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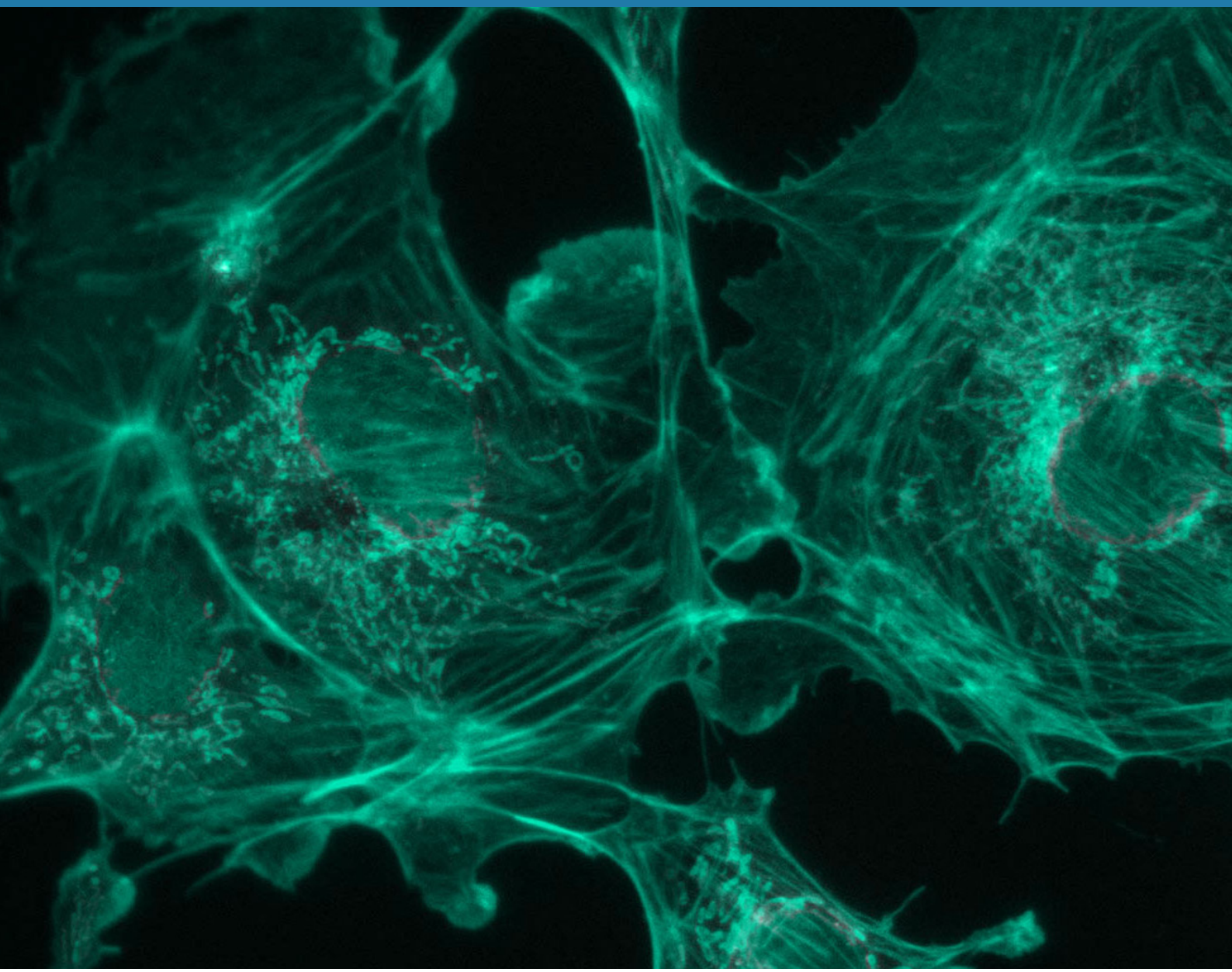

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MICROTUBULES AND MITOCHONDRIA NANOTUNNELS

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ABSTRACT

Mitochondrial function strictly depends on mitochondrial structure, location and dynamics. Mitochondria and cytoskeleton interaction is crucial for normal mitochondrial morphology distribution and motility. In cardiac muscle, microtubules sustain muscle contraction and mitochondria disposition; the latter is crucial for localized uptake of calcium (Ca^{2+}) and consequentially energy production needed for cardiac function.

Few studies have been conducted on understanding mitochondria and microtubules interaction in cardiomyocytes. In cardiac muscle, recent studies have shown that cardiac mitochondria do not need to migrate to communicate with each other, but they can extend protrusions called nanotunnels to reach and communicate with other mitochondria in long-distance. This process does not involve mitochondria movement, but possibly involves a pulling action along microtubules. The association of mitochondria nanotunnels and microtubules have been identified using 2D and 3D electron microscopy [1]. These new findings highlight the need to investigate the action of microtubules on cardiac mitochondria and its effect on mitochondria dynamics and cardiac function.

Keywords: mitochondria nanotunnels, microtubules, cardiac muscle.

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Introduction

Mitochondria represent the center of energy production in the cell, regulating cell life or death. They play a crucial role in several processes: production of ATP, regulation of apoptosis, membrane potentials, Ca^{2+} homeostasis. Mitochondrial calcium uptake through the mitochondrial calcium uniporter (MCU) regulates mitochondrial function with an important role in ATP production in skeletal and cardiac muscles [2, 3], in mitochondrial motility, morphology, in fission and fusion dynamics [4, 5]. In several types of cells, mitochondria are dynamically interconnected and continuously fuse and divide in order to repair damaged mitochondria and maintain mitochondrial function. Mitochondrial activity and cell maintenance depends on mitochondria structure, distribution, motility, dynamics, fission and fusion balance. Mitochondria shape and disposition vary depending on the cell and organism of origin, but in all cases, they are indispensable for effective organelle function within the cells requirements. The disposition of mitochondria in skeletal and cardiac muscle is highly stereotyped because it is strictly related to the cross striation of myofibrils and in turn to the location of Calcium Release Units (CRUs) [6, 7], the sites where calcium is released from the sarcoplasmic reticulum during muscle activation. This location is essential because minor Ca^{2+} entry into mitochondria activates ATP production [2].

The strategic distribution of mitochondria is determined by elements of the cytoskeleton network that coordinate mitochondrial movement, distribution, mitochondrial morphology, and may secondarily influence mitochondrial communication [8, 1]. Several studies have shown the organism and cell specific association between mitochondria and cytoskeleton components and that mitochondrial movement is driven by actin filaments and microtubules [9, 10, 11, 12]. The organization of mitochondria in the myocardium of vertebrates is well established and recent works reveal interesting details in short and long distance interactions among mitochondria with active exchange of matrix proteins. Specific elongated protrusions called nanotunnels [13] allow mitochondria to interact and exchange matrix protein in long distances with slow mixing kinetics [1].

While the organization of mitochondria in cardiac muscle is well described, the interaction of mitochondria and microtubules and its effect on mitochondria dynamics is not well investigated. This review focuses on the interaction between mitochondria and microtubules and in particular, on the newly discovered association of mitochondrial nanotunnels and microtubules interaction in cardiomyocytes and its consequences on inter-mitochondrial communication [1].

Mitochondria and nanotunnels in cardiac muscle

In mammalian, cardiac mitochondria are strategically located between myofibrils and close to the CRUs in the form of dyads (association of junctional sarcoplasmic reticulum with transverse T-tubules). Mitochondria occupy 40% of the cardiac volume and are arranged among myofibrils in a longitudinal fashion. Their diameter, length and shape can substantially vary and they rarely present protruding elongations called nanotunnels [13, 1].

In rat isolated cardiomyocytes, Huang et al. [13] discovered that mitochondria extend membrane nanotubes to reach other mitochondria in long distance allowing inter-mitochondrial communication. Using live confocal imaging these nanotubes of 90-210 nm diameter were observed connecting mitochondria in adult cardiomyocytes exchanging mtPAGFP (photoactivatable matrix-targeted soluble fluorescent protein). Transmission electron microscopy confirmed the existence of a nanotubular structure bridging long-distance mitochondria. These structures were further observed in cardiac muscle of mice using 2D electron microscopy, tomography, 3D reconstruction and live confocal imaging [1, 14].

Mitochondrial nanotunnels are composed of a double mitochondrial membrane and fewer cristae aligned to the longitudinal axes. Their diameter and length detected in thin sections of 2D electron microscopy is respectively between 0.04–0.20 μm and 0.7-3.6 μm [1] comparable to those detected in bacteria [15]. Similarly, mitochondria tubulations were detected in rat kidney cells [16].
In multicellular organisms, tubular formations allow communication between cells, giving the possibility to exchange cellular content, DNA and proteins [17, 18]. In cytotoxic T lymphocytes membrane bridges were observed with a diameter of 50-100 nm [19] and bacteria developed protrusions to exchange molecules between cells [15].
Mitochondria nanotunnels allow slow inter-mitochondrial matrix proteins exchange [1]. The mechanism involved in the inter-mitochondria communication though nanotunnels is not clear; it is possible that this process involves a fusion event with direct matrix mixing or that the interaction between membrane kissing junctions is involved between the nanotunnel tip and a second mitochondrion [13, 1]. Ca^{2+} homeostasis may play a role in mitochondrial nanotunnels formation and dynamics. Ca^{2+} imbalance can affect mitochondria morphology and distribution in skeletal and cardiac muscle [20, 7] and Ca^{2+} levels also control mitochondrial dynamics via fission by targeting Drp1 (dynamin-1-like protein), a GTPase that regulates mitochondrial fission [21] and via motility by targeting Miro (Mitochondrial Rho) proteins, outer mitochondrial membrane GTPases involved in mitochondrial trafficking [22].
Malfunction of the cardiac Ca^{2+} release channel, RyR2, induces an increase in mitochondrial nanotunnels frequency and secondarily slower inter-mitochondrial protein exchange [1]. Cardiac inter-mitochondria long-distance communication allows the exchange of matrix proteins without mitochondrial movement, thus keeping the original position of the organelle, while the elongations seem to be driven by microtubules [1].

Microtubules and mitochondria

The cytoskeleton contributes to the cell architecture and acts as a transport system of vesicles, molecules and organelles. Microtubules are constituted by polymers of tubulin with diameter of about 12 nm - 24 nm [23] and play a fundamental role in maintaining cellular structure together with intermediate filaments and microfilaments. Several proteins bind to microtubules, such as dynein and kinesin, defined as motor proteins. Mitochondria continuously remodel their shape and size going through fission and fusion events and their movement and rearrangement is based on the cytoskeleton transportation system [24] which depends on both actin microfilaments and microtubules [25, 26]. Mitochondrial transportation has been extensively studied, however little is known about the mechanism involved. In *S. cerevisiae*, disruption of the yeast microtubule network [27, 28] does not affect mitochondrial morphology but instead, actin plays an important role for mitochondrial movement [29]. On the contrary, microtubules play a relevant role for mitochondrial distribution in fission yeast [30], in filamentous fungi [31] and in mammalian cells where mitochondrial transport depends on kinesin-like motor proteins in mice [32]. Kinesin-1 (KIF5B) and Kinesin-3 (KIF1B) have been reported to be implicated in mitochondrial distribution [33] and inhibition of dynein affects mitochondrial distribution in axons [34]. Furthermore, inhibition of Kinesin-1 in Drosophila inhibits mitochondrial movement [35] demonstrating that kinesin and dynein are implicated in mitochondrial movement and consequently in mitochondria dynamics, playing a significant role in mitochondrial function. Two proteins, Miro and Milton have been reported to link kinesin-1 to mitochondria [36, 37]. In mammalian neurons, in vivo experiments show that Milton and Miro localize to mitochondria in brain [38, 22]. Evidence of direct interaction between Miro and Milton was detected in

Drosophila providing evidence in understanding the mechanism, which is involved in microtubules-based mitochondria movement [39, 40]. As already reported, Ca^{2+} plays an important role in mitochondrial function and mitochondria morphology, distribution, dynamics and mitochondrial movement. Mitochondrial recruitment in areas of increased Ca^{2+} level is important for accomplishing energy demand. Mitochondrial axon transport is regulated by Ca^{2+} and mitochondrial arrest can be caused by increased concentration of calcium and Ca^{2+} mobilizing agonists [41, 42, 4]. Furthermore, several studies concentrate on understanding the role of Ca^{2+} on Miro GTPases function showing that Ca^{2+} impacts Miro function and mitochondrial motility and dynamics [43, 44, 22].

Microtubules and mitochondria nanotunnels

Fewer studies have been conducted on microtubules and mitochondria in cardiac muscle.
Adult cardiomyocytes display an organized cytoarchitecture that grants synchronized muscle contraction and its maintenance is established by the cytoskeleton which is able to hold sarcomeres and couple myofibrils. Mitochondria are also part of this fine architecture. Microtubules in cardiac muscle have been observed in thin sections of electron microscopy in 1979 in dog and guinea pig hearts [45]. In this report, microtubules were observed near the nucleus and between myofibrils, especially located at the Z-line and mostly close to mitochondria. The majority of microtubules profiles were detected diagonally across the sarcomere, where the sarcoplasmic reticulum is located between mitochondria and microtubules. Confocal imaging reveals that microtubules form an orthogonal network around myofibrils and mitochondria are localized within this fine architecture (Fig. 1, Lavorato, unpublished data).

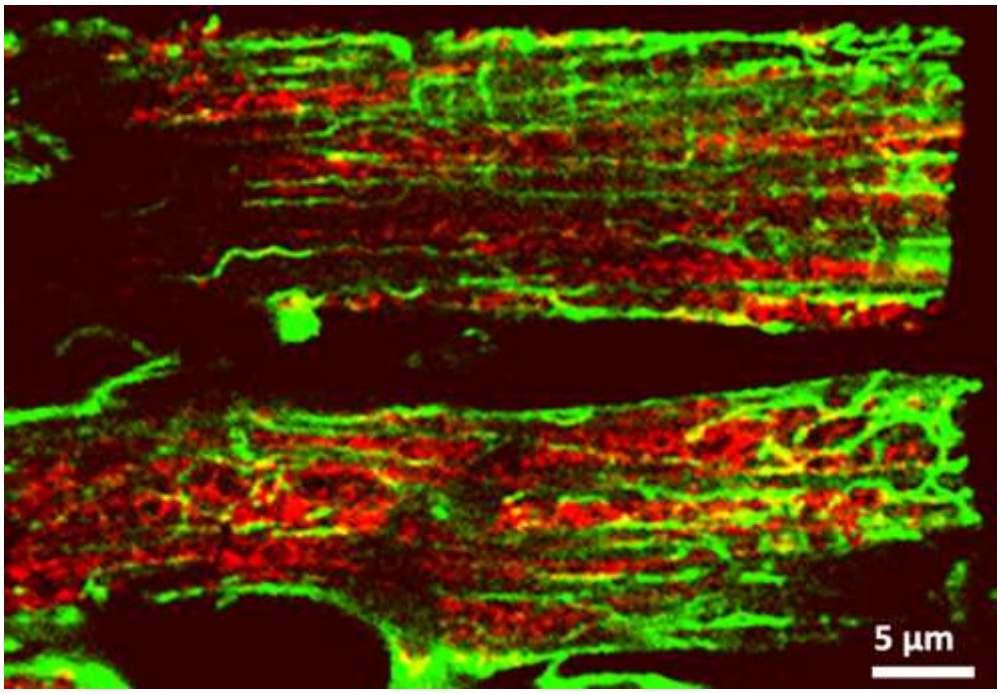

Figure 1. Confocal image of microtubules (green) and mitochondria (red) in mouse cardiac cardiomyocytes. Microtubules and mitochondria are co-immunostained with anti-α-tubulin (green) and anti-mitoneet (red) primary antibodies and alexa 488 and Cy3 secondary antibodies. Microtubules are well organized in a net that "traps" mitochondria.

The structure of microtubules network in cardiac muscle plays a crucial role in cardiac function. During contraction, microtubules need to be able to contain muscle architecture changes. Robinson et al. [46] demonstrate that microtubules detyrosination, followed by microtubules network deformation, affects mechanical properties of cardiac muscle during contraction. Furthermore, loss of the normal cytoskeletal structure causes heart failure [47] and structural remodeling affects T-tubules and sarcoplasmic reticulum arrangement involving the ryanodine receptors (RyRs2), which are crucial proteins for muscle contraction [48, 49]. Recently, studies have defined the role of BIN1, anchoring microtubules to the T-tubules membrane system and delivering the L-type calcium channels to the T-tubules [50]. During heart failure and malfunction of RyR2 with consequent abnormal Ca^{2+} homeostasis, mitochondria disposition, morphology and dynamics can be affected [51, 7, 1]. In non muscle cells mitochondrial migration is essential for the accomplishment of fusion and fission events [52]. Contrarily, in adult cardiomyocytes, mitochondria are very close to each other and the extensive amount of myofibrils may block mitochondrial movement. In our recent study [1] we reported that a Catecholaminergic Polymorphic Ventricular tachycardia-linked RyR2 mutation (A4860G) causes Ca^{2+} imbalance by depressing RyR2 channel activity during excitation–contraction coupling [53] and increases the frequency of mitochondrial nanotunnels. In this model, Using 2D and 3D transmission electron microscopy approach, we reported the initial evidence of mitochondria and nanotunnels association with microtubules in cardiac muscle. Ultrathin sections of rapidly frozen cardiomyocytes and electron tomography show abundance of microtubules in proximity of mitochondrial nanotunnel extensions (Fig. 2A, C-D). Intermediate filaments are also, but rarely observed in the same area (Fig. 2A, C-D). 3D reconstruction of mitochondrial nanotunnels and microtubules (Fig. 2 B) better show the association between the two, suggesting that microtubules may contribute to nanotunneling dynamics. In particular, in figure 2, C-D, microtubules detected in sequential images obtained from electron tomography, follow mitochondrial nanotunnels profiles through the thickness of the section and intermediate filaments are also observed in crowded mitochondrial nanotunnels area in the cell thickness (supplementary video, S1).

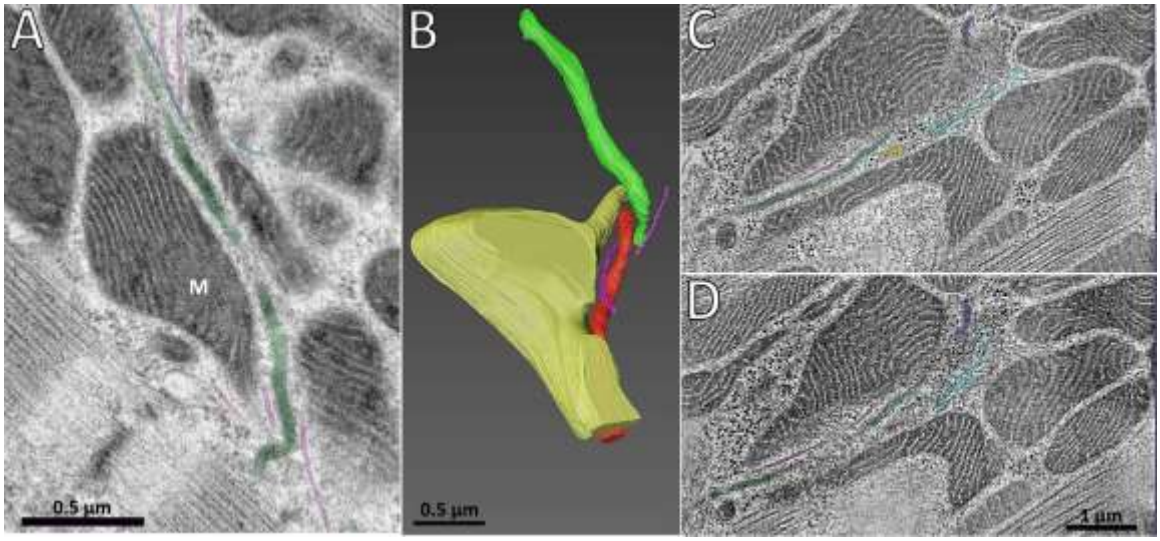

Figure 2. Microtubules and mitochondria nanotunnels. A) Microtubules (purple) close to mitochondrial profiles (M) and nanotunnels (green) in thin section of rapidly frozen cardiomyocites. Intermediate filaments (pale blue) are also observed in proximity of mitochondria nanotunnels. B) 3D reconstruction of microtubules (purple) and

mitochondria nanotunnels (yellow, green, red). C-D) Images extrapolated from electron tomography, where microtubules (purple) follow mitochondria nanotunnels profiles (green) through the thickness of the section and intermediated filaments (pale blue) are close to the nanotunnels crowded area. Images were adapted from Lavorato et al, 2017.

The association of mitochondrial extensions to microtubules may imply a transport mechanism that allows long-range distance communication between mitochondria. Inter-mitochondrial communication via nanotunnels does not involve obvious mitochondrial migration and transmission electron microscopy shows that nanotunnels cristae are aligned parallel to the longitudinal axis of the extensions [1]. This strongly suggests that microtubules participate in the growth of nanotunnels from immobile mitochondria with a pulling action, possibly involving motor proteins. In kidney cells, mitochondrial extensions (not nanotunnels) have been correlated to microtubule-directed movement and mitochondrial fission process [54]. Recent studies also showed that tubular extensions from chloroplasts (stromules) protrude along microtubules and their architecture affects stromule dynamics [55]. Since Ca^{2+} imbalance in the CPVT model increases mitochondrial nanotunnels frequency, it is possible to suppose that the driving force of microtubules on mitochondria depends on Ca^{2+} concentration or that unbalanced Ca^{2+} levels arrest mitochondrial movement; thus increased mitochondria nanotunnels extend from immobile mitochondria, are pulled by the action of microtubules to accomplish inter-mitochondrial communication.
Lavorato et al. [1], and this review initiate the investigation on mitochondria nanotunnels and microtubules association in cardiac muscle and highlight the need for further investigation to better explain the mechanism involved in mitochondria-microtubules interaction in cardiomyocytes.

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Manuela Lavorato is a Research Associate at the Dept. of Genetics, Mitochondria Medicine Center at the Children's Hospital of Philadelphia. She received her PhD in animal biology in Italy at the University of Calabria (2011). Most part of her research project was conducted at the Dept. of Environmental Sciences, Behavioral Biology Unit at the University of Liège in Belgium. She started her career analyzing the effect of neurotoxic pesticides on amphibian behavior and morphology, using video-tracking analysis systems and electron microscopy. In 2012, she joined Clara Franzini-Armstrong laboratory as postdoctoral researcher at the University of Pennsylvania, where she focused on analyzing skeletal and cardiac muscle in myopathies. In particular she investigated the effects on calcium release channels (RyRs, DHPR) mutations on muscle ultrastructure and on mitochondria dynamics. In Clara Franzini-Armstrong laboratory, she became expert in examining muscle and mitochondria ultrastructure using transmission electron microscopy, tomography and scanning electron microscopy. Her interest in cardiac muscle and mitochondria strongly increased when she started analyzing the effects of a Cardiac Ryanodine Receptor (RyR2) mutation linked to Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) on cardiac muscle, in collaboration with Dr. Hector H. Valdivia, finding a unique mitochondrial response. Her interest in mitochondria led her to Dr. Marni Falk's laboratory (2017) at the Mitochondria Medicine Center of the Children's Hospital of Philadelphia (2017), where she started as a Research Associate. Her work presently focuses on understanding several mitochondrial multi-complex diseases affecting children.