2011; Thornton et al., 2011). In 2021, we showed a lack of the structural elements that allows assembly of CEUs in muscle of sedentary aged mice (i.e. SR-stacks and elongated TTs at the I band) that could explain dysfunctional SOCE (Boncompagni et al., 2021). However, we also showed that CEUs could be rescued in mice exercising voluntarily in wheel cages, suggesting that regular exercise while ageing could maintain SOCE functional longer in life.

c) Presence of CEUs, hence gain of SOCE function, may increase the risk of heat stroke in extreme climatic conditions. Indeed, their constitutive assembly in CASQ1-null mice resulted in an exertional-environmental heat stroke (EHS) phenotype, i.e. mice are susceptible to trigger lethal episodes in conditions of high temperature and/or strenuous exercise (Boncompagni et al., 2017; Dainese et al., 2009; Michelucci et al., 2015; Protasi et al., 2009, 2022). Also, exercise-induced assembly of CEUs in wild type mice contributed to the increase in body temperature during exertional stress, predisposing to a possible heat illness (Girolami et al., 2023).

Chapter 2: Previous Work relevant to the Thesis

2.1 Formation of Tubular Aggregates (TAs) and loss of Ca^{2+} Entry Units (CEUs).

Sedentary ageing compromises the architecture of mitochondrial network and sarcotubular system: using EDL muscles of ageing male mice in 2021 our group demonstrated a correlation between SOCE dysfunction and rearrangement of the SR to form tubular aggregates (TAs, Figure 3), regular arrays of long tubes, which have been found in a variety of human disorders, including TA myopathy (TAM, see next paragraph) (Apostol et al., 2009; W. K. Engel et al., 1970; Morgan-Hughes, 1998; Pierobon-Bormioli et al., 1985; Rosenberg, 1985; Salviati et al., 1985).

Mechanisms underlying formation of TAs, are still under investigation: several years ago, it was demonstrated that formation of TAs may be induced by anoxia (Schiaffino et al., 1977), while - in our experience - inactivity (which also result in anoxia) is a contributing factor (Boncompagni et al., 2021).

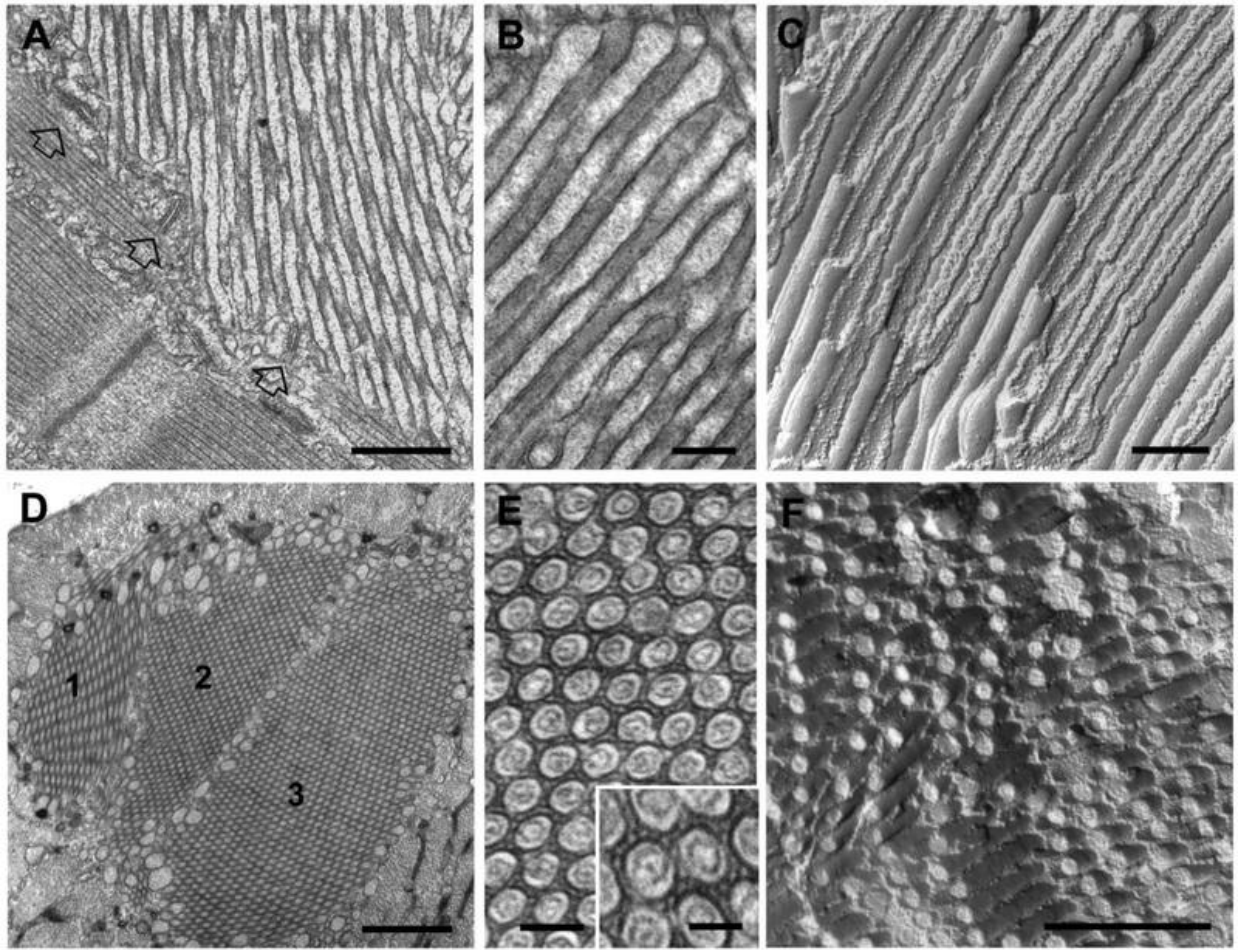
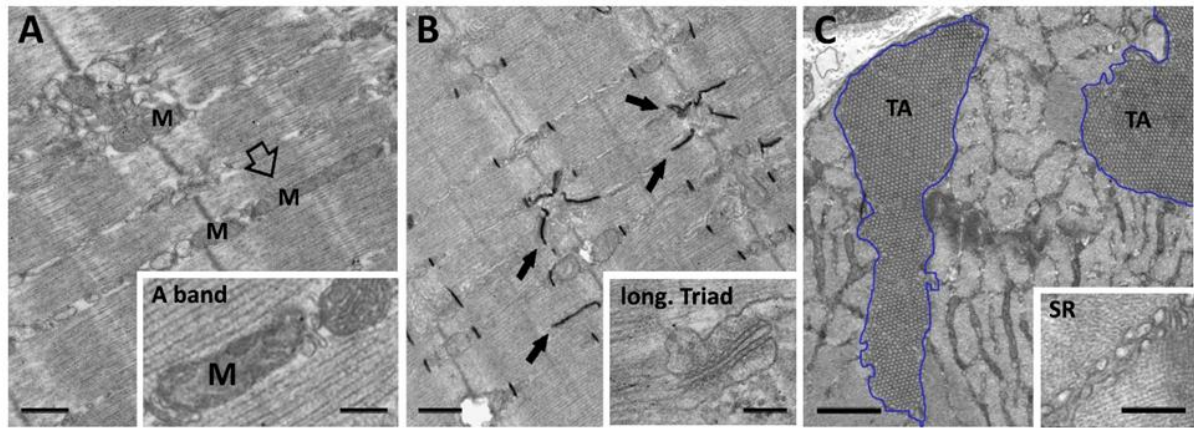


Figure 3: Tubular aggregates (TAs) in extensor digitorum longus (EDL) fibers from 2-year-old male wild type mice. A-C) EM images of longitudinal views of TAs in thin sections (A and B) and freeze fracture (C). Empty arrows in panel A point to jSR-TT junctions (or triads) located at the periphery of a TA. Tubes of TAs are typically filled with electron-dense material, most likely CASQ1 (B). The regular and straight shape of the tubules are emphasized in freeze fracture replicas, where tubules appear as long cylinders showing alternated views of the luminal and cytoplasmic leaflets. D-F) EM images of cross-sectional views of TAs in thin sections (D and E) and freeze fracture (F). Large TAs often result from the association of multiple TAs of smaller size (panel D: 1–3). In the core of each domain, tubes forming the TA display uniform diameters and appear ordered in a hexagonal pattern (E). Note the presence of small linkages that bridge the gap between membranes of adjacent tubules (E, inset). Bars: A, D and F: 0.5 μm ; B and C: 0.2 μm ; E: 0.1 μm (inset: 0.05 μm) From Boncomoagni et al. 2012.

Aged



Aged-trained

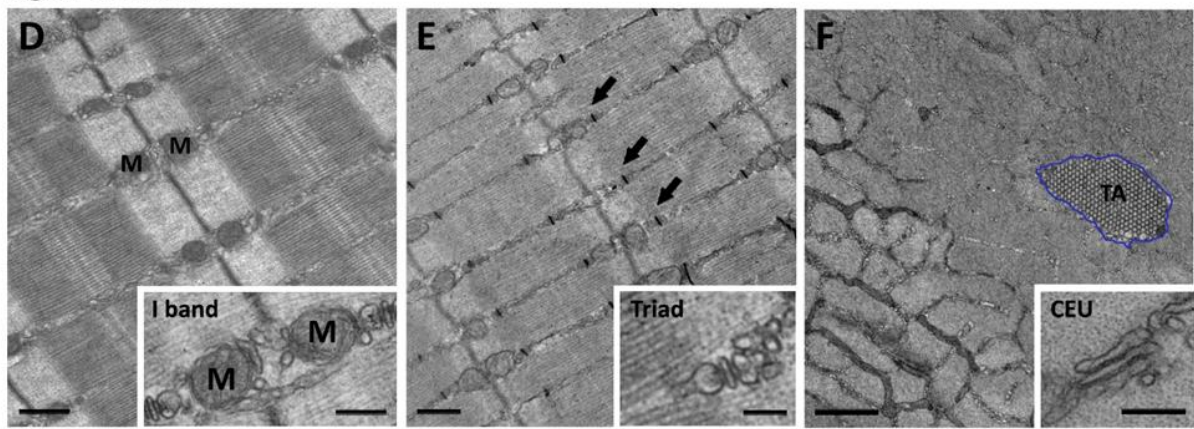


Figure 4. Longitudinal (panels A and B; panels D and E) and transverse (panels C and F) EM sections of skeletal muscle fibers from aged (panels A-C) and aged-trained (panels D-F) mice. (A-C) In fibers from aged mice: a) some mitochondria (M) are misplaced at the A band of the sarcomere (empty arrow in panel A, and inset); b) TT network (identified by a dark precipitate in panel B) and triads (inset) may become oblique or longitudinally oriented (small arrows in panel B, and inset); c) SR may form TAs (outlined in blue in panel C) and rarely form SR-stacks at the I band (inset in panel B). (D-F) In fibers from aged-trained mice: a) mitochondria (M) are correctly positioned at the I band (M in panel D, and inset); b) TT network (identified by a dark precipitate in panel C) and triads (inset) are transversally oriented (small arrows in panel E, and inset); c) TAs (outlined in blue in panel F) are diminished in number and size, while SR at I band more often forms SR stacks (inset in panel F). Scale bars: A, B, D and E = 0.5 μm ; C and F = 1 μm , insets A, D = 0.25 μm , insets B, E = 0.1 μm , insets C, F = 0.3 μm .

In two papers published respectively in 2012 and 2021, Boncompagni and colleagues demonstrated that TAs in ageing mice stain positive for STIM1, ORAI1 and CASQ1, but they are negative for RYR staining (Boncompagni et al., 2012, 2021), as TTs and triads are excluded from TAs. Accumulation of STIM1, ORAI1, and CASQ1 in TAs of ageing mice correlates to functional experiments showing an increased fatigability of muscles during repetitive stimulation, and dysfunctional SOCE (Boncompagni et al., 2021). Of note, mice lacking CASQ1 abnormal aggregates of SR tubules have different conformation and never develop into ordered aggregates of straight cylinders, possibly due to lack of CASQ1 accumulation (Boncompagni et al., 2012). Structurally, presence of TAs also correlated with the absence of SR-stacks and elongated TT at the I band (inset in Figure 4, panel C), the two structural components forming the junctions (i.e. CEUs) dedicated to SOCE (see above Chapter 1, Section 2.3).

The conclusion that emerged from these studies (Boncompagni et al., 2021) was that TAs could represent a dysfunctional remodeling of the SR that traps (also) SOCE proteins in a dysfunctional compartment. This SR remodeling could be the consequence of ageing with reduced muscle activity (i.e. mice were kept in standard cages, with no access to wheels for voluntary training), which means mice were ageing with reduced opportunity to keep active, fatigue muscle, hence recruit SOCE (which possibly could be inactivated, because not necessary for everyday life). These findings are in line with previous publications by other investigators showing how a reduced SOCE activity contributes to age-related muscle weakness (Brotto, 2011; Thornton et al., 2011; X. Zhao et al., 2008).

2.2 Exercise reduces Tubular Aggregates (TAs) and maintains SOCE function.

Boncompagni et al. demonstrated that TAs contain STIM1, ORAI1, and CASQ1 apparently trapped in a dysfunctional compartment (Boncompagni et al., 2012, 2021). Interestingly, accumulation of TAs, presence of membrane elements needed for assembly of CEUs, and the function of SOCE were then studied in mice that have had access to running wheels for 12 months (from 1 to 2 years of age). The results of this effort demonstrated that long-term voluntary wheel running (VWR) for 1 year was quite effective in: a) reducing formation of TAs (outlined in blue in Figure 4, panel F), b) preventing loss of SR-stacks and TTs at the I band (inset in Figure 4, panel F), and c) rescuing functional SOCE, showed by gain-of-function during a fatigue protocol in presence of external Ca^{2+} which could be blocked in 0 Ca^{2+} or by presence of a SOCE inhibitor (Boncompagni et al., 2021). The authors concluded that, as SOCE is a mechanism that normally is recruited during muscle activity (which induces fatigue and SR depletion), in sedentary people/mice it would be rarely activated leading to complete inactivation of the mechanism (reduced presence of CEUs and improper accumulation of STIM1, ORAI1, and CASQ1 in TAs), while in muscle that is regularly exercised will remain active during life-time (presence of CEUs, reduced formation of TAs, and maintained SOCE function).

Chapter 3: the Project of my Thesis

3.1 Tubular Aggregate Myopathy (TAM).

TAM is a rare myopathy predominantly linked to mutations in the main players of SOCE: STIM1, ORAI1 and CASQ1. TAM primarily affects skeletal muscle with different degrees of symptoms (e.g. progressive muscle weakness, elevated levels of creatine kinase, hypocalcemia, exercise intolerance) and diverse forms of severity (Chevessier et al., 2005). Additional multi-systemic manifestations, including thrombocytopenia, and hyposplenism are observed in some but not all individuals with TAM, which results in a clinical continuum with Stormorken and York Platelet Syndrome (Böhm et al., 2013, 2017; Y. Endo et al., 2015; Garibaldi et al., 2017; Harris et al., 2017; Hedberg et al., 2014; Misceo et al., 2014; Stormorken et al., 1985; Walter et al., 2015). Despite variations in their symptoms, a consistent histological feature is observed in muscle biopsies from all TAM patients: the presence of TAs in skeletal muscle fibers (Figure 5) (Böhm et al., 2013, 2017; Y. Endo et al., 2015; Garibaldi et al., 2017; Harris et al., 2017; de Groot & Arts, 1982; Pierobon-Bormioli et al., 1985; Rohkamm et al., 1983).

The most common form of TAM is the autosomal dominant heritable, but it can be autosomal recessive (N. R. Kim & Suh, 2003). Moreover, there are cases of idiopathic TAM that could be secondary to alcoholism and endocrinopathies (del Villar Negro et al., 1982).

Almost all the mutations in STIM1 and ORAI1 reported in TAM are gain-of-function mutations: mutated STIM1 can be activated without SR Ca^{2+} depletion, resulting in improper ORAI1 channel opening. Most of the autosomal dominant mutations in TAM are located in the EF-hand domain of STIM1, which is considered to be a hotspot for mutation. So far, ten different mutations have been reported (Böhm et al., 2013, 2014; Walter et al., 2015).

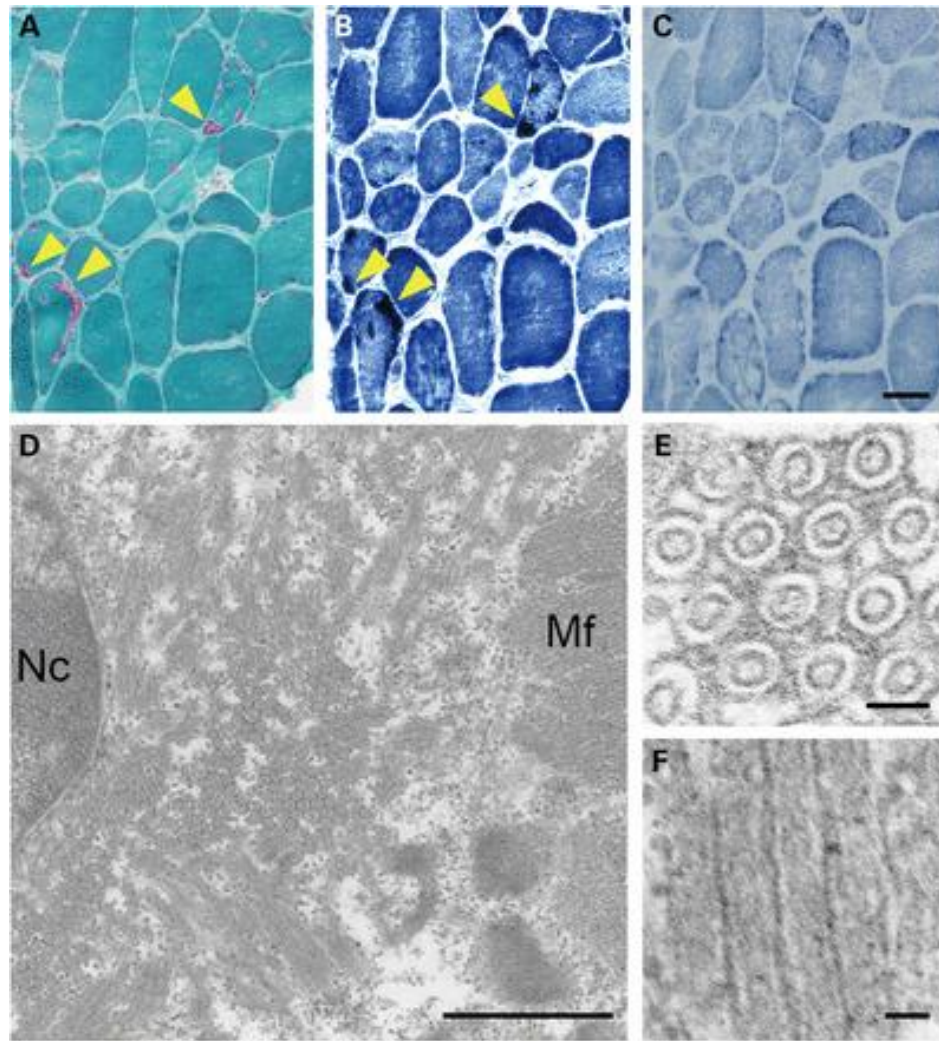


Figure 5. Histology and electron microscopy of a muscle biopsy sample from affected individual. Histological analysis of transverse sections revealed aggregations with Gomori trichrome (mGt) (A) and NADH-tetrazolium reductase (NADH-TR)(B) but not succinate dehydrogenase (SDH) (C) staining. Ultrastructural analysis demonstrated prominent tubular aggregation with double 27 walled membranes in transversal (D and E) and longitudinal (F) sections. Nc, nucleus; Mf, myofibrils. Arrows, tubular aggregates (TAs). Scale bars, 50 μ m (C); 1 μ m (D); 100 nm (E); 50 nm (F). (Modified from Endo et al., 2015).

An example of STIM1 mutation can be the one in Asp84Gly: in myoblast of patients harboring this mutation, there is a constitutive Ca^{2+} influx and marked increases in cytosolic Ca^{2+} upon treatment with thapsigargin, a SERCA inhibitor (Walter et al., 2015), supporting the idea of a self-activation of mutated STIM1. For Orai1, three different autosomal dominant mutations have been reported to cause the TAM phenotype (Y. Endo et al., 2015; Nesin et al., 2014). The Gly98Ser and Leu138Phe mutations result in constitutive external Ca^{2+} influx without SR Ca^{2+} depletion (Y. Endo et al., 2015). The altered inactivation of the mutated ORAI1 may contribute to the prolonged elevated cytosolic Ca^{2+} (Nesin et al., 2014). In humans, gain-of-function mutations in ORAI1 gene allow extracellular Ca^{2+} influx to the cytosol, which is independent of the SR Ca^{2+} concentration or STIM1 activation (Böhm et al., 2013, 2014; Walter et al., 2015). More recently mutations in the gene encoding CASQ1 were recently found in a smaller fraction of patients with TAM (Barone et al., 2017). These CASQ1 mutations affected conserved amino acids in position 44 (p.(Asp44Asn)), 103 (p.(Gly103Asp)) and 385 (p.(Ile385Thr)). The authors of the manuscript characterized these novel CASQ1 mutations, demonstrating that the mutated CASQ1 have altered Ca^{2+} -dependent polymerization and reduced ability to store Ca^{2+} . The ability of CASQ1 mutations to inhibit Ca^{2+} entry pathways was maintained in only one of the three mutations, suggesting a further potential level of complexity in the patho-mechanisms of TAM due to mutations in CASQ1.

To date, the underlying governing mechanisms linking these human mutations to Ca^{2+} imbalance and development of TAM are still unknown. Moreover, the detailed explanation of the differentiation into a variety of phenotypes by the STIM1, ORAI1 and CASQ1 mutations is unclear; more the cause of the phenotypical differences between TAM and other syndromes or myopathies.

TAM mouse models were generated to described to improve the understanding of the physiopathology of TAM, though none of the STIM1 models of TAM exhibit TAs (Cordero-Sanchez et al., 2020; Gamage et al., 2018; Grosse et al., 2007; Silva-Rojas et al., 2019).

Dirksen and colleagues (N. Zhao et al., 2024) recently generated a knock-in mouse with a pore mutation in the mouse ORAI1 protein (glycine to serine mutation at amino acid 100 or G100S), analogous to the G98S mutation in ORAI1 found in humans which results in a severe, childhood onset form of TAM, and identified in at least three unrelated TAM families (Böhm et al., 2017; Y. Endo et al., 2015). Our lab participated in the characterization of this first ORAI1 TAM mouse model: significant muscle weakness and exercise intolerance are observed in GS mice based on both behavioural tests (grip strength, rotarod, treadmill) and ex vivo muscle contraction measurements. GS mice also exhibit a significant hypocalcaemia and increase in serum CK levels. However, skeletal muscle from GS mice showed no evidence of major muscle damage or necrosis since significant changes in fiber size/type, central nucleation and/or fibrosis were not detected. Most importantly, we found that multiple fast-twitch muscles from 8 months old GS mice (EDL and FDB muscles) exhibit a robust presence of honeycomb-like structures of TAs that stain pink in Gomori trichrome. Coincidentally, the mutation resulted revealed substantial mitochondrial damage and dysfunction. suggesting a connection between ORAI1 activity and mitochondrial structure/function. Finally, in a recent paper (O'Connor et al., 2025) we collected evidence that physical training, in the form of voluntary wheel running (VWR) is able to counteract TAs formation and to improve other signs of pathology in GS mice (O'Connor et al., 2025).

Finally, systematic review of the current literature failed to identify any CASQ1 model of TAM: this gap of knowledge opened a new opportunity of research which prompted us to characterize a novel in vivo model of TAM possibly replicating the pathological features of the disease in order to facilitate the study of the pathophysiological mechanisms and to better address future therapeutic testing.

3.2 Main objective of the Thesis work.

So far, no animal model of TAM carrying a CASQ1 mutation has been reported in literature.

Here, we characterized a newly generated knock-in mouse model of TAM (in collaboration with the laboratory of Dr. RT. Dirksen at the University of Rochester, NY) harbouring the mutation p.(Asp44Asn) in the *Casq1* gene (*Casq1*^{GD44N/+} or D44N/+ mice). Patients with the D44N mutation exhibit primarily skeletal muscle manifestations, including myopathy, exercise intolerance, and a robust presence of TAs upon electron microscopy analysis of muscle biopsies. Interestingly, expression of the D44N mutant in eukaryotic cells reduced the amount of Ca²⁺ in the SR and additionally showed a defect in the ability to inhibit SOCE (Barone et al., 2017), indicating that CASQ1 participates in the modulation of store operate calcium entry (SOCE)-mediated Ca²⁺ homeostasis via a reverse-directional signal.

Here, we primarily focused on the skeletal muscle aspects of heterozygous D44N mice, by characterizing their structure, function and Ca²⁺ handling ability.

3.3 Materials and Methods.

-Animals. Knock-in *CASQ1*^{D44N/+} (D44N/+) mice were generated using CRISPR/Cas9 gene editing by the Mouse Genome Editing Resource Facility at University of Rochester Medical Center, in the laboratory of Robert T. Dirksen. Heterozygous D44N mice were backcrossed with wild-type (WT, *Casq1*^{+/+}) mice of the same genetic background (C57Bl/6 J) for more than seven generations to establish the congenic line and select against potential off-target editing. Heterozygous D44N mice were then breed siblings to generate also WT mice from the same colony which were used as control. All experiments were conducted according to the Directive of the European Union 2010/63/UE and were approved by the Animal Ethical Committee of the University of Chieti-Pescara and by Italian Ministry of Health (n. 780/2022-PR).

D44N/+ male mice were housed in microisolator cages at 20 °C in a 12 h light/dark cycle and provided free access to standard food and water. All animals were sacrificed by cervical dislocation, as approved by the Italian D. lgs. n.26/2014.

-Age-dependent survival analysis. The rate of spontaneous mortality of D44N/+ vs. WT male and female mice housed under standard conditions was monitored throughout their lifespan over an observational period of 30 months. The results were analysed according to Kaplan–Meier survival curve.

- In vivo mice weight and grip strength analyses. As a standard procedure, whole body weight and force developed by mice during instinctive grasp (i.e. grip strength) were determined in 4–8 mo. male and female WT and D44N/+ mice, as described in (Di Fonso et al., 2024). Briefly, for grip test, mice were held by the tail and lowered to a metal grating connected to the shaft of a Shimpo Fgv 0.5x force transducer (Nidec-Shimpo America Corporation, Itasca, USA). Once the mouse had firmly grabbed the grating, a steady, gentle pull was exerted on the tail. Measurements of peak force generated by each mouse using fore- and hind-limbs were repeated three times with appropriate intervals (at least 30 sec.) to avoid fatigue and the highest value of peak force (both normalized to total body mass and the absolute value) measured before each experiment was used.

-Histology and electron microscopy (EM). Extensor digitorum longus (EDL) muscles were carefully dissected from sacrificed mice, pinned on Sylgard dishes, fixed at RT with 3.5% glutaraldehyde in 0.1 M NaCaCO buffer (pH 7.2) and kept at +4 °C in fixative solution until further use. Fixed muscles were then postfixed in 2% OsO₄ in the same buffer for 2 hrs, then block-stained in uranyl acetate replacement. After dehydration, specimens were embedded in an epoxy resin (Epon 812; Electron Microscopy Sciences, Hatfield, PA, USA), as previously described (Pietrangelo et al., 2015, 2019). For TT staining, specimens were post-fixed in a mixture of 2% OsO₄ and 0.8% K₃Fe(CN)₆ for 1–2 hrs followed by a rinse with 0.1M NaCaCO

buffer with 75 mM CaCl₂. Ultrathin sections (~50 nm) were cut using a Leica Ultracut R microtome (Leica Microsystem, Vienna, Austria) with a Diatome diamond knife (Diatome, Biel, Switzerland) and double-stained with uranyl acetate replacement and lead citrate. Sections were viewed and photographed in a 120 kV JEM-1400 Flash Transmission Electron Microscope (Jeol Ltd, Tokyo, Japan) equipped with CMOS camera Matataki and TEM Center software (Jeol Ltd, Tokyo, Japan). For histological examination, 700 nm thick sections were stained in a solution containing 1% Toluidine blue O and 1% Sodium Borate Tetra in distilled water for 3 min on a hot plate at 55–60 °C. After washing and drying, sections were mounted with DPX mounting medium for Histology (Sigma-Aldrich, Milan, Italy) and observed with a Leica DMLB light microscope (Leica Microsystem, Vienna, Austria).

For all quantitative EM analyses, micrographs of non-overlapping regions were randomly collected from longitudinal and transversal sections of internal areas of EDL fibers. The following ultrastructural parameters were determined:

a) Tubular aggregates (TAs). Percentage of fibers presenting TAs, number of TAs per fiber and the average size of TAs were evaluated in micrographs collected from EDL muscle fibers in transverse sections and reported as mean values, as in Boncompagni et al. 2021 (Boncompagni et al., 2021);

b) SR stacks. Incidence of fibers presenting SR stacks (expressed as percentage) and number of SR stacks (per 100 μm^2 of section) were evaluated in micrographs collected from EDL muscle fibers in transverse sections. In each specimen, 15–20 fibers were analysed, and in each fiber, 5 micrographs were taken at 12,000 $\times$ magnification.

c) Non-triadic TT network at the I band. We determined both (i) the total network of the TT at the I band of sarcomere and (ii) the extension of the SR in close association with the TT. The non-triadic TT network was evaluated in micrographs collected from EDL muscle fibers either stained or not with ferrocyanide in transverse sections and reported as average number per area

of section ($100\ \mu\text{m}^2$). In each specimen, 15–20 fibers were analysed, and in each fiber, 5 micrographs were taken at $12,000\times$ magnification.

d) Density of mitochondria (per $100\ \mu\text{m}^2$ of section) was evaluated in micrographs collected from EDL muscle fibers in longitudinal sections. In each specimen, 15–20 fibers were analyzed, and in each fiber, 5 micrographs were taken at $6,000\times$ magnification. In each EM image, we also determined (i) the mitochondrial positioning with respect to the A band and (ii) the number of severely altered mitochondria, and mean values were reported both as number/ $100\ \mu\text{m}^2$ of section and as percentage of total number.

- ***Ex-vivo contractility.*** Ex-vivo assessments of contractile function during repeated high-frequency stimulation were performed on intact EDL and soleus muscles isolated from 4–6-month-old male and female mice. Following careful dissection from the hindlimbs, muscles were placed in a dish containing standard Krebs–Henseleit (KH) buffer (18 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1 mM KH_2PO_4 , 1 mM MgSO_4 , 25 mM NaHCO_3 , and 11 mM glucose; pH 7.4). Each muscle was secured at both tendons using fine silk sutures. Muscles were then vertically mounted in an organ bath filled with KH solution, positioned between platinum electrodes, and connected to a servo motor and force transducer (model 1200A; Aurora Scientific, Aurora, ON, Canada).

Optimal muscle length (L_0) was established by delivering 80-Hz tetanic stimuli at 1-min intervals and adjusting length until maximal force (F_0) was achieved while minimizing fatigue. After determining L_0 , muscles were equilibrated with three tetanic contractions (150 Hz, 500 ms) separated by 1-min rest periods. Force–frequency curves were obtained by stimulating EDL and Soleus muscles across frequencies ranging from 1 to 250 Hz. Muscle fatigability was evaluated using a repeated high-frequency protocol consisting of 60 trains of 50-Hz, 500-ms contractions delivered every 2.5 s.

Force production was recorded with Dynamic Muscle Control software (v.5.415; Aurora Scientific) and analyzed using Dynamic Muscle Analysis software (v.5.200; Aurora Scientific).

Specific force (mN/mm²) was calculated by normalizing absolute force (mN) to physiological cross-sectional area (mm²), determined as: wet weight (mg) / [Lo (mm) × 1.06 (mg/mm³) × 0.44]. All measurements were conducted at room temperature.

- ***Voluntary wheel running (VWR) training.*** Selected D44N/+ mice were housed in wheel cages for VWR to track activity over a 4-month period (from four to eight months of age). During the training period, animals were individually accommodated in a 16-station home cage running wheel (Columbus Instruments, Columbus, OH, USA) and had free access to standard chow and water. Each individual wheel cage was connected to a computer through a magnetic indicator and a sensor. Running activity of mice was monitored via CMI software (Columbus Instruments, Columbus, OH, USA) and number of rotations performed each hour in each cage was recorded. Terminal experiments were performed immediately after removing mice from cages with wheels.

- ***Statistical analysis.*** Statistical significance was determined using either unpaired, two-tailed Student's t test (when comparing means between two groups) or two-way ANOVA followed by the post hoc Tukey test for repeated measures (when comparing across more than 2 groups) using Prism 9 software (GraphPad Software). In all cases, differences were considered statistically significant at *p<0.05. Data are presented as mean ± SEM.

3.4 Results.

- ***WT and D44N/+ mice display similar survival curve, body mass and in vivo force in adult animals.*** D44N/+ mice were fertile, born at a normal Mendelian ratio and, in adulthood, mice did not show any obvious physical abnormalities (Figure 6, panel A). The rate of spontaneous mortality under standard housing conditions was determined for D44N/+ mice during an observational period of about 30 months. Both male and female D44N/+ animals survived for up to 800 days. As a result, the survival curves of D44N/+ mice do not significantly differ from that of control wild type (WT) mice (Figure 6, panel B). At 4-6 months of age size and weight

(Figure 6, panels A and C) were comparable to that of the age-matched WT mice. We also assessed *vivo* muscle force using a grip-strength test in 4-6 months old (mo.) D44N/+ mice vs. WT animals, and found that both relative grip strength normalized to body mass (Figure 6, panel D) and absolute force (Figure 6, panel E) were non significantly different to those of WT mice, even if a slight trend to decrease was present.

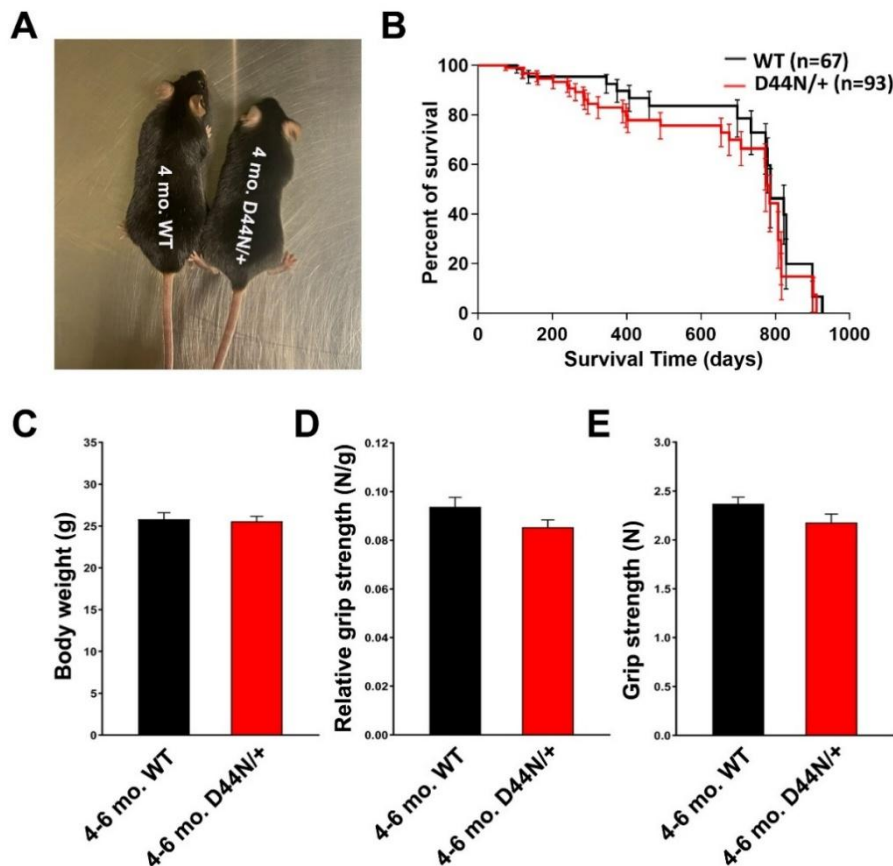


Figure 6. D44N/+ mice have a normal lifespan and adult (4-6 months old) animals do not present defects in body weight and in vivo force. (A) Physical appearance of D44N/+ mice is comparable to that of WT mice at 4 months of age. (B) Age-dependent survival analysis of male and female D44N/+ vs. wild type (WT) mice housed under standard conditions, evaluated using the Kaplan–Meier method. *n* = number of animals. (C–E) In vivo measurements in 4-6 months old (mo.) male and female D44N/+ vs. WT animals of: average body weight (C), relative force normalized to body weight (D) and absolute grip strength (E). Data are shown as means $\pm$ SEM. *n* = 24 animals for each group.

- *Ex vivo force–frequency relationship and fatigue resistance are not impaired in adult D44N/+ male and female mice.* To assess the impact of the D44N mutation on force output of adult (4–6 mo.) male and female mice, EDL and soleus muscles were subjected to ex vivo contraction protocols. As shown in Figure 7, no significant differences were detected between muscles from WT and D44N/+ mice. Though, a slight-reduction in force output was observed in soleus muscles of D44N/+ mice in both the force-frequency and fatigue protocol (Figure 7, panels D and E).

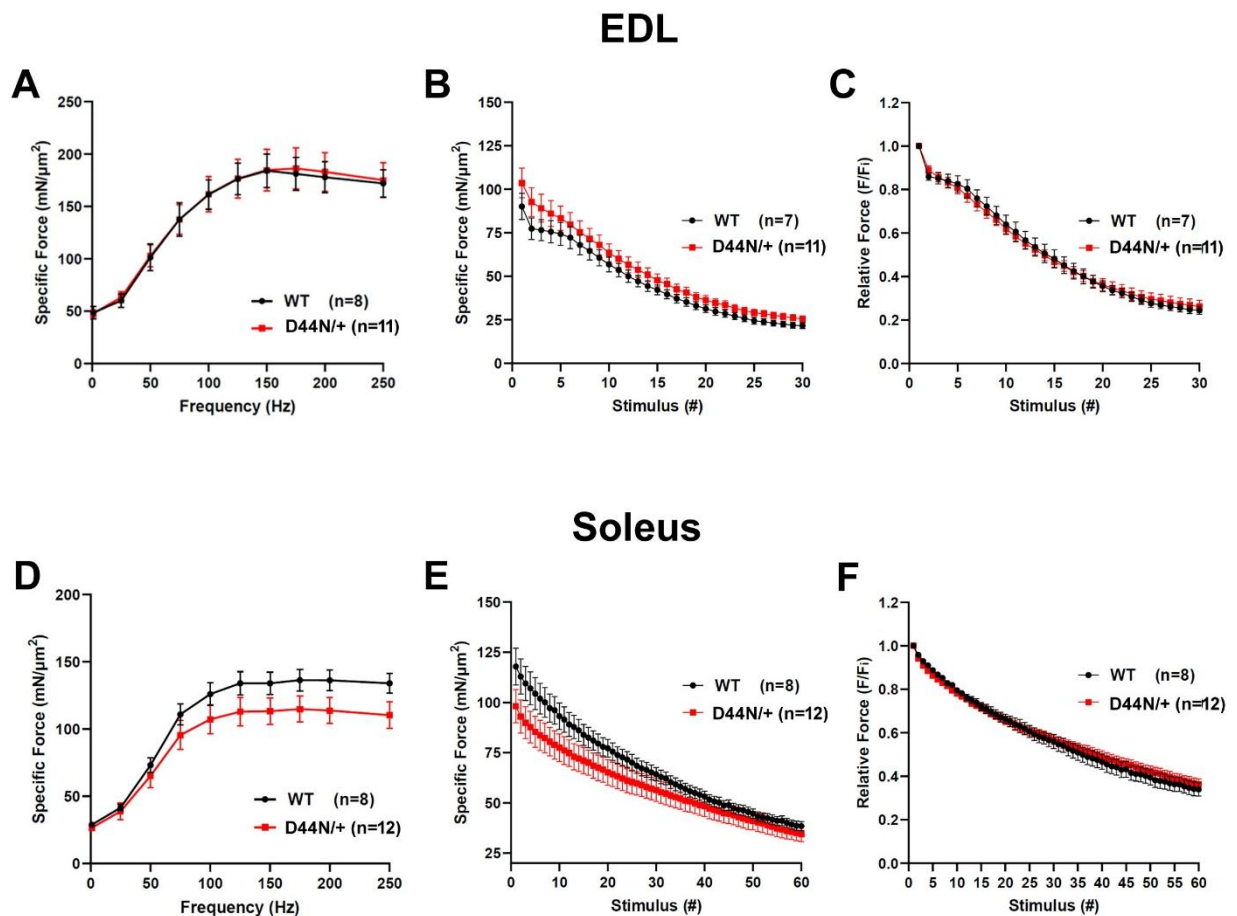


Figure 7. Ex vivo force–frequency relationship and fatigue resistance are not impaired in 4–6 mo. (A and D) Specific force in EDL (A) and soleus (D) muscles evoked by stimulation frequencies ranging from 1 to 250 Hz. (B and E) Fatigue-resistance protocol (60 Hz, 1-s trains, 60 stimuli) normalized to physiological cross-sectional area (CSA) in EDL (B) and soleus (E). (C and F) Fatigue-resistance protocol normalized to the first stimulus in EDL (C) and soleus (F). Data are presented as mean ± SEM. n = number of muscles tested.

- Size and incidence of tubular aggregates (TAs) in EDL muscles of D44N/+ male mice increase with age. TAs are tightly packed straight tubes of SR origin. TAs are the main histopathological hallmark in the skeletal muscle biopsies of patients affected by TAM, but are also found in fast-twitch muscle fibers of ageing WT male mice (Apostol et al., 2009; Boncompagni et al., 2012; W. K. Engel et al., 1970; Morgan-Hughes, 1998; Pierobon-Bormioli et al., 1985; Rosenberg, 1985; Salviati et al., 1985). Presence of TAs during ageing has been associated with dysfunction in SOCE mechanism (Boncompagni et al., 2021).

Based on our previous experience studying TAs in ageing muscle of WT mice (Boncompagni et al., 2012 and 2021), we designed a time-course study: EM transverse sections were obtained from fast-twitch EDL muscles of WT and D44N/+ male mice at 8, 13 and 16 months of age (Figure 8, panels A-D) and analyzed.

Qualitative evaluation revealed that, while TAs are nearly absent in our 8 months old WT male mice (Figure 8, panel A), they are found in 13 and 16 mo. WT animals (Figure 8, panel B). The enlarged details in panel A and B of Figure 8 show SR appearance at the I band, in fibers from WT mice, with either typical organization in vesicles and tubes (Figure 8, inset in panel A) in non-triadic regions of younger animal, or tightly packed straight tubes organized to form the classical structure of a TA, in ageing muscles (Figure 8, inset in panel B).

The same analysis was done in fibers from D44N/+ mice and revealed that classical TAs are frequently seen already at 8 months of age (Figure 8, panels C and D) and that, in 13-16 months old D44N/+ mice they appeared structurally different from those observed in age matched WT mice, as TAs presented abnormal vacuole-like tubes near tightly packed straight tubes in ageing D44N/+ mice (asterisks in Figure 8, panel D).

Visual observations were corroborated by EM quantitative analysis in muscle fibers of 8, 13 and 16 mo. WT and D44N/+ male mice, aiming to evaluate (i) the average size of TAs (Figure 8, panel E) and (ii) the percentage of fibers containing TAs (Figure 8, panel F). Data obtained showed that whilst size of TAs did not change with increasing age in EDL muscles of WT male

mice, on the other hand, the average area of single TAs progressively increased with age in fibers from D44N/+ mice.

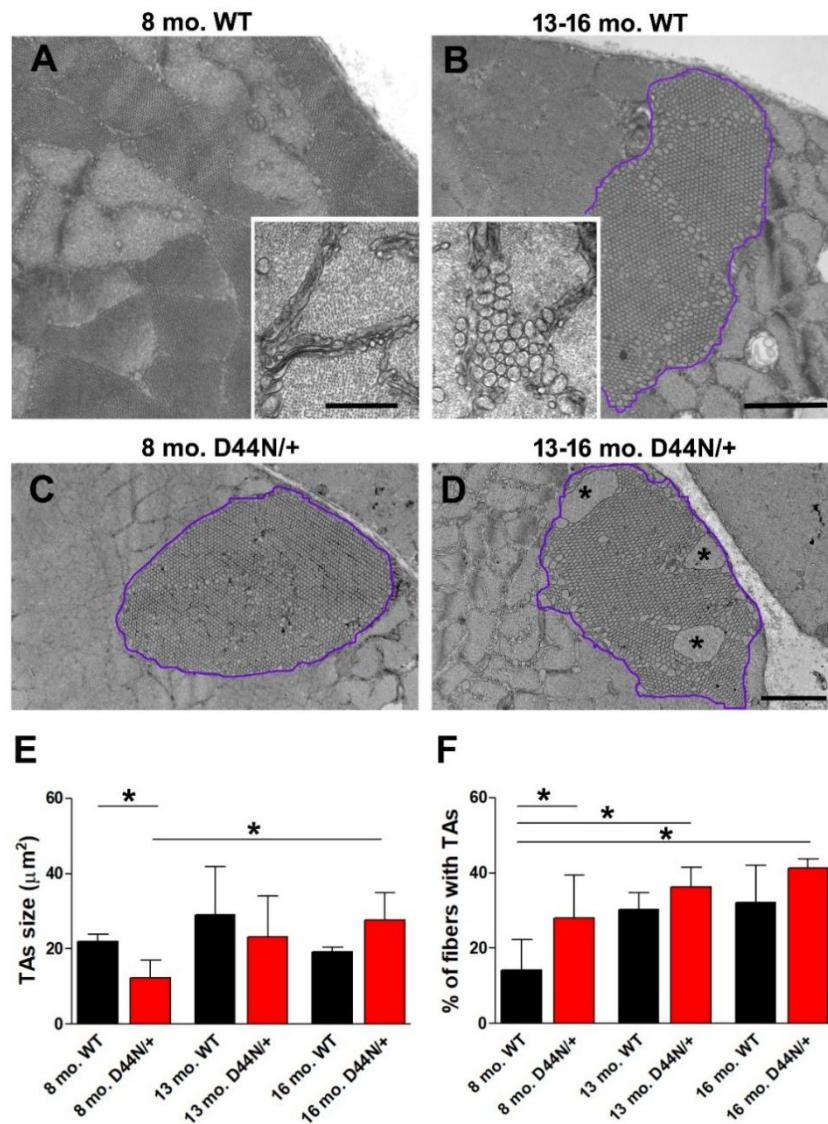


Figure 8. In fast-twitch muscles of male mice, D44N/+ mutation causes accumulation of tubular aggregates (TAs) starting from 8 months of age. (A-D) Representative EM images taken from transversal sections of skeletal muscle fibers of 8 (panels A and C) and 13-16 mo. (panels B and D) mice, both WT (panels A and B) and D44N/+ (panels C and D). At higher magnification (insets in panels A and B), different arrangements of the sarcoplasmic reticulum (SR) at the I band of sarcomere are visible. In panels B, C and D three TAs are outlined in violet. Asterisks mark irregular TAs with vacuoles (panel D). Scale bars: A-D = 1 μm; insets in A and B = 0.5 μm. (E-F) Quantitative EM analyses of TAs size (panels E) and incidence (panel F) in the fast-twitch extensor digitorum longus (EDL) muscle dissected from 8, 13 and 16 mo. male WT and D44N/+ mice. Data are shown as mean ± SEM. *p < 0.05.

EM also revealed that TAs were not present in EDL muscles of 8 mo. female D44N/+ mice (Figure 9, panels A and D), but they started to accumulate at 13 months of age (Figure 9, panels B and C). In female mice, nascent TAs (Figure 9, panel D) were few and often very small, and they did not progressively accumulate nor became bigger with increasing age, as demonstrated by EM quantitative analyses (Figure 9, panel D).

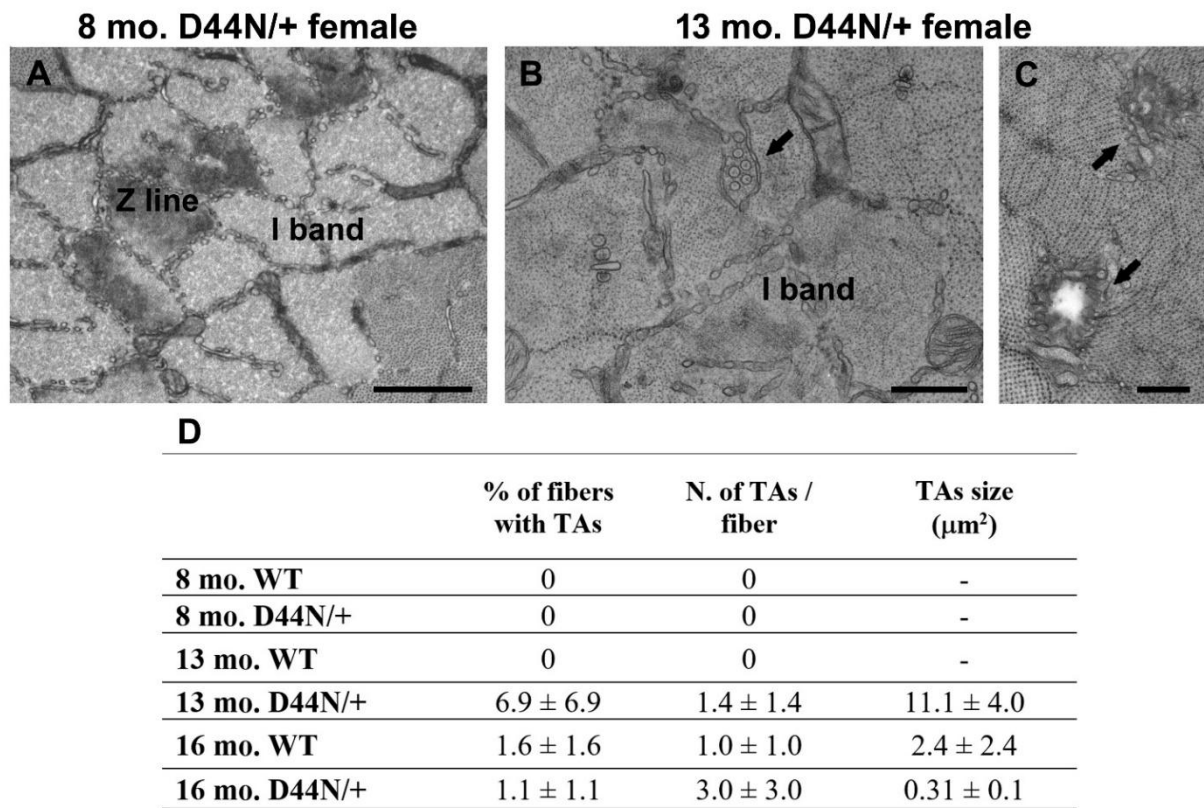


Figure 9. TAs start to develop later in female D44N/+ animals. (A-C) Representative EM images taken from transverse sections of EDL muscle fibers of 8 (panel A) and 13 mo. (panels B and C) female D44N/+ mice showing the appearance of SR at the I band of sarcomere. In panels B and C black arrows point to nascent TAs. Scale bars: A = 1 μm ; B and C = 0.5 μm . (D) Quantitative EM analyses of TAs in the fast-twitch EDL muscle dissected from 8, 13 and 16 mo. female WT and D44N/+ mice. Data are shown as mean $\pm$ SEM. * $p < 0.05$.

- Increased number of enlarged SR vesicles and SR stacks (without TT extensions) in EDL muscles from D44N/+ mice. Presence of TAs in different conditions, including ageing, correlates with a reduced ability to maintain contractile force during repetitive high-frequency stimulation, as the result of the lowered ability to refill internal Ca^{2+} stores *via* SOCE (Boncompagni et al., 2021; Rastelli et al., 2025). Therefore, here, we evaluated using EM, SR and TT elements, the two structural components needed for the assembly of CEUs, the intracellular junctions required for SOCE mechanism.

If compared to WT, in 8 mo. male D44N/+ mice was often noticed the presence of SR vesicles larger in size (Figure 10). This qualitative observation was confirmed by EM quantitative analysis (Figure 10, panel C): in fact, in 8 mo. male D44N/+ mice 40% of fibers presented enlarged SR vesicles (vs. only 10% in WT animals). Moreover, multiple flat cisternae of free SR were also present (Figure 10, inset in panel A). We then quantitatively assessed the presence of the two structural components needed for the assembly of functional CEUs, i.e., SR stacks and TT extension at the I band, and verified that, if compared to WT, in 8 mo. male D44N/+ mice frequency of SR stacks was increased, although the total number of SR stacks per area of section was significantly decreased (Figure 10, panel C). Moreover, TT length and SR-TT contact length were identical to those obtained in muscles from WT mice (Figure 10, panel C).

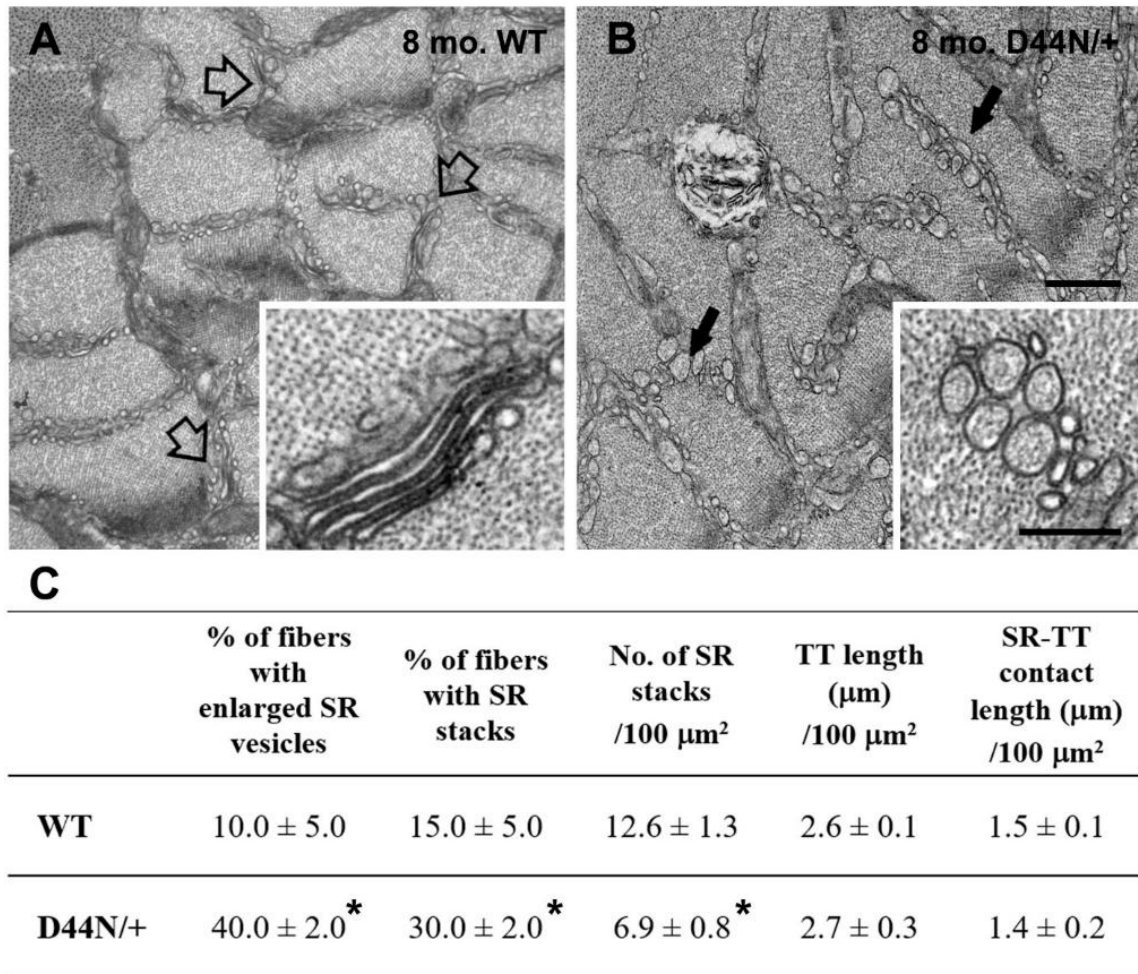


Figure 10. In EDL muscle fibers from 8 mo. male D44N/+ mice rearrangement of sarco-tubular system membranes at the I band is present. (A-B) Representative traversal EM images of EDL fibers from 8 mo. male WT (A) and D44N/+ (B) mice. Empty arrows in panel A point to I band areas with stacks of flat cisternae, and the higher magnification image (inset in panel A) shows an enlargement of a SR stack. Black arrows in panel B point to I band with enlarged SR vesicles, also shown in the inset in panel B. Scale bars: A and B = 0.5 μm ; insets in A and B = 200 nm. (C) EM quantitative analyses of: i) percentage of fibers presenting enlarged SR vesicles; ii) percentage of fibers with SR stacks; iii) number of SR stacks in 100 μm^2 ; iv) extension of T-tubules (TT) network in 100 μm^2 ; v) SR-TT contact length in 100 μm^2 . Data are shown as mean values $\pm$ SEM; * p < 0.05 vs. WT. n = 3 animals for each group.

- ***Mitochondria are affected in D44N/+ mice.*** Mitochondrial loss, damage, and mis-localization are phenomena that are widely described in literature, both in aging and in muscle diseases, including myopathies (Boncompagni et al., 2009; Durham et al., 2008; Paolini et al., 2015; N. Zhao et al., 2024).

EM analysis of intermyofibrillar population of mitochondria in both WT and D44N/+ mice were performed: in fast twitch fibers of WT mice, mitochondria were regularly placed at the I band of sarcomere, at both sides of the Z lines (Figure 11, inset in panel A) and healthy mitochondria (Figure 11, panel A) usually exhibited an electron-dense matrix, with clearly visible parallel internal cristae (see Figure 11, panel A and inset)(Bolaños et al., 2008; Boncompagni et al., 2009; Dirksen, 2009; A. E. Rossi et al., 2009). Although most mitochondria were placed in the correct position also in D44N/+ samples, in some areas of fibers from D44N/+ animals, the mitochondrial ultrastructure was compromised (Figure 11, panel B). As shown by the enlargement in panel B of Figure 11, severely altered mitochondria were seen more often than in WT samples and they were characterized by the presence of one or more of the following features: disrupted external membrane, vacuolation of internal membranes, myelin figure formation, etc. Visual observations were confirmed by quantitative analysis of EM images taken from EDL muscle fibers from 8 mo. WT and D44N/+ mice. We evaluated: (i) total number of mitochondria, (ii) number (and percentage of total number) of mitochondria placed incorrectly at A band of sarcomere; and (iii) percentage of damaged mitochondria (Figure 11, panel C). These quantitative analyses revealed that percentage of damaged mitochondria was significantly increased in the EDL muscles of 8 mo. male D44N/+ mice compared to age-matched WT mice, while the number (and the percentage) of mitochondria misplaced was not different from that measured in WT samples (Figure 11, panel C).

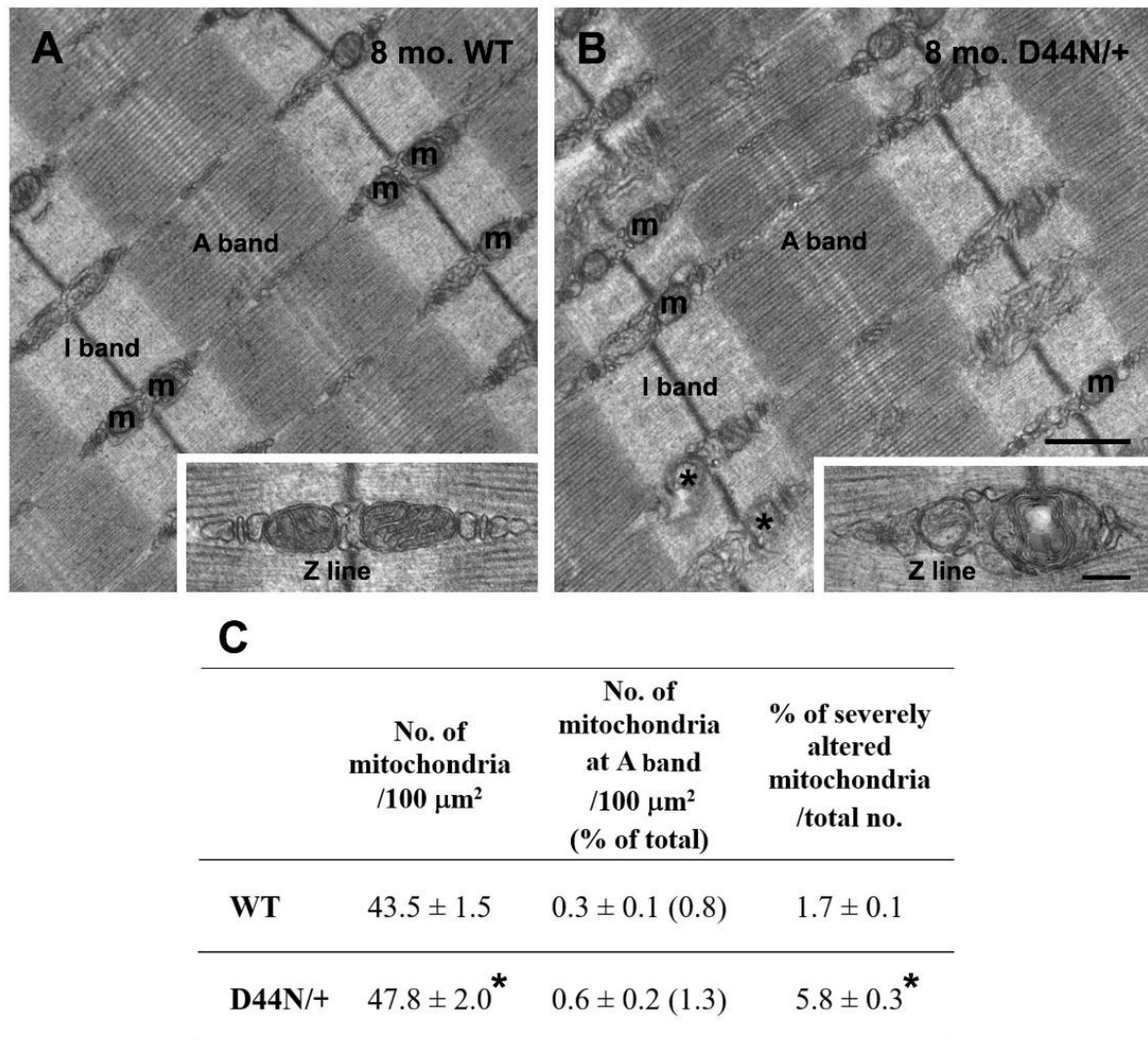


Figure 11. Qualitative and quantitative EM analyses of EDL muscles dissected from 8 mo. D44N/+ male mice reveal a higher incidence of severely altered organelles. (A-B) Representative longitudinal EM sections of EDL fibers from WT (panel A) and D44N/+ (panel B) mice showing distribution and ultrastructure of mitochondria (marked by m letter). Asterisks in panel B indicate severely altered mitochondria. Insets in panel A and B point to position of mitochondria with respect to the Z line. Scale bars: A and B = 0.5 μm ; insets in A and B = 200 nm. (C) EM quantitative analyses of: i) number/area of mitochondria, ii) number/area and percentage of A band mitochondria, iii) percentage of severely altered mitochondria/total number. Data are shown as mean values $\pm$ SEM; * p < 0.05 vs. WT. n = 3 animals for each group.

- ***Voluntary Wheel Running (VWR) prevented accrual of TAs and reduced mitochondrial damage.*** Two recent papers demonstrated that sustained VWR: (i) in aging mice restored SOCE function and prevented the formation of TAs (Boncompagni et al., 2021, Rastelli et al., 2025); and (ii) in *Orai1*G100S/+ (GS) mice, a TAM mouse model harboring a gain of-function mutation in the ORAI1 pore, reduced both the incidence of TAs in the EDL muscle and normalized mitochondrial protein expression and morphology in the slow twitch soleus (Zhao et al., 2024). Here, we assessed the therapeutic potential of VWR in mitigating the multiple skeletal muscle phenotypes observed in D44N/+ mice. First, we tested if 4 months of VWR, from 4 to 8 months of age, a time point in which D44N/+ have significantly more TAs than WT mice (Figure 8), was able to reduce the frequency of TAs. Qualitative light microscopy and EM quantifications (Figure 12) pointed out that after the 4 months of VWR, less TAs were visible in EDL muscles of D44N/+ mice (Figure 12, panels B) if compared to sedentary D44N/+ mice (Figure 12, panel A), and that frequency and number of TAs per fiber were both decreased (Figure 12, panels C and D, respectively). Again, using EM, we also evaluated the effect of VWR on mitochondrial number and ultrastructure. Qualitative and quantitative EM analysis (Figure 13) revealed that, although total number of mitochondria was unchanged (Figure 13, panel C), the number of severely altered organelles was significantly reduced following VWR (Figure 13, panel D).

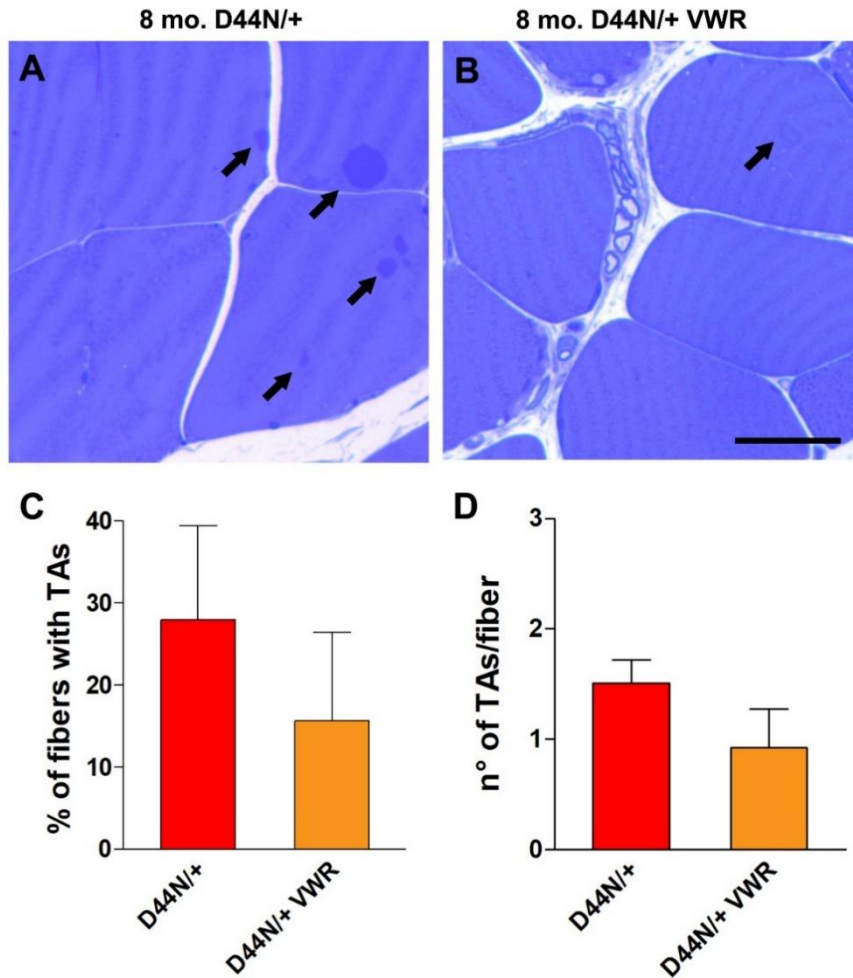


Figure 12. Voluntary wheel running (VWR) from 4 to 8 months of age is able to protect against accumulation of TAs. (A-B) Histological transverse section images of EDL muscle fibers from 8 mo. male D44N/+ vs WT mice. TAs are pointed by black arrows. Scale bar: A and B = 25 μ m. (C-D) Bar plots showing the EM quantitative analyses of the percentage of fibers containing TAs (C), and the number of TAs/fiber (D) in EDLs from 8 mo. male D44N/+ mice either sedentary or trained in cages for VWR. Bar plots show mean values $\pm$ SEM. $n = 3$ animals for each group.

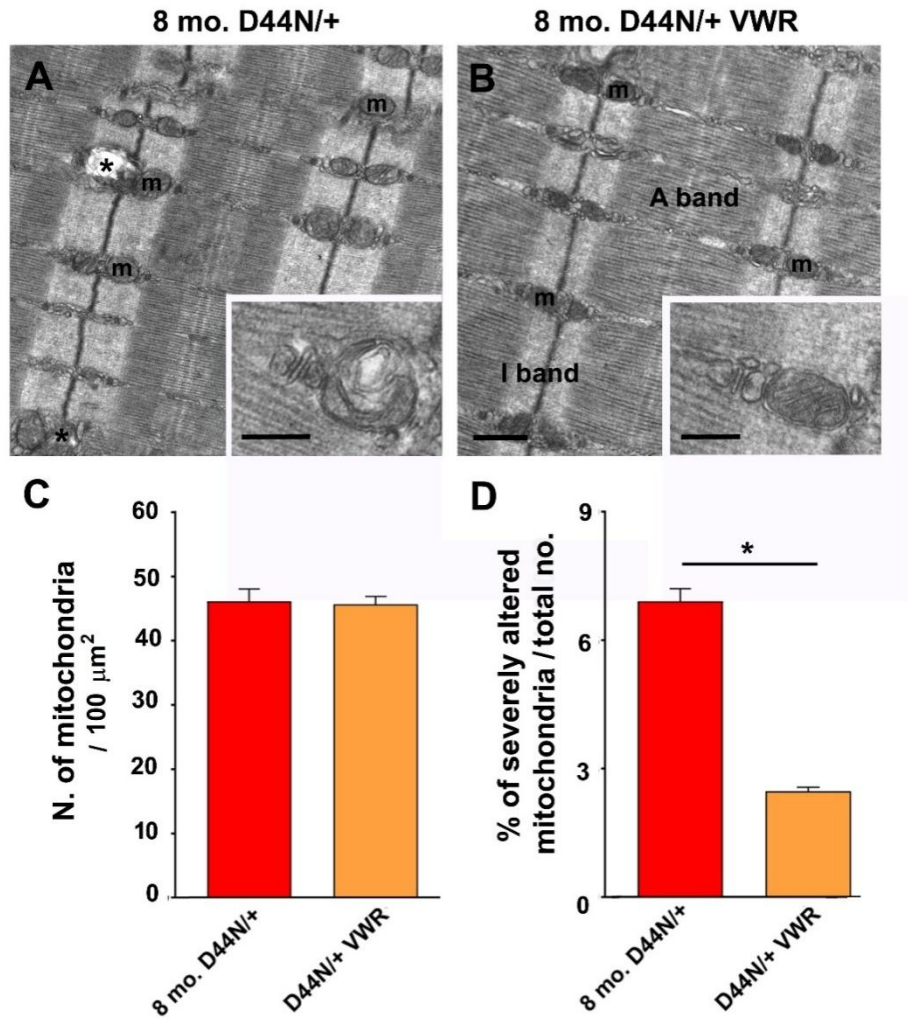


Figure 13. VWR is effective in mitigating also mitochondria damage in skeletal muscle fibers of male animals with D44N/+ mutation. (A and B) Representative longitudinal EM images of EDL fibers from D44N/+ mice either sedentary (panel A) or trained in VWR cages (panel B) with most mitochondria (indicated by m letter) positioned within the I band of the sarcomere. Asterisks point to severely altered organelles. Insets in panel A and B show mitochondria appearance at higher EM magnifications. Scale bars: A and B = 0.5 μm ; insets in A and B = 200 nm. (C and D) EM quantification of number of mitochondria in 100 μm^2 (panel C) and percentage of severely altered mitochondria/total number (panel D). Data are shown as mean values $\pm$ SEM; * p < 0.05 vs. WT. n = 3 animals for each group.

3.5 Discussion.

TAM is a rare disease linked to mutations in the genes involved in Ca^{2+} handling. This disease often shows a mild phenotype, primarily characterized by progressive muscle weakness, elevated levels of CK, hypocalcaemia, exercise intolerance, and the presence of TAs (Barone et al., 2017; Böhm et al., 2013, 2017; Chevessier et al., 2005; Y. Endo et al., 2015; Garibaldi et al., 2017; Harris et al., 2017; Morin et al., 2020; Sallinger et al., 2020). TAs are ordered arrays of SR tubules observed in muscle biopsies from patients affected by different myopathies, and they represent the primary morphological alteration in TAM patients (Apostol et al., 2009; W. K. Engel et al., 1970; Morgan-Hughes, 1998; Pierobon-Bormioli et al., 1985; Rosenberg, 1985; Salviati et al., 1985). Notably, TAs are also found in fast twitch muscle fibers of aged WT male mice, not in females (Boncompagni et al., 2012).

The lack of a robust animal model accurately mirroring the human TAM condition has been a significant barrier for years to elucidate the underlying pathophysiological mechanisms and to develop effective treatments. As a consequence, an accurate disease model continued to be a significant challenge to improve research progress in the field in the last decade: different preclinical models of TAM have been developed, particularly those carrying STIM1 mutations, which exhibited several clinical hallmarks of TAM but lacked TAs (Gamage 2018; Silva-Rojas 2019; Cordero-Sanchez 2020; Grosse 2007).

Recently, we collaborated on the characterization of a mouse model of TAM based on a mutation in the ORAI1 pore that phenocopied multiple key aspects of TAM in humans, including TAs, and suggests an important role for mitochondria in relation to SOCE activity (Zhao 2024). However, to date TAM models associated with mutations in CASQ1 were not available.

Our study, introducing the first CASQ1 TAM model has the potential of valuable insights into progression of the disease and potential therapeutic avenues.

- **Main Findings.** In the present work, we showed that (i) heterozygous D44N mutation did not cause premature death of animals, did not impair body mass, and did not affect significantly in vivo/ex vivo force in adult mice. Nevertheless: (ii) in EDL muscles of D44N/+ male mice TAs started to accumulate earlier than in age-matched WT (by 8 months of age in males), but slightly later in females, and in 13-16 mo. male D44N/+ mice, TAs contained also vacuoles, resembling those of patients with vacuolar myopathies; (iii) other structural changes were observed in skeletal muscle of D44N/+ mice, i.e. increased percentage of fibers presenting enlarged SR vesicles and SR stacks; (iv) VWR in D44N/+ mice reduced formation of TAs, and preserved mitochondrial ultrastructure confirming long term exercise as a potent intervention in counteracting the development of TAs, as already demonstrated in ageing and in GS mice (Boncompagni et al., 2021; O'Connor et al., 2025).

Our work demonstrated that the defining hallmark of TAM, i.e. the accumulation of TAs, appeared at 8 months in EDL muscles of D44N/+ male mice (Figure 8) and that later in life, in 13-16 mo. male animals, the ultrastructure of TAs changed, from being well organized tightly packed straight tubes into abnormal vacuole-like structures disposed near orderly SR tubes (Figure 8, panel D). In addition, TAs started to form also in EDLs of female D44N/+ animals at 13 months of age but they were never found in soleus muscles. We have previously provided evidence that in the ageing condition, TAs started to form in fast twitch EDL muscle at 12 months only in WT male animals (not in female mice) and they presented SR straight tubules with highly regular arrangement also in 24 mo. WT male mice (Boncompagni et al., 2012, 2021). In a previous TAM mouse model, Dirksen and colleagues (Zhao et al., 2024) have recently shown that multiple fast-twitch muscles from 8 mo. GS mice (EDL and FDB muscles, but not the slow twitch soleus) exhibited a robust presence of ordered TAs with honeycomb-like structures and that once started, the accrual of TAs was age dependent (Zhao et al., 2024) showing no significant changes in their morphology. Besides, no sex variation was observed in EDL muscles from both 8 months male and female GS animals

(Zhao et al., 2024). To resume, when comparing EM analysis of skeletal muscle fibers from D44N/+ mice to data obtained from the GS TAM model and the ageing condition, it emerged that in all the three conditions the slow twitch muscle soleus showed a complete absence of TAs, confirming type I fibers being somehow protected from TAs accumulation. However, our CASQ1 related TAM model presented some peculiar features: (i) TAs started to develop in D44N/+ male mice, similarly to the GS model but earlier than in WT mice, at 8 months of age; (ii) in D44N/+ female animals TAs were not seen before 13 months of age. These aspects make of D44N/+ mice a midpoint between the GS model - characterized by increased TAs incidence in both male and female mice - and WT animals, in which TAs naturally occurred only in ageing male mice. From the structural point of view, TAs became irregularly shaped in ageing D44N/+ male mice, a finding that can be related both to the previously reported (i) formation of "atypical" TAs in muscles of CASQ1-ko mice, in which the absence of the CASQ1 protein led to the abnormal aggregation of SR tubules with different conformations which never developed into ordered aggregates of straight cylinders (Boncompagni et al. 2012) and to (i) the presence of a rare human muscle disorder linked to mutations in CASQ1, called vacuolar aggregate myopathy. Protein aggregates and an increased number of vacuoles were present in muscles of affected patients, similar to those observed in our 13-16 mo. D44N/+ male mice (Angelini, 2018; Böhm & Laporte, 2018; Di Blasi et al., 2015; Semplicini et al., 2018; Tomelleri, 2006). All these observations suggest that CASQ1 is essential for maintaining the linear symmetry and uniform diameter of tubules forming TAs.

In our TAM model, other significant structural alterations were observed in muscles of D44N/+ mice, i.e. enlarged SR vesicles and the formation of SR stacks (Boncompagni et al., 2017).

These observations support the prevailing hypothesis that TAM pathogenesis involves a fundamental disruption of SR membrane integrity and Ca^{2+} homeostasis, leading to a compensatory, yet ultimately pathological, proliferation of the SR membrane system into TAs (Engel A. G., & Franzini-Armstrong. C., 1994). Moreover, the finding that in muscles of

D44N/+ mice was detected a remodelling of the SR to form stacks of membranes like those observed when SOCE is activated, could represent the initial step of a possible compensatory attempt to maintain a structural / functional coupling between STIM1 and ORAI1 to boost Ca^{2+} entry and to maintain SOCE function.

Coincidentally, the D44N/+ mutation resulted in substantial mitochondrial damage in EDL muscles of 8 mo. male mice, as revealed by EM (Figure 11). Morphological and functional alterations of mitochondria were reported previously in multiple mouse models with a broad range of skeletal muscle disorders, including Duchenne muscular dystrophy, and cachexia (Carson et al., 2016; Padrão et al., 2013; Pant et al., 2015). It was also reported in literature (Sánchez-González et al., 2022) that chronic inhibition of mitochondrial ATP synthase in skeletal muscle was a direct driver of TA formation. This process involved a metabolic switch toward glycolytic fibers and increased mitochondrial fission. Moreover, TAs appeared to result from an "over-response" of the mitochondrial fission controller DRP1, which increased interactions between mitochondria and the SR. Under prolonged oxidative stress or hypoxia, TAs were observed to cluster specifically around defective mitochondria. The interaction between these organelles served as a mechanism to buffer Ca^{2+} overloads when mitochondrial oxidative phosphorylation (OXPHOS) was impaired. Finally, some histochemical studies have detected mitochondrial enzymes (e.g., NADH-tetrazolium reductase) within or immediately adjacent to these structures, suggesting joint participation in their genesis (Novotová et al., 2002; Schiaffino, 2012). Recent research further confirmed a significant structural and functional relationship between TA and mitochondria in skeletal muscle: by 8 months of age, skeletal muscles in GS mice showed robust mitochondrial damage, concomitant to TAs accrual (N. Zhao et al., 2024).

Our results are in lines with these findings as the heterozygous D44N mutation, beyond the SR, also impaired the mitochondrial network. As the importance of maintaining the correct the cross-talk between the SR and mitochondria (to match energy production to mechanical work

and maintaining cellular health in physiological conditions) is well known (A. E. Rossi et al., 2009, 2011), in our model mitochondrial dysfunction would probably be a mechanism leading to further impairment of muscle metabolism, potentially exacerbating the structural remodelling and contributing to the overall disease phenotype at advanced stages.

Perhaps the most encouraging finding of our work was that VWR successfully rescued some skeletal muscle signs in D44N/+ mice: it mitigated TAs formation and mitochondrial defects.

The beneficial effect of VWR in D44N/+ mice is consistent with the exercise-induced reduction in TAs observed in muscles of WT aged male and in 8 mo. GS mice, both exhibiting depressed SOCE function (Boncompagni et al., 2021; O'Connor et al., 2025). Boncompagni and colleagues (Boncompagni et al., 2017, 2018) demonstrated also that in adult mice exercise VWR drove the formation of CEUs, the sites of functional and dynamic STIM1-ORAI1 association that promoted SOCE and limited muscle fatigue during repetitive stimulation. Moreover during aging (Boncompagni et al., 2021; Rastelli et al., 2025) VWR guaranteed both (i) the maintenance of the structural elements required for the assembly of functional CEUs, i.e., SR stacks and TT at the I band and (ii) a better functional activation of SOCE, which was typically reduced in sedentary mice (Boncompagni et al., 2021; Rastelli et al., 2025).

Thus, we hypothesize that in our CASQ1 related TAM model the beneficial reduction of TAs induced by VWR could probably be also mediated by the fact that, by activating SOCE with long term exercise, correct STIM1 and ORAI1 interaction promote the maintenance of muscle architecture.

- *Implications of our Findings.* Mutations in CASQ1 in humans are associated to either vacuolar myopathy or TAM, a milder form of myopathy (Angelini, 2018; Barone et al., 2017; Böhm & Laporte, 2018; Di Blasi et al., 2015; Lewis et al., 2015; Rossi et al., 2014; Semplicini et al., 2018; Tomelleri, 2006). For this point of view, the mild phenotype of D44N mice is not surprising, and somehow recapitulate the human disease linked to this specific mutation.

- ***Final Remarks.*** In summary, the D44N/+ mice may represent in future years a good tool to study those form of TAM (and vacuolar myopathy) associated to mutations in CASQ1. As shown by our results, the existence of a mouse model of the pathology may help to identify the time course of the key pathological events, including age-dependent TA accumulation and mitochondrial damage, and try to develop e therapeutic strategies to limit muscle weakness and the progress of the disease.

Future research efforts should focus on both deciphering (i) the role of mitochondria in TAM to clarify whether they actively contribute to the pathogenesis or merely represent a downstream consequence of the disease; (ii) the molecular mechanisms by which VWR confers protection against TAs accrual, to identify specific pharmacological mimetics that could be used as a treatment strategy for TAM patients.

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