ACTA MYOLOGICA

(Myopathies, Cardiomyopathies and Neuromyopathies)

Vol. XLI - June 2022

Official Journal of
Mediterranean Society of Myology
and
Associazione Italiana di Miologia

Founders: Giovanni Nigro and Lucia Ines Comi

Three-monthly

G. CONTE ACADEMY - NAPOLI

PACINI EDITORE MEDICINA

Established in 1982 as Cardiomyology

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Acta Myologica publishes 4 issues per year in March, June, September, December. The Journal is available in OPEN ACCESS at: www.actamyologica.it

Acta Myologica is cited in Index Medicus, PubMed/MedLine, Scopus, Open-J Gate, Free Medical Journals, Socolar. The Journal is available on PubMed Central (http://www.ncbi.nlm.nih.gov/pmc/journals/1221/).

Journal Citation Reports: SJR 2019 0.616; SNIP 2019 0.818
Acta Myologica is available on Google Scholar

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Tribunal Authorization, Napoli N. 3827, January 10, 1989 - Journal registered at "Registro pubblico degli Operatori della Comunicazione" (Pacini Editore srl registration n. 6269 - 29/8/2001).

Publisher

Pacini Editore

Via A. Gherardesca - 56121 Pisa, Italy

Published by Pacini Editore Srl, Pisa, Italy, June 2022

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doi:10.36185/2532-1900-070

LETTER TO THE EDITOR

It's time to measure disability in spinal muscular atrophy

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Dear Editor

In recent years we have actioned a revolution in the nosography of spinal muscular atrophy (SMA). SMA is a severe inherited neuromuscular disease representing the most common genetic cause of infant mortality (incidence 1:11,000). SMA is caused by mutations in the Survival Motor Neuron 1 *(SMN1)* gene, resulting in insufficient expression levels of the SMN protein with motor neuron degeneration and muscle weakness [1]. As of 2017, gene-base therapies (GBT) capable of acting on the genetic defect directly on the DNA or its transcript, have radically changed the functional profile, natural history and prognosis of SMA (especially in children). Despite fatal and incurable in the past, SMA is now a non-life-threatening, treatable disease, with a significant improvement in its clinical course [2]. This enables treatments that are no longer palliative/supportive but aimed at reducing functional limitations and minimizing disability. Disabilities with varying severity from case to case become the central theme of care pathways in the era of GBT.

In line with the International Classification of Functioning, Disability, and Health (ICF), speaking of disability means speaking of a multidimensional concept that includes 'body function/structure', 'activities/participation', and 'personal/environmental factors'. It also includes the psychological, social/vocational dimensions of person's life. Disability denotes the negative aspects of the individuals (with a health condition) interaction with 'their contextual factors (environmental/personal factors) and is opposed to the concept of functioning, which denotes the positive aspects of the individuals interaction with their environment [3].

Undoubtedly, reducing levels of disability is one of the greatest unmet needs of people with SMA. To date, several scales are used to assess SMA for both clinical and research purposes that served primarily to monitor patient deterioration.

However, they do not provide us with information to assess, by adopting the ICF language, how 'impairments in muscle function' (e.g. impairments in muscle power function and muscle endurance function) or 'impairments in mental function and in sensory functions and pain' (e.g. sleep function impairments, fatigue, emotional function thinking) may lead to 'restrictions on participation in 'community, social and civic life' (restrictions in mobility, relationships and recreation and leisure), or how contextual factors (environmental and personal factors) such as 'lack of support from close family' and 'lack of support from the social security system or health professionals', are key predictors of the impact of disease-related disabilities on quality of life (QoL).

Received: May 16, 2022
Accepted: June 13, 2022

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How to cite this article: Trabacca A, Ferrante C, De Rinaldis M. It's time to measure disability in spinal muscular atrophy. Acta Myol 2022;41:57-58. https://doi.org/10.36185/2532-1900-070

In the era of GBT, which significantly alter the functional levels of SMA patients, none of the functional scales is currently designed to measure residual disability, or rather to assess levels of functioning in daily life because they were developed for non-treated patients.

These scales are used specifically to assess: global motor skills (Motor Function Measure), fine and gross motor function (Bayley-III Scales), motor function (Children's Hospital of Philadelphia Infant Test), development of motor function (Hammersmith Infant Neurological Examination – Part. 2), gross motor skills (Hammersmith Functional Motor Scales Expanded), upper limb function (Revised Upper Limb Module) and measures of physical endurance (6-Minute Walk Test) [4]. Each scale measures patients differently at different life stages (some are age-specific), in different clinical types, sometimes with contradictory results, allowing no transferability of results from scale to scale, nor comparisons of the changes magnitude [5]. At current times, we need more and more specific measures capable of capturing the functional improvements reported by patients or their parents, and still un-detected by the current assessment scales despite their relevant role in improving the SMA-related QoL. Undoubtedly, the new SMN-dependent treatments are changing the natural course of the disease. Despite this, the management of disability is still an unmet need and another import issue is the assessment of residual disability by more specific measures. Today, more importantly, an assessment of nosological impairments should go hand in hand with an assessment of the disability and functional profile through the ICF framework, which offers a picture of human functioning. A deeper understanding of ICF-based functioning relies on all aspects related to person functioning, and also considers the impact of the environment on the various SMA-related disability levels. Measuring disability is thus the new challenge we face in developing new standards of care for people with SMA in the era of disease-modifying therapies.

Acknowledgements

This work was supported by the Italian Ministry of health RC 2019-2021.

Conflict of interest statement

The Authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

AT reviewed the literature, and wrote the manuscript with support from MD and CF. All Authors discussed the results and contributed to the final manuscript.

Ethical consideration

Ethical approval for research involving human participants and/or animals: not applicable

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ACTA MYOLOGICA 2022; XLI: p. 59-75
doi:10.36185/2532-1900-071

ORIGINAL ARTICLES

The role of BAG3 in dilated cardiomyopathy and its association with Charcot-Marie-Tooth disease type 2

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Bcl2-associated athanogene 3 (BAG3) is a multifunctional cochaperone responsible for protein quality control within cells. BAG3 interacts with chaperones HSPB8 and Hsp70 to transport misfolded proteins to the Microtubule Organizing Center (MTOC) and degrade them in autophagosomes in a process known as Chaperone Assisted Selective Autophagy (CASA). Mutations in the second conserved IPV motif of BAG3 are known to cause Dilated Cardiomyopathy (DCM) by inhibiting adequate removal of non-native proteins. The proline 209 to leucine (P209L) BAG3 mutant in particular causes the aggregation of BAG3 and misfolded proteins as well as the sequestration of essential chaperones. The exact mechanisms of protein aggregation in DCM are unknown. However, the similar presence of insoluble protein aggregates in Charcot-Marie-Tooth disease type 2 (CMT2) induced by the proline 182 to leucine (P182L) HSPB1 mutant points to a possible avenue for future research: IPV motif. In this review, we summarize the molecular mechanisms of CASA and the currently known pathological effects of mutated BAG3 in DCM. Additionally, we will provide insight on the importance of the IPV motif in protein aggregation by analyzing a potential association between DCM and CMT2.

Key words: Chaperone Assisted Selective Autophagy (CASA), Dilated Cardiomyopathy (DCM), Charcot-Marie-Tooth disease (CMT), Bcl2-associated athanogene 3 (BAG3), protein quality control (PQC), IPV motif, oligomerization, protein aggregation

Received: April 26, 2022
Accepted: June 13, 2022

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How to cite this article: Yerabandi N, Kouznetsova VL, Kesari S, et al. The role of BAG3 in dilated cardiomyopathy and its association with Charcot-Marie-Tooth disease type 2. Acta Myol 2022;41:59-75. https://doi.org/10.36185/2532-1900-071

Abbreviations

AAA+ family: ATPases associated with various cellular activities; ACD: alpha-crystallin domain; ADP: adenosine diphosphate; ALP: autophagy lysosomal pathway; ATP: adenosine triphosphate; BAG: Bcl2-associated athanogene; BAG1: Bcl2-associated athanogene 1; BAG3: Bcl2-associated athanogene 3; CASA: Chaperone Assisted Selective Autophagy; CHIP: C-terminus of Hsc70 Interacting Protein; CMT: Charcot-Marie-Tooth disease; CMT1: Charcot-Marie-Tooth disease type 1; CMT2: Charcot-Marie-Tooth disease type 2; CTR: C-terminal region; DCM: Dilated cardiomyopathy; ER: endoplasmic reticulum; HOPS: homotypic fusion and protein sorting; Hsc70: heat shock cognate protein 70; HSP: heat shock protein; Hsp70: heat shock protein 70; HSPB8: small heat shock protein B8; IPV motif: Ile-Pro-Val motif; JDP: J domain protein; LIR: LC3-interacting region; MTOC: microtubule-organizing center; NBD: nucleotide binding domain; NSF: N-ethylmaleimide-sensitive factor; NTR: N-terminal region; P209L: proline 209 to leucine muta-

tion; P182L: proline 182 to leucine mutation; PQC: protein quality control; PxxP: proline-rich center of BAG3; SBD: substrate-binding domain; sHSP: small heat shock protein; SNARE: soluble NSF (N-Ethylmaleimide-Sensitive Factor) attachment protein receptor; SYNPO2: synaptopodin-2; TPR: tetratricopeptide repeat; UBA: ubiquitin-associated (domain); UPP: ubiquitin-proteasome pathway; UPS: ubiquitin–proteasome system; VPS18: vacuolar protein sorting-associated protein 18.

Introduction

Protein quality control (PQC) is an essential cellular function that is responsible for maintaining protein homeostasis [1-3]. Preserving protein stability has special significance within myocytes as muscle contractions increase heat and tension within cells [1,4-6]. The contractions induce thermal and oxidative stresses within myocytes, which make proteins more prone to becoming unfolded or mutated [3]. Chaperones play a role in cellular maintenance by inhibiting aggregation of non-native proteins and refolding misfolded proteins to their native state [1,2,5]. However, if a non-native protein cannot be remodeled, the chaperone system directs the substrate to the ubiquitin proteasome system (UPS) or the autophagy lysosome system [2,3,5-7]. These pathways maintain the stability of proteins within the cell by eliminating waste that can damage cellular structure and functions [2,7]. Short-lived misfolded proteins can be degraded in UPS, but when non-native proteins aggregate under acute stress, they are relocated to the autophagy lysosome system [3,7].

Chaperone Assisted Selective Autophagy (CASA), or BAG3-mediated macroautophagy, is a selective type of macroautophagy within the autophagy lysosome system that serves to maintain protein stability within myocytes, neurons, and other mechanically strained tissue cells [3]. The CASA complex consists of the co-chaperone BAG3, heat shock protein 70 (Hsp70), small heat shock protein B8 (HSPB8), E3 ubiquitin ligase CHIP, cytoskeletal motor protein dynein, autophagy receptor p62, and autophagosome-forming synaptopodin-2 (SYNPO2) [2,3,5]. Upon failure of a chaperone's refolding ability and the impairment of the ubiquitin proteasome pathway, CASA will be activated in order to degrade non-native proteins within the cell [1,3,6,8]. Maintaining protein homeostasis is essential within myocytes in order to prevent the accumulation of misfolded proteins, which can interfere with the cell's function and ultimately result in apoptosis [1,4-6]. Muscle contractions increase heat and tension within the cell, and as a result, the thermal and oxidative stresses within myocytes can cause proteins to become unfolded or misfolded through mutations [3]. The chaperone system works to prevent the excessive buildup of misfolded proteins by either assisting with refolding or directing the proteins towards the ubiquitin proteasome pathway or the autophagy lysosomal pathway [1,3,5,6]. The role of protein quality control (PQC) has been essential in preventing myopathy and neuropathy [3-5].

The Bcl2-associated athanogene (BAG) family of proteins are a group of co-chaperones that are essential to chaperone activity [1,9]. The BAG family includes six BAG proteins with the conserved BAG domain in each one [1]. These proteins are involved in many cellular pathways including apoptosis, macroautophagy, cytoskeleton organization, and motility [10,11]. Bcl2-associated athanogene 3 (*BAG3*) is a 575 amino acid co-chaperone from the BAG family that participates in the autophagy lysosomal pathway by interacting with Hsc70/Hsp70 and HSPB8 and similar small chaperones [1,3,4,9]. This protein has a WW domain involved in binding to diverse proteins, the PxxP motif responsible for microtubule-based retrograde transport, two Ile-Pro-Val (IPV) motifs involved in macroautophagy through interaction with small heat shock proteins (sHSPs), and the conserved BAG domain involved in apoptosis and the inhibition of proteasomal degradation [2,3,12,13] (Fig. 1). Within the CASA complex, Hsp70 binds to the BAG domain of BAG3 to redirect the misfolded proteins to the autophagy lysosomal pathway [5,9,10]. Similarly, HSPB8, along with other small heat shock proteins like HSPB6, binds to the two IPV motifs for the purpose of maintaining protein stability [1,2,4,9,14,15]. Dynein, a cytoskeletal motor protein, binds to the PxxP motif of BAG3 and facilitates the transportation of the CASA complex to the microtubule organizing center [5]. SYNPO2 binds to the WW domain and plays a role in autophagosome formation [12].

The interaction of BAG3 with chaperones is essential for chaperone activity [9]. Under physiological stress within the cell, the co-chaperone interacts with the chaperone Hsp70 as a cellular response to maintain protein homeostasis [1,3,5,13]. Hsp70 is part of the family of heat shock proteins (HSPs), which are ATP-dependent chaperones that are involved in the refolding of misfolded proteins within the cell [16,17]. HSPs consist of three domains including the N-terminal nucleotide binding domain (NBD), substrate-binding domain (SBD), and C-terminal region (CTR) [3,16]. Hsp70 also has an interdomain linker, which facilitates communication between the NBD and SBD, allowing for conformational changes in each domain [16]. The N-terminal region contains a nucleotide binding cleft for ATP and ADP binding, which dictates the protein's ability to bind to substrates [3,9,16]. BAG3 functions as a nucleotide exchange factor by regulating the ATPase cycle of Hsp70 [1,5,9]. The co-chaperone binds to the nucleotide binding domain and facilitates ADP to ATP exchange in order to release the refolded client and allow a new client to

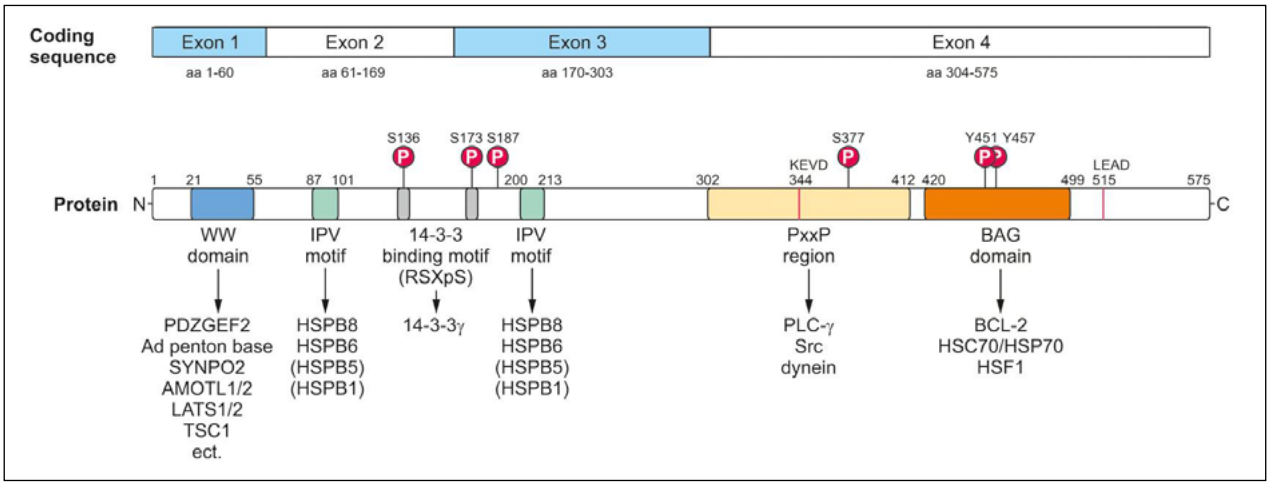

Figure 1. BAG3 consists of many domains, allowing the multifunctional protein to attach to various proteins and participate in several cellular functions. The different regions of BAG3 include the WW domain, two conserved IPV motifs, two 14-3-3 binding motifs, PxxP region, and the conserved BAG domain, which are encoded by four exons. The WW domain is located within the N-terminal region, while the conserved BAG domain is located within the C-terminal region. The phosphorylation sites of BAG3 have been marked in six locations at serine and tyrosine amino acids. The PxxP region contains a caspase cleavage site (KEVD) along with the C-terminal region (LEAD) (reproduced from the open source, from Stürner, Behl, 2017, mod.) [3].

enter the cleft [1,9,16,17]. BAG3 also interacts with the class of small heat shock proteins (sHSPs), which differ from other heat shock proteins since they function as "holdases" rather than enzymes [2,4,9,10]. In other words, sHSPs bind to and stabilize misfolded proteins, preventing aggregation [2,4,10]. There are ten proteins in the sHSP family (HSPB1 to HSPB10), which all contain the conserved alpha-crystallin domain (ACD), a flexible N-terminal region (NTR), and a C-terminal region (CTR) containing the IXI (IPV) motif [2,4-6]. BAG3 binds to the β4/β8 hydrophobic groove within the ACD of HSPB8 during autophagy [2,9].

CASA is prone to errors when the BAG3 gene contains a mutation, resulting in the production of a mutant BAG3 protein [3]. Mutations of proline in codon 209 within the second IPV motif have been identified to cause forms of myopathies, including dilated, distal, and peripheral neuropathy [1-3,13-15,18]. The P209Q, P209S, and P209L missense mutations have been associated with the development of myofibrillar myopathy [13]. Biopsies of patients with myopathy and peripheral neuropathy reveal the aggregation of misfolded proteins within the cells [13]. The proline 209 to leucine (P209L) BAG3 mutant has been specifically associated with the development of the clinical conditions Dilated Cardiomyopathy (DCM) and Charcot-Marie-Tooth (CMT) disease [11,13,19]. Patients with this disease are observed to have an accumulation of misfolded proteins in cardiomyocytes, which clinically leads to weakening of the heart muscle [3,11]. This toxic gain-of-function mutant is disrupting CASA, resulting in the aggregation of insoluble misfolded proteins and P209L mutants at aggresomes [13,20]. The relocation of the CASA complex to these aggresomes is preventing the degradation of misfolded proteins and reducing availability of essential proteins within the CASA complex [13].

Chaperone system

HSPB8 is known to serve as the "first line of defense" in the chaperone system that is activated in response to heat stress [2,17]. Functioning as "holdases," these ATP-independent chaperones form sHSP-substrate complexes [2,4,17]. These complexes have a different morphology and a smaller size compared to the aggregates that form when sHSPs are not present [17]. When a protein becomes denatured, its hydrophobic segments are no longer hidden deep within the 3D structure of the protein but instead become vulnerable to its surroundings [17]. As a result, HSPB8 is able to use its hydrophobic ACD to interact with the hydrophobic segments of denatured proteins [21]. This interaction serves to stabilize misfolded proteins and prevent them from aggregating before Hsp70 processing or degradation [4,17].

Subsequently, ATP-dependent Hsp70 displaces sHSPs from the sHSP-substrate complexes by competitively binding to the substrates [17]. It has been discovered that Hsp70 plays a role in permanently disassociating the complexes [17]. By outcompeting sHSPs in binding to the substrates, Hsp70 initiates protein refolding and contrib-

utes to disaggregation efforts within the cell [16,17]. Hsp70 and Hsp100, belonging to the AAA+ family ("ATPases associated with various cellular activities"), commonly work together in order to refold substrates from the sHSP–substrate complexes [8,17]. The scheme of these interactions is presented on Figure 2.

Chaperone-assisted selective autophagy

Formation of HSPB8-Hsp70-BAG3-CHIP complex

When the chaperones are unable to refold the denatured proteins back into their native state, these proteins need to be degraded before they interfere with the cell's function [1,2,5,22]. Ubiquitination is an essential process by which misfolded proteins can be selectively identified for degradation [22]. This post-translational modification involves tagging proteins that need to be degraded with the molecule ubiquitin, signaling the protein's degradation [22]. E3 Ubiquitin Protein Ligase CHIP is an Hsp70-interacting protein that is significant for ubiquitination [2,5,19,22]. CHIP is a member of the U-Box-containing E3 ubiquitin ligases family in which the conserved U-Box domain is responsible for the formation of multi ubiquitin chains [1,5,22]. The protein also contains an N-terminus tetratricopeptide repeat (TPR) domain, enabling E3 ubiquitin ligase to interact with chaperones like Hsp70 and Hsp90 [22]. CHIP binds to the CTR of Hsp70 and selectively ubiquitylates the substrates of the chaperone, directing the misfolded proteins toward Ubiquitin-Proteasome System (UPS) or Autophagy Lysosomal pathway (ALP) [1,2,4,22]. As a result, CHIP functions as a link between the proteasome and autophagy systems [1,2,22].

CASA requires the association of CHIP with chaperone Hsp70 and the ubiquitination of the chaperone's substrate [1,5,22]. The ubiquitination of protein aggregates recruits co-chaperone BAG3 and chaperone HSPB8 to the complex [19]. BAG3 binds to Hsp70 at its nucleotide binding domain, allowing it to regulate the ATPase cycle of the chaperone which controls substrate interaction [1-3,9]. The attachment of BAG3 to Hsp70 triggers ADP to ATP exchange, which changes the conformation of the substrate-binding domain of Hsp70 to an open state, promoting substrate release [2,3,9,16,17]. N-terminal conserved domain (J domain) proteins (JDPs) are responsible for ATP hydrolysis, which favors the closed state of the substrate-binding domain, allowing misfolded proteins to be held in place [3,9,16,17] (Fig. 3).

The co-chaperone BAG3 simultaneously binds to the hydrophobic groove in the ACD of HSPB8 and the NBD of Hsp70, but BAG3 does not directly bind to CHIP or the protein aggregate within the CASA complex [2,3,9]. Instead, these components are bound to Hsp70. Ubiquitination is also essential for autophagy receptor p62 recruitment at the microtubule-organizing center (MTOC) for phagophore expansion [1,5,14,15,23]. Co-chaperone BAG3 is crucial for the direction of the tagged substrates to autophagic degradation, and it competes directly with BAG1 for binding to Hsp70 [1,2,5]. Co-chaperone BAG1, also a member of the conserved BAG domain family, binds to

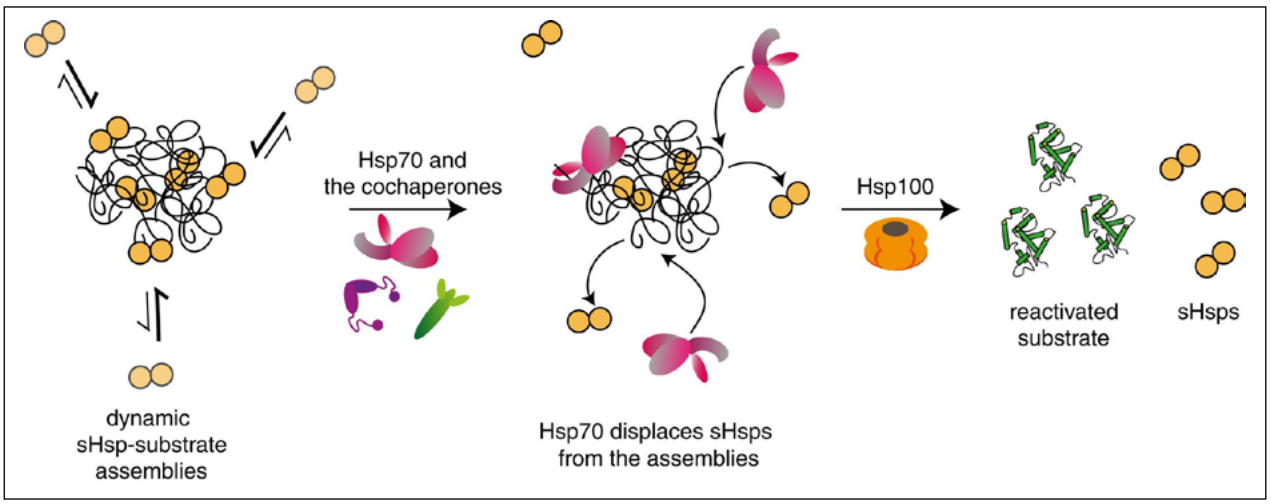

Figure 2. sHSP-substrate complexes are formed in which the outer surface consists of dynamic sHSPs in equilibrium shifted toward sHSPs being bound. These assemblies contain a stable sHSP-substrate center, which initially remains intact when Hsp70 and cochaperones displace the dynamic sHSPS in the outer surface. After successfully outcompeting the superficial sHSPs in binding to substrates, Hsp70 and Hsp100 identify the misfolded proteins and begin refolding, causing the sHSP–substrate assemblies to disassociate (reproduced from the open source, from Żwirowski et al., 2017, mod.) [17].

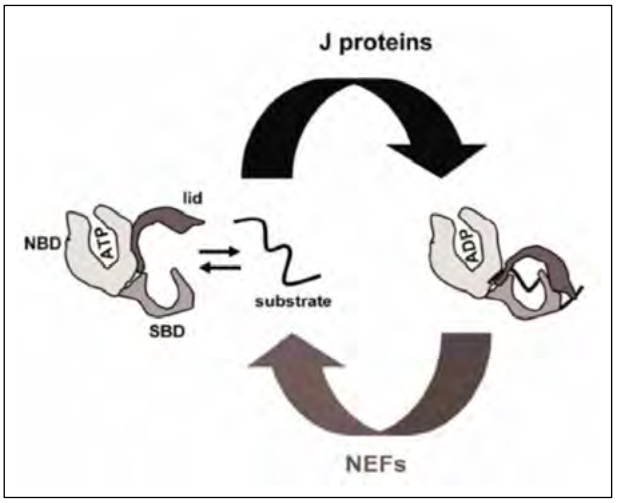

Figure 3. ATPase cycle of Hsp70. When ADP binds to Hsp70, SBD is configured to its closed conformation and has higher affinity for substrates. The lid domain closes onto the substrate that is bound to the SBD. NEFs catalyze the release of ADP and the binding of ATP to the NBD, which induces an open configuration of the lid domain. Hsp70 now has lower affinity for the substrate, leading to substrate release from the SBD. J-domain proteins are important for facilitating ATP hydrolysis to produce ADP. The ADP-bound-state once again configures for the lid domain to close upon a new substrate within the cleft (reproduced from the open source, from Kabani, Martineau, 2008, mod.) [24].

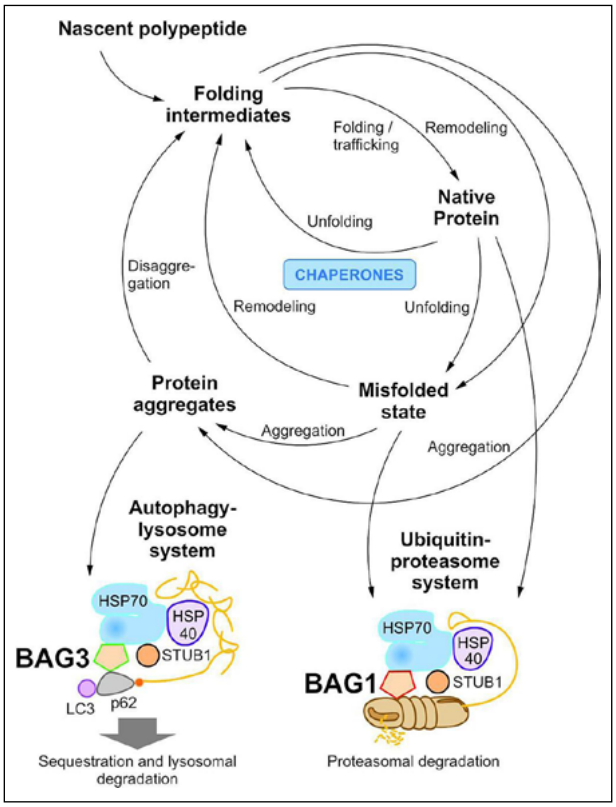

Figure 4. Under proteotoxic stress conditions, native proteins can become unfolded into a misfolded protein state. Molecular chaperones, such as the HSP family, bind to the misfolded proteins inhibiting aggregation and initiating refolding. If successfully remodeled, the misfolded protein will become a native protein. If refolding fails, non-native proteins may also be degraded by UPS, involving the binding of BAG1 to the Hsp70-substrate complex. Upon aggregation of misfolded proteins, protein aggregates are directed to the autophagy system with the recruitment of BAG3 (reproduced from the open source, from Stürner, Behl, 2017, mod.) [3].

Hsp70-CHIP complexes in a similar fashion as BAG3 and transports ubiquitinated substrates for proteasomal degradation [1,2,5,22]. Removal of proteins by proteasome is most common under physiological conditions in which UPS maintains PQC within the cell [1,25]. However, upon acute stress, the proteasomal pathway is inhibited as it is ineffective at degrading insoluble and larger protein aggregates [1,7]. As a result, during proteasome inhibition, BAG3 is upregulated and facilitates removal of protein aggregates by autophagy, preventing the accumulation of more aggregates [1,2,7,8,14,15]. This inverse relationship between the expression of BAG3 and BAG1 is known as the "BAG1–BAG3 switch" [1,2,7]. Overall, when cells are under oxidative stress or heat, the expression of BAG3 increases, causing misfolded proteins to be directed towards CASA more frequently [1,5,7,8,14,15] (Fig. 4).

Complete assembly of CASA complex

After HSPB8, BAG3, and Hsp70 have assembled, the misfolded proteins need to be transported to the Microtubule-Organizing Center (MTOC) located near the nucleus of the cell [3,5,8]. Substrates for autophagy are transported and sequestered at the MTOC, where autophagosome nucleation occurs [3,5]. Therefore, the relocation of substrates to MTOC allows for the efficient removal of the insoluble proteins in a space concentrated with autophagosomes [13]. The HSPB8 BAG3 Hsp70 complex interacts with the dynein motor complex in order to be transported along microtubules to the MTOC [3,5,8,25]. First, the 14-3-3 adaptor protein binds to BAG3 in regions RSQS136 and RSQS173 (between two IPV motifs) and it functions as a bridge between BAG3 and motor protein dynein [3,8,25]. 14-3-3 adaptor protein is from a family of multifunctional, cytosolic, ubiquitous proteins that bind to over 200 different proteins [25]. It is important for PQC due to its role in transporting the CASA complex via microtubules [25]. 14-3-3 proteins form homo- and hetero-dimers with monomers arranged in an antiparallel direction [25]. As a

result, in a 14-3-3 dimer, the ligand binding grooves of both monomers are on opposite ends, allowing more than one protein to bind to the dimer [25]. Therefore, the adaptor protein binds to BAG3 while simultaneously binding to dynein [3,25].

Dynein, a member of a family of cytoskeletal motor proteins, hydrolyzes ATP, generating a force that will move cargo protein towards the minus-end of a microtubule [26]. As a result, dynein facilitates the retrograde transport of BAG3 and its cargo protein along microtubules to the MTOC [3,5,8,25]. At the MTOC, autophagy receptor p62 binds to the ubiquitin chain on the misfolded protein [3,5,23]. p62/SQSTM1 (sequestosome 1) is a multifunctional protein and an autophagy substrate that delivers misfolded proteins to both UPS and lysosomal autophagy [23]. p62 is involved in many other cellular pathways including cell signaling, cell division, and redox processes [27]. Upon inhibition of UPS, the expression and phosphorylation of p62 significantly increases, which allows the receptor to bind to ubiquitinated proteins and deliver them for degradation via autophagy [23] (Fig. 5). p62 is degraded along with other misfolded proteins in autophagy, and as a result, impaired autophagy restricts p62 and ubiquitinated protein degradation [5,23]. The autophagy receptor uses its C-terminal ubiquitin-associated (UBA) domain to interact with ubiquitin chains on misfolded proteins, and then delivers these proteins to either proteasomes using its PB1 domain or autophagy using its LC3-interacting region (LIR) [2,5,23,28] (Fig. 5).

Additionally, autophagosome-forming SYNPO2 attaches to the WW domain of BAG3, completing the assembly of the CASA complex [12]. BAG3 and SYNPO2 interacting is important for the maturation of phagophores and the fusion of autophagosomes and lysosomes [12]. SYNPO2 uses its PDZ domain to physically connect BAG3 client complexes with membrane fusion complexes in autophagosomes [12]. Overall, p62 and SYNPO2 both allow for CASA complexes to interact with autophagosomes for protein degradation [12].

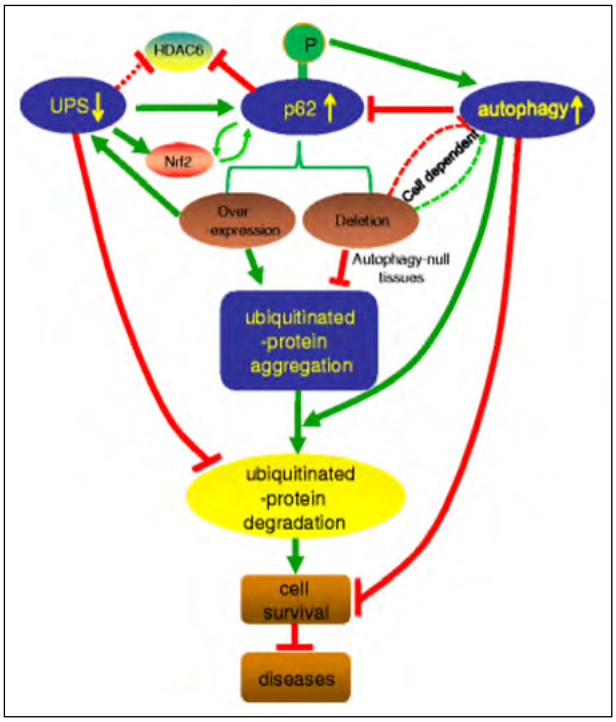

Figure 5. p62 overexpression protects the proteasome stability of a cell by increasing the number of ubiquitinated protein aggregates that are sent to the autophagy lysosomal pathway. A deficiency in UPS decreases the number of ubiquitinated proteins degraded by UPS, leading to increased p62 expression and phosphorylation on S405 and S409. Upon UPS inhibition, the levels of Nrf2 increases, inducing p62 overexpression. The greater concentration of p62 better enables it to compete with Nrf2 for Kea1, forming p62-Keap1 complexes, which assist with the formation of ubiquitinated protein aggregates for degradation by autophagy. HDAC6 is critical in the autophagy lysosomal pathway, but its synthesis is directly inhibited by UPS inhibition and p62 overexpression. However, p62 overexpression can be inhibited by an increase in autophagy. Therefore, the ratio of p62 to HDAC6 is essential for cellular stability (reproduced from the open source, from Liu et al., 2016, mod.) [23].

Autophagosome and autolysosome formation

Once the entire CASA complex has been assembled, autophagosome nucleation is initiated at MTOC, enabling the misfolded proteins to be degraded in autolysosomes [23,28]. An autophagosome is a spherical double-layered structure that is responsible for engulfing damaged cellular components and fusing with lysosomes (autolysosome) to break down the components [3,28]. Prior to autophagosome formation, phagophores, crescent-shaped double membrane that fuses to form a double-membrane vesicle, must form [3,28]. A phagophore is formed by vesicle precursors that fuse together into a double-membraned sac coated with LC3-II proteins, which are common biomarkers for autophagosomes due to their role in substrate selection and autophagosome formation [1,5,12,23,29]. LC3 proteins are accessible in two forms in the cell: LC3-I is in the cytoplasm and assists with the elongation of the autophagosome during formation and LC3-II is membrane-bound and helps with docking cargo proteins to the autophagosome for degradation [23,28-30].

Autophagosome formation involves triggering the expansion of a phagophore (premature autophagosome) via interaction between SYNPO2 and VPS18 and SNARE containing protein complex, which are membrane fusion machinery attached to phagophores [12,31] (Fig. 6). SNARE

proteins are known to mediate vesicle fusion into a phagophore by connecting the two membrane sites [31]. VPS18 is a subunit from the homotypic fusion and protein sorting (HOPS) complex that functions as a membrane tether by facilitating the docking and fusion process of autophagosomes [12,31]. Attached to BAG3, SYNPO2 initiates autophagosome nucleation by interacting with this membrane fusion machinery on the phagophore [12]. Autophagy receptor p62 is responsible for delivering the cargo protein to the autophagosome for degradation [1,5,23,28]. p62, bound to the ubiquitin chain of the misfolded protein, loads the cargo protein into the autophagosome by attaching itself to membrane-bound LC3-II on the phagophore [1,2,5,23,28]. After this interaction, p62 and the ubiquitinated misfolded protein detach from the CASA complex and bind to the maturing phagophore using LC3-II [1,2,5,23]. Once the phagophore has fully transformed into an autophagosome, p62 and the misfolded protein are sequestered in the autophagosome, ready for degradation [1,3,5,14,15]. Eventually, the autophagosome will fuse with a lysosome, forming an autolysosome [1,3,14,15]. Inside this structure, the hydrolytic enzymes of the lysosome will degrade the misfolded protein and p62 [1,3,5]. The CASA complex will disintegrate into its individual proteins until it's signaled to form again by the presence of protein aggregates. The proper functioning of this pathway is needed to maintain the structure of the Z disc by regulating the removal and production of filamin within cardiomyocytes [1,3,14,15,28] (Fig. 6). Without sufficient degradation, protein aggregates would accumulate within the cell and interfere with its functions, eventually leading to cell death [3,5]. The complete scheme of interactions in the functional CASA pathway is depicted in Figure 7.

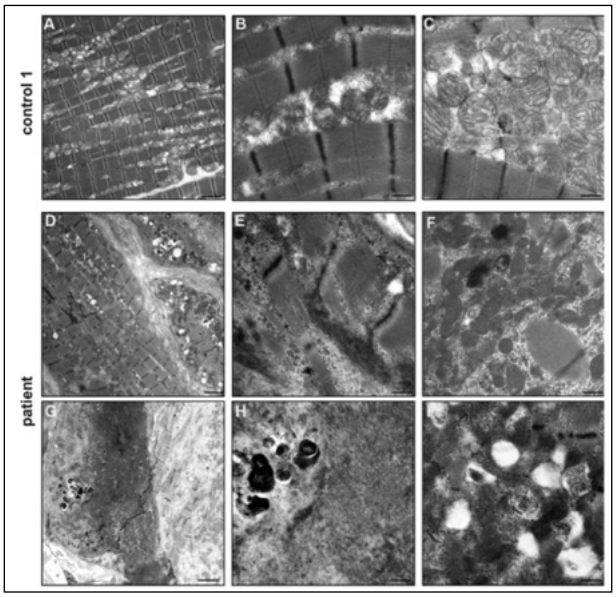

Figure 6. Ultrastructural analysis of cardiac tissue from control 1 (A-C) and BAG3 P209L-mutant patient (D-I). (D) BAG3-mutant cardiac tissue has altered Z-disk structure and extreme myofibrillar disintegration with cells of varying diameters and vacuoles. (E) Recurring Z-disk alterations and substantial electron dense material originating from Z-disks. (F) Central accumulation of mitochondria and visible lipofuscin granules. (G,H) Bundles of electron-dense material are abundant and partly surrounded by electron dense vacuoles. (I) In spaces between myofibrillar bundles, empty vacuoles and glycogen or electron dense vacuoles are present. Scale: 2.5 µm (A,D,G) and 0.5 µm (B,C,E,F,H,I) (reproduced from the open source, from Schänzer et al., 2018, mod.) [33].

The effect of BAG3 P209L mutant on CASA

The BAG3 P209L mutant is inhibiting the cell's innate response to protein instability, resulting in cells with an abundance of protein aggregates [11,20]. Specifically, the mutant is preventing the delivery of misfolded proteins to the autophagosomes at MTOC, causing the accumulation of these proteins as well as the unavailability of CASA components [11,13,19,20]. It has been discovered that the point mutation in codon 209 of the second IPV motif is not impairing the ability of BAG3 to interact with other proteins in the CASA complex [13,32]. In vitro, human studies found that mutant BAG3 retains its ability to bind to HSPB8 and Hsp70 but has reduced binding affinity for HSPB8 [13,32]. One such study found an increase in the interaction between autophagy receptor p62 and the CASA complex with mutant BAG3 although there is no direct interaction between the mutant and p62 [13]. These results suggest that the entire CASA complex is able to fully assemble with or without the P209L mutant [13]. It is unclear whether BAG3 P209L can impair autophagosome formation. A mice model of BAG3 variants observed higher levels of SOD1_G923A in the insoluble fraction of cells with Pro209 mutation compared to other BAG3 variants, indicating that the CASA complex recognizes misfolded proteins but is unable to degrade them [13]. Despite the proper assembly of the CASA complex, cells with mutant P209L are associated with the clustering of misfolded proteins into insoluble aggregates [11,20,32]. It appears that the complexes with mutant BAG3 are unable to deliver the cargo protein to the autophagosomes, significantly decreasing the effectiveness of CASA [19,32]. The accumulation of CASA complexes at MTOC results from the inefficient degradation of misfolded proteins [13]. As a gain-of-function mutation, the proline to leucine alteration in codon 209 is known to decrease the solubility of BAG3, as observed in human, in-vitro studies, and therefore may be responsible for the aggregation of BAG3 and other misfolded proteins [13,20]. BAG3 co-aggregates with other components in

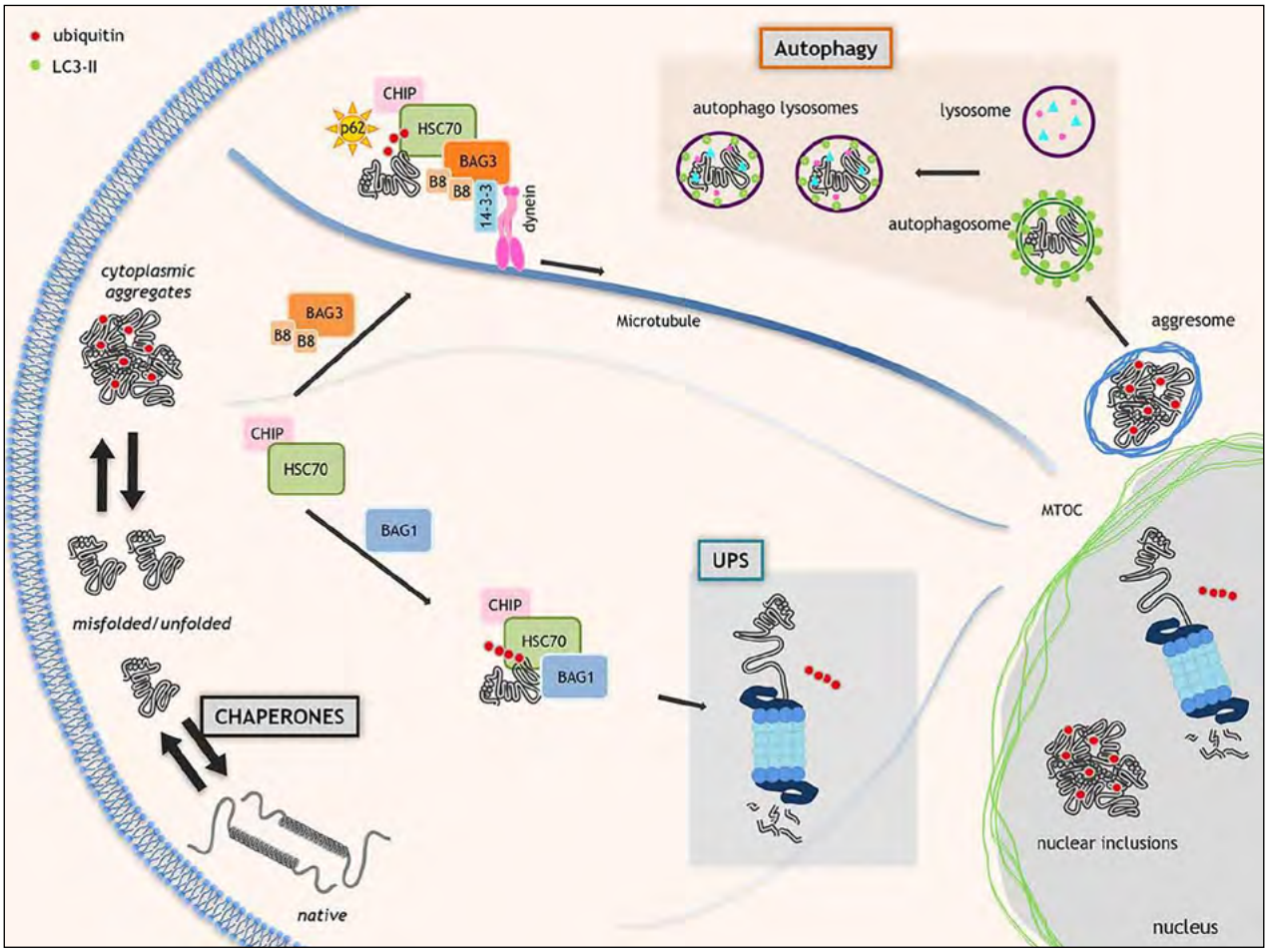

Figure 7. Protein quality control in neurons and other mechanically strained tissue cells. Upon the misfolding or unfolding of a protein, molecular chaperones bind to these non-native proteins and facilitate the process of refolding. However, if refolding is unsuccessful, the HSC70 chaperone is responsible for routing the misfolded protein to one of two pathways for degradation. The binding of BAG1, a nucleotide exchange factor from the Bcl2-associated athanogene family to the HSC70 CHIP complex allows for the inhibition of HSC70's chaperone activity and the polyubiquitination of the client by CHIP. This interaction directs the misfolding protein to UPS, in which a proteasome breaks down the client. Conversely, the binding of BAG3 and HSPB8 to the complex reroutes the substrate to CASA. In this pathway, 14-3-3 protein and dynein are responsible for facilitating the transportation of the CASA complex along microtubules to MTOC, site of autophagosome formation. Here, autophagy receptor p62/SQSTM1 attaches to the polyubiquitin chain of the misfolded protein and directs the client to the autophagosome through its interaction with the LC3 receptor. The misfolded protein is degraded once a lysosome fuses with the autophagosome, forming an autolysosome (reproduced from the open source, from Cristofani et al., 2019, mod.) [5].

the CASA complex, contributing to the large aggregates found within affected cells [11,13.32]. It has been determined that the collective aggregation of PQC proteins can be attributed to the interaction between BAG3 and Hsp70 as eliminating the BAG domain ceases the accumulation of misfolded proteins [13,32] (Fig. 7).

In addition to the excessive formation of aggregates, the P209L mutant condition is also classified with trapping PQC proteins, including Hsp70 and HSPB8, into aggresomes [13,32]. Aggresomes are a cellular response to the overload of misfolded proteins that involves the formation of circular bodies that hold protein aggregates in an attempt to concentrate them in one location [13]. These inclusion bodies can be toxic to the cell when they accumulate, which can ultimately lead to cell death. Besides storing misfolded proteins, aggresomes in human cell lines with mutant P209L BAG3 have also been shown to relocate BAG3 and other essential chaperones, reducing the number of available chaperones in the cell [13,32]. As a result, misfolded proteins have less access to functional chaperones, promoting unregulated aggrega-

tion [13,32]. One possible explanation for the formation of insoluble aggregates and sequestration of PQC components is that BAG3 is unable to properly function as a nucleotide exchange factor [13,34]. As previously mentioned, BAG3 is responsible for the ADP to ATP exchange within the ATPase cycle of Hsp70, which signals client release [9,13]. When ADP is bound to the nucleotide binding cleft in the NTR, P209L mutant BAG3 may not be able to exchange the nucleotide for ATP, resulting in no conformational change to the open state of the substrate-binding cleft [13,16]. As a result, the continuous closed state of this cleft will not allow the misfolded protein within the SBD to be released into the autophagosome, disrupting the process of CASA [13]. This may delay the dissociation of PQC components in the CASA complex, and ultimately increase the likelihood of the complex becoming engulfed by an aggresome [13] (Fig. 8).

HSPB1 IPV motif, Charcot-Marie-Tooth disease, and their association with dilated cardiomyopathy

Charcot-Marie-Tooth (CMT) disease is the most common form of inherited peripheral neuropathy, a group of neurodegenerative diseases characterized by damaged motor and sensory neurons in the peripheral nervous system [35-38]. More than 80 genes with mutations linked to CMT disease have been identified, contributing to the diversity of the genetic disorders classified as CMT disease [39]. Individuals with CMT disease experience sensory and motor deficits that progress over time [35,36,40,41]. CMT can be divided into two types, CMT1 and CMT2: CMT1 (demyelinating CMT) is caused by impaired myelin sheath while CMT2 (axonal CMT) is caused by damaged neuronal axons [35-37,40,42,43]. Mutations within the genes of small heat shock proteins, including HSPB1, HSPB8, and HSPB3, are commonly associated with causing CMT2 or distal hereditary motor neuropathy (dHNM) [37,39-45]. CMT2 disease-causing mutations within the sHSP family affect self-interaction among sHSPs, impairing oligomerization and the chaperone capacity of these chaperones [36,42,46].

Like BAG3, HSPB1 has a conserved IPV (also called IXI) motif located within its C-terminal region that also interacts with the β4/β8 groove of another HSPB1 dimer [6,44,46-48]. Mutations in HSPB1 are known to cause Charcot-Marie-Tooth disease type 2 (CMT2), but the mutation proline 182 to leucine (P182L) within the IXI motif is similarly associated with protein insolubility and protein aggregates [38,41,42,45,49,50]. The HSPB1 P182L variant is

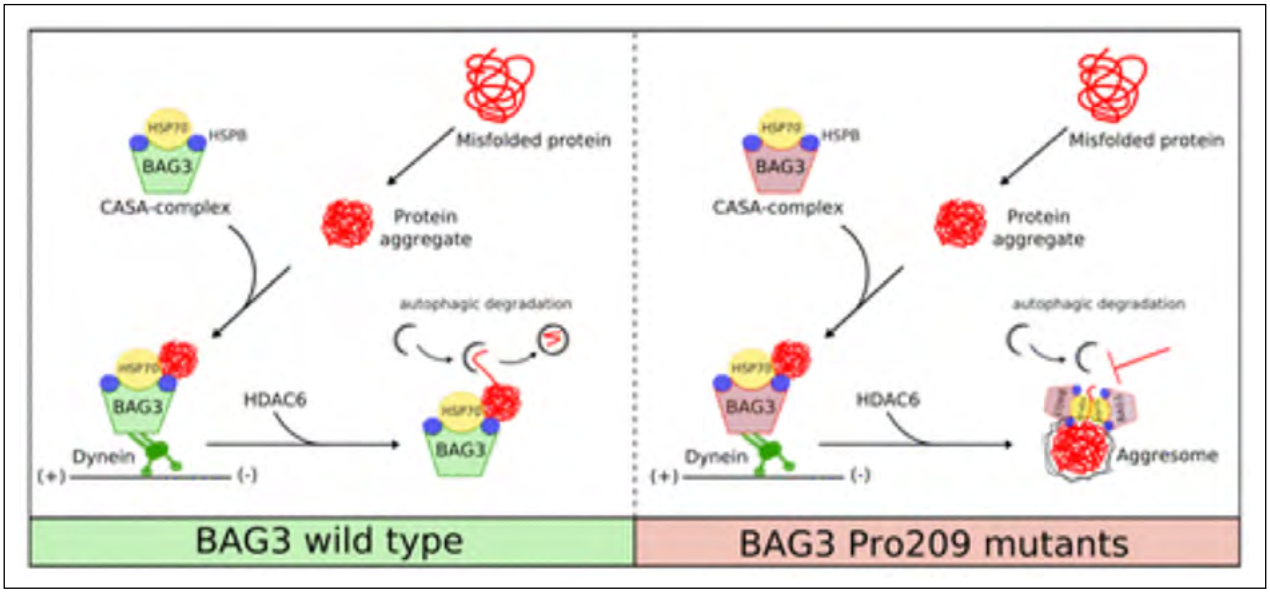

Figure 8. In the presence of BAG3 wild type, the CASA complex assembles, attaches to a protein aggregate, to an autophagosome in MTOC, where the aggregate becomes degraded. The P209L mutation increases the aggregation propensity of BAG3 and may impair the ATPase cycle of Hsp70, causing Hsp70 to be unable to release the protein aggregate. As a result, the protein aggregate may remain bound to the CASA complex and not be delivered to an autophagosome, leading to the accumulation of CASA complexes in MTOC. These complexes become relocated to aggresomes, decreasing the accessibility of essential and functional proteins, including Hsp70, HSPB8, and p62 (reproduced from the open source, from Adriaenssens et al., 2020, mod.) [13].

classified as rare but severe as onset occurs within the first five years of life compared to adult onset of HSPB1 variants with mutations in the ACD [42,49]. It was discovered that the P182L variant promotes aggregation of client proteins and overexpression can lead to the formation of aggresomes with HSPB1 [38,45,49,50]. This may lead to speculation of whether there is a possible similarity between DCM, caused by mutant P209L BAG3, and CMT2, caused by mutant P182L HSPB1.

Self-interaction among small heat shock proteins

Point mutations associated with CMT2 within sHSPs may have toxic effects on self-interaction and self-assembly, which are considered unique and essential properties among the sHSP family [41]. All sHSPs form dimers and/or oligomers through intradimer or interdimer interactions [46-48]. sHSP oligomerization is dynamic with each subunit in equilibrium between dimeric and oligomeric states, enhancing sHSP chaperone activity [21] (Fig. 9).

HSPB1 and HSPB5 are known to form larger homooligomers (oligomers formed from dimers of the same protein) compared to the rest of the sHSP family which form smaller protein structures [6,46,47]. Heterooligomeric complexes (oligomers formed from dimers of different proteins) can be formed as well, especially between HSPB2 and HSPB3, HSPB5 and HSPB6, and HSPB1 and HSPB5 [6,40,46,47]. sHSPs common to CASA, HSPB6 and HSPB8, mostly exist as homodimers due to their lack of an IXI motif but can selectively bind to dimers of other sHSPs [6,46,47].

The structure of a monomer in a small heat shock protein consists of three domains: NTR, ACD, and

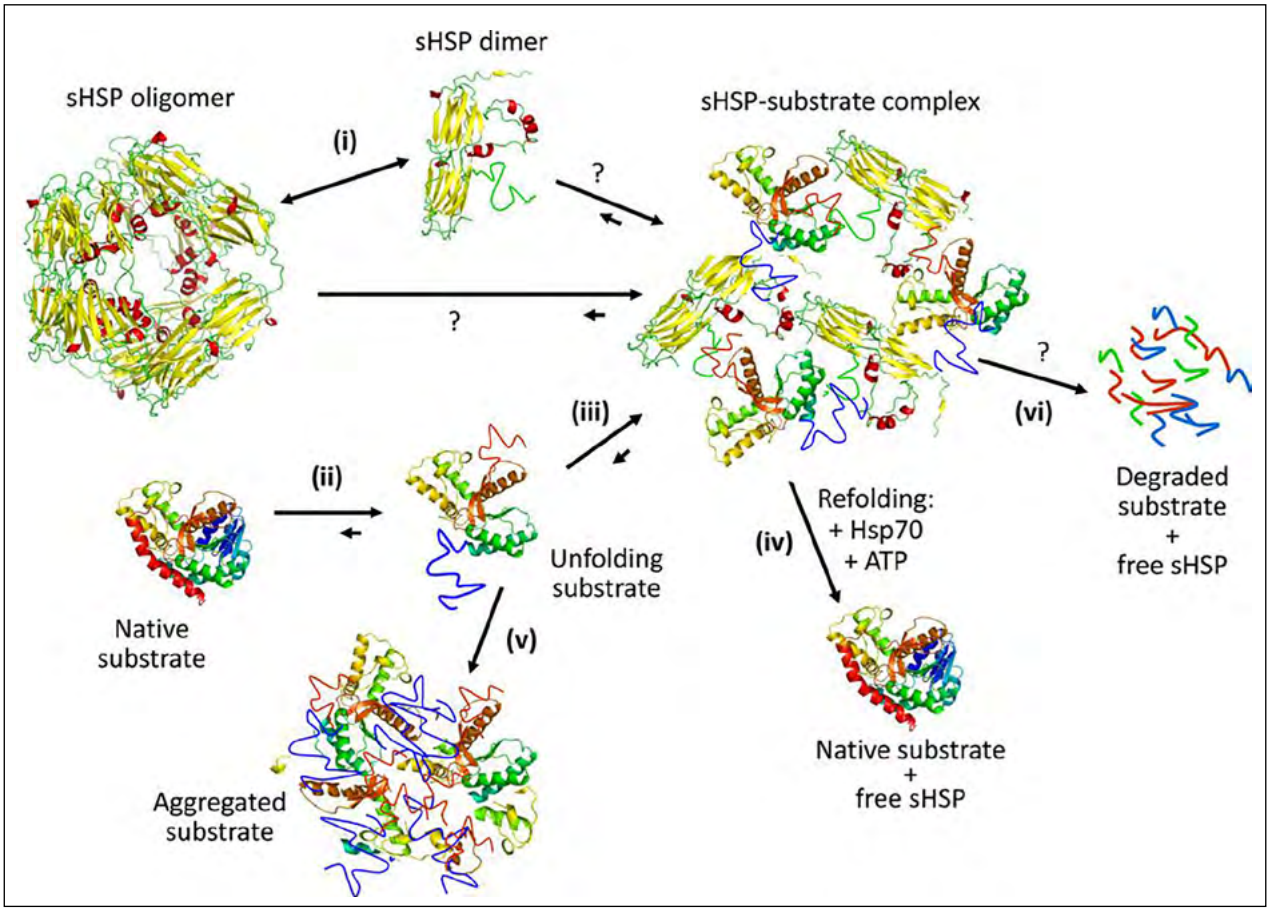

Figure 9. Model for sHSP chaperone activity. (i) sHSP dimers and monomers continuously alternate between being bound in an oligomer or released into the environment. (ii) Misfolded and unfolded proteins expose hydrophobic regions to which sHSPs bind using a hydrophobic groove in the ACD, forming large substrate-sHSP complexes (iii). (iv) The denatured substrate can be refolded by Hsp70, ATP, and a co-chaperone which displace the sHSP-substrate complex and attach to the substrate. (v) Lack of adequate sHSPs can result in the formation of protein aggregates, which are harder to eliminate. (vi) Cellular proteases can also degrade the substrate from an sHSP-substrate complex, but this pathway is less well-known. This model does not elucidate all possible sHSP proteins actions (reproduced with the permission of the publisher, from Basha et al., 2012, mod.) [21].

CTR [6,21,44,46-48]. The disordered NTR and CTR have binding sites that adhere to the grooves of the structured and conserved ACD [6,21,44,47]. Competition for binding to the grooves within ACD is high but each interaction is weak, creating "tethered" and "untethered" states among the disordered regions [6,46]. ACD is known to serve as a binding region for many different proteins, including BAG3, as well as segments of other sHSPs [6]. There are three main types of self-interactions that the ACD facilitates: ACD ACD, CTR ACD, and NTR ACD [6,21,44,46-48]. These interactions are essential for oligomer assembly and subunit exchange, which contribute to chaperone activity [21,44,46]. ACD to ACD interaction forms the basis of dimerization among all sHSPs [6,21,44,46-48]. The ACD β6/β7 strands of two monomers weakly interact in an antiparallel alignment [6,21,46,48]. The formation of a sHSP dimer creates two β4/β8 groove binding sites for the NTR and CTR segments of sHSPs or for aggregation-prone proteins, with each groove located within a monomer [6,21,46,48]. The hydrophobic chains within these grooves interact with the hydrophobic regions of the denatured proteins, reducing their propensity for aggregation until the proteins can be refolded or degraded [21]. The structure of the identical edge grooves are distinctive because the compacted side chains within the β4 and β8 strands form a binding site that looks like two holes separated by a wall [6]. In addition, the monomer-to-monomer interaction among sHSPs forms one dimer interface groove near the β6 and β7 strands [6,24,46] (Fig. 10).

The CTR is a less conserved region of small heat shock proteins that can serve as a "solubility tag" [38,46]. Its length varies from protein to protein, but it shares the common features of being flexible and disordered [44,46,49]. Some sHSPs contain a short IXI motif within the CTR that is most commonly associated with the formation of higher order oligomers [6,44,46,47]. HSPB1, HSPB2, HSPB4, and HSPB5 are all known to have this 3-letter sequence, allowing them to assemble into larger homoligomers due to the motif's crucial role in oligomerization [6,46,49]. CTR to ACD interaction takes place at the inter-dimer level, meaning from the IXI motif of one sHSP dimer to the ACD of another dimer [6,44,47,49]. Due to the knob-like structure of the β4/β8 groove, the IXI motif binds to the edge groove of the ACD in a knob-into-hole fashion [6,46]. Besides forming larger order oligomers, the CTR ACD interaction is also known to recruit and exchange monomers within an oligomer [44,46,48,51] (Fig. 10). One study divided this process into two steps [51]. First, the unbound CTRs within a sHSP oligomer bind to the β4/β8 groove of a subunit within a free dimer [44,51]. Then, the free dimer dissociates into two monomers [51]. One of the dimers within the oligomer dissociates and forms a heterodimer with a subunit from the new dimer [51]. Therefore, the CTR's IXI

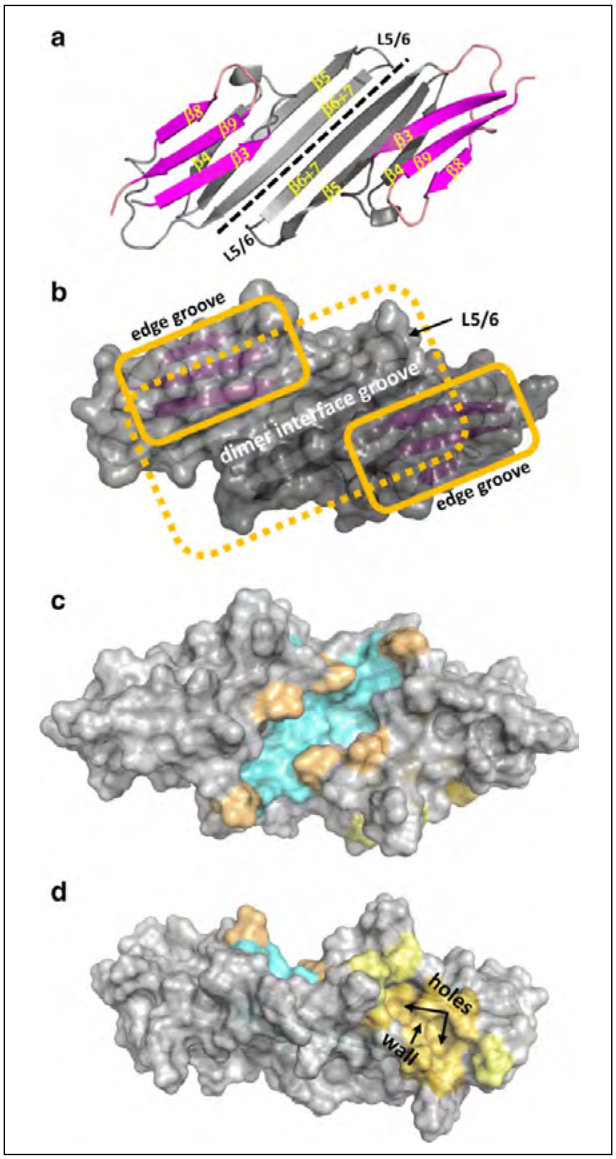

Figure 10. Structure and grooves of alpha-crystallin domain (ACD) within sHSPs. (A) ACD dimer with β-strand and L5/6 loops connecting β5 strands to β6+7 strands. The dashed line represents the dimer interface. The β-strand is divided into a "bottom" sheet with 6β strands (in gray) consisting of β4, β5, and β6+7 from both sHSP monomers [6]. The "top" sheet is composed of 3β strands (in magenta), including β3, β8, and β9 [6]. (B) Surface front view of the dimer interface groove formed by the β6+7 strands. The L5/6 loops on either side of the groove are in "loop-up" position [6]. The 6-stranded β sheet is indicated by the dashed yellow line and the 3-stranded β sheets are indicated by the solid yellow line. (C) Color coded portions of the dimer interface groove from the same front view. Cyan residues represent the floor of the groove and buff residues represent the sides of the groove. (D) Top view of ACD structure shows hydrophobic β4/β8 groove (edge groove) with the two holes and wall identified (reproduced from the open source, from Klevit, 2020, mod.) [6].

motif interacts with the hydrophobic groove of the ACD, which is essential for oligomerization and monomer exchange [51] (Fig. 11). This study's conclusions and models were made based on observations from the expression of sHSP constructs in *Escherichia coli* and evidence from previous studies [51]. As a result, it is important to note that there are limitations in applying the process described above to humans. Small heat shock proteins that lack the IXI motif in the CTR, like HSPB6 and HSPB8, mostly exist as small homodimers, but they can also form heterooligomers with other sHSPs that have the IXI motif [6,9,51].

The NTR of sHSPs is longer than the CTR, and as a result, it has more well-defined regions that bind to the ACD's hydrophobic grooves [6,44]. One study divided the NTR into six regions with the distal region, aromatic region, conserved motif, tryptophan-rich region, insertion, and boundary region [46]. These regions were defined by applying hydrogen deuterium exchange mass spectrometry (HDXMS) to human HSPB1 dimer and nuclear magnetic resonance (NMR) to short segments of NTR, which revealed specific interactions between the ACD and NTR [46]. NTR interactions with the ACD include the distal region bound to the edge groove, the aromatic region bound to L3/4 and L5/6, and the conserved and boundary regions bound to the dimer interface groove [6,46]. Due to the highly disordered state of the NTR, this domain is able to bind to the ACD at the intramonomer, intradimer, and interdimer levels [46,47] (Fig. 12). The NTR has been identified as crucial for oligomerization due to its flexible interactions [21,44,46-48]. Additionally, this domain exists in a bound and unbound state, which may regulate client binding among sHSPs [6,46]. The tethered state is proposed to restrict the binding of misfolded proteins and limit chaperone function, while the untethered state may enhance chaperone function by allowing client proteins to bind [6]. A schematic of the role of sHSP self-interactions in oligomer assembly is depicted in Figure 13.

Mutations in HSPB1 and their effect on protein stability

Seven to eight percent of mutations causing CMT2 are located in HSPB1, which equates to about 30 HSPB1 mutants [50]. In-vitro and in-vivo studies of HSPB1 mutants have noted the formation of larger HSPB1 oligomers, whether the mutation is located in the NTR, CTR, or ACD [36-38,41,49,50]. All mutants are more likely to aggregate at lower temperatures [50]. Larger HSPB1 oligomers are only formed at higher concentrations when the mutation is located in the ACD [37]. At lower concentrations, HSPB1 ACD mutants are known to disassociate from the oligomeric structure [37]. Additionally, HSPB1 has three sites of phosphorylation in its NTR that are associated with the dissociation of an HSPB1 oligomer [50,52] (Fig. 14). In-vitro and mass-spectrometry studies have discovered that phosphorylation-induced disassembly is resisted by HSPB1 NTR mutants, whereas HSPB1 ACD mutants more readily dissociate upon phosphorylation compared to wild type HSPB1 [50,52]. This can be explained by the fact that the NTR of HSPB1 is phosphorylated in response to stress and serves as an instigator of oligomer disassembly [50,52]. The larger oligomeric structure of wild type HSPB1 dissociates into smaller complexes with increased availability to bind to misfolded proteins and therefore increased chaperone function [41,50,52]. However, when HSPB1 has a mutation in the NTR, the stress-induced phosphorylation is disrupted, which prevents the reduction in oligomer size [50]. As a result, the β4/β8 groove will likely remain occupied and not be able to bind to as many misfolded proteins [52]. Lastly, mutations in any domain alter the protein structure of HSPB1, leading to a change in chaperone capacity [49].

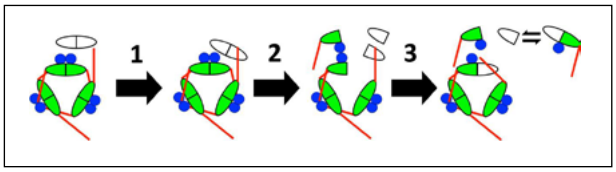

Figure 11. Model for subunit exchange within sHSPs. Unbound ACD dimer is white, and within the oligomer, NTR is blue, CTR is red, and ACD is green. (1) Free CTR within the oligomer recruits an unbound ACD dimer to the oligomer. (2) A subunit within the oligomer and the recruited subunit dissociates. (3) A heterodimer is formed with one new subunit and one original subunit (reproduced from the open source, from Delbecq et al., 2015, mod.) [51].

P182L-induced protein aggregation

Like other HSPB1 mutants, in-vitro and in-vivo studies have established that the P182L mutant forms larger oligomeric complexes than wild type HSPB1 and other HSPB1 mutants do [38,41,42,49]. These massive structures may contain only P182L mutants or possibly a combination of mutant and wild type HSPB1 [42]. Despite the formation of larger structures, a lower affinity between the IXI motif and ACD has been observed in P182L mutants [38,42]. One study noted that the amino acid Pro182 in the wild type contributes to a rigid binding-friendly conformation more often than the mutation with codon Leu 182 [42]. As a result, lack of this rigidity has made it easier for the P182L mutant to escape from the binding-friendly conformation, which lowers the interaction between IXI motif and ACD [42]. This conclusion was reached using 200 ns of all-atom molecular dynamics (MD) simulations performed on fragments of wild type and P182L HSPB1 [42]. Another paper explained the contradiction of a lower binding affinity yet a larger oligomeric structure through discoveries of oligomerization at the molecular level [38,42].

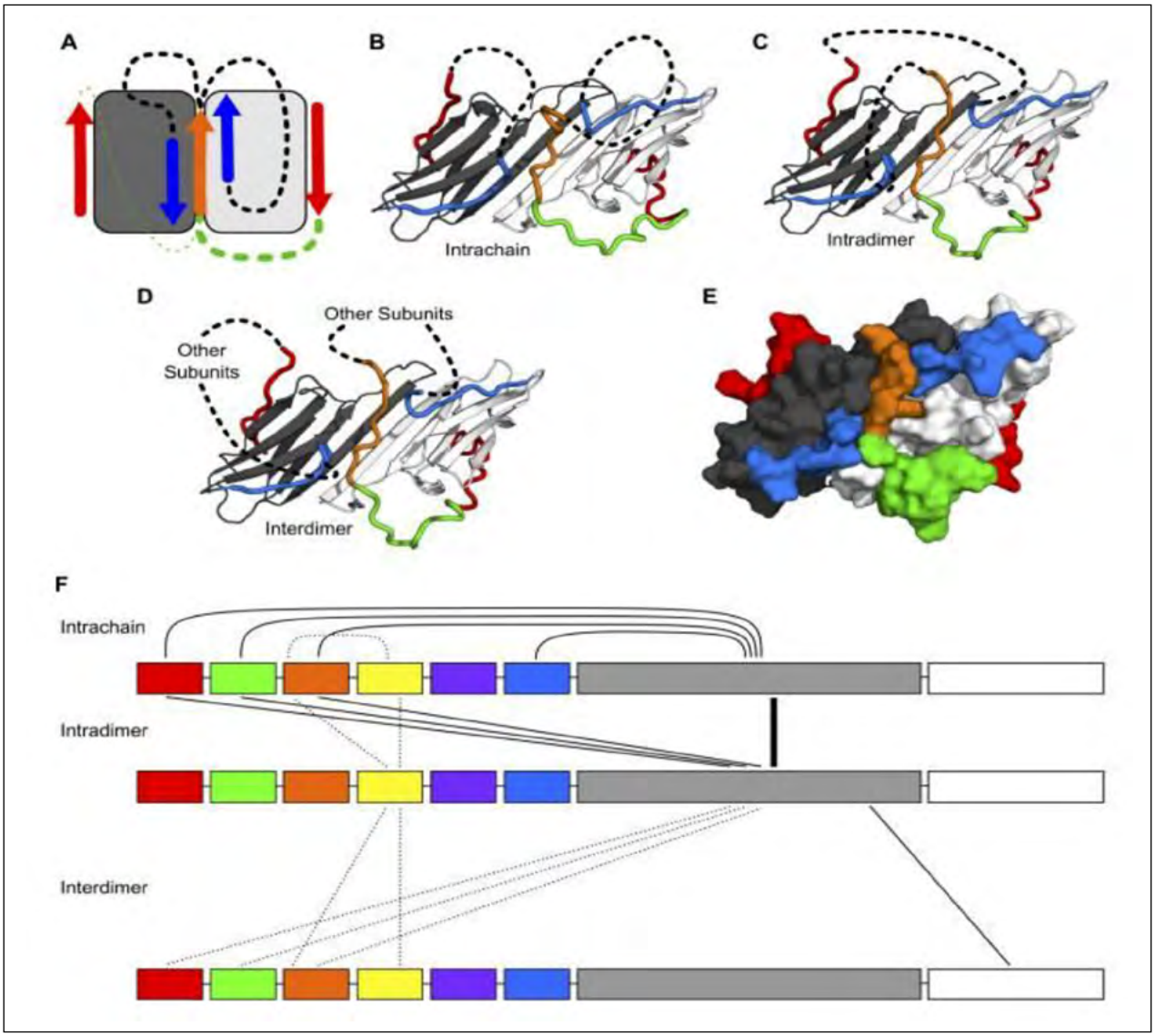

Figure 12. Representation of the NTR-ACD interactions. (A,B) intrachain, (C) intradimer, and (D) interdimer interactions between sHSPs. (E,F) Structure of NTR with distal region in red, aromatic region in green, conserved region in orange, Trp-rich region in yellow, insertion region in purple, and boundary region in blue. ACD is in gray and CTR is in white. Interactions indicated by solid lines are supported by evidence from the study and the dotted lines are interactions that the researchers "believe are likely to occur" [46]. This figure demonstrates the degree of flexibility in NTR's interactions within an sHSP or with other sHSPs (reproduced from the open source, from Clouser et al., 2019, mod.) [46].

Using NMR spectroscopy, it was found that although the IXI motif and β4/β8 groove are located very close to each other, the IXI motif is generally unbound and unstructured in sHSP oligomers [38,42]. As a result, in a wild-type oligomer, the binding of two IXI motifs causes the exchange of a subunit within the oligomer [35,40]. When considering this model for mutant HSPB1, the P182L mutation likely causes slower subunit ejection [38,42]. Therefore, the faster recruitment of subunits compared to removal causes an increased oligomeric size [38,42]. Additionally, the same study identified the amino acid side chains of Ile181 and Val183 in the IXI motif as important for proper binding to the β4/β8 hydrophobic groove, and a lack of one of these side chains can disrupt binding [42]. The reduced binding affinity between the IXI motif and β4/β8 hydrophobic groove results in the CTR binding less frequently to the ACD of HSPB1 [38,42]. As previously stated, there are many sequences within the NTR that are competing with the CTR to bind to the β4/β8 groove [38,42]. As a result, it is likely that the CTR will become outcompeted by the

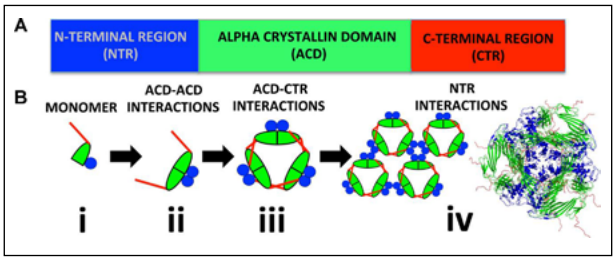

Figure 13. sHSP domains. (A) Schematic representation of sHSP with three domains: NTR in blue, ACD in green, and CTR in red. (B) Model of sHSP oligomerization. sHSP monomer (i) binds to another sHSP monomer via ACD–ACD interaction, forming a dimer (ii). A higher-order structure (iii) is formed by inter-dimer interactions between the CTR and ACD. NTR interactions between subunits contribute to the formation of an oligomer (iv). The structure of a final 24mer HSPB5 oligomer (reproduced from the open source, from Delbecq et al., 2015, mod.) [51].

NTR in binding to the ACD, significantly changing the structure of the HSPB1 oligomer [38,42].

Similar to DCM, cells infected with CMT2 have large insoluble aggregates that result from the failure to refold or degrade misfolded proteins, as observed in transgenic mice models [41,45,49,50]. Within these cells, wild type HSPB1 can form a heterooligomeric structure with P182L HSPB1 [38,42,45]. As a result, when mutant HSPB1 is relocated to cytoplasmic aggregates, wild type HSPB1 may become recruited as well [38,42,45]. This results in reduced availability of wild type HSPB1 proteins, causing more damage to the cell [38,42,45]. As a catalyst for a neurodegenerative disease, the P182L mutation in HSPB1 is also known to impair axonal transport in neurons [40,41,45,49,50]. The subunit of dynactin, p150, is essential for retrograde transport within cells as it mediates the binding between dynein and cargo protein [41,45]. p150 is observed to be less soluble in cells with mutant P182L, resulting in p150 becoming sequestered into aggresomes along with HSPB1 [45]. With an increasing amount of p150 becoming isolated, transport of cargo within the cell becomes infeasible, contributing to the buildup of aggregates and the inhibition of essential processes [45]. Therefore, the sequestration of p150 induced by mutant HSPB1 aggregation can impact the transport of certain molecules along the axon [45].

Conclusions and prospects

The similarities in the pathological mechanisms of BAG3-induced DCM and CMT2 can imply a possible association between the two diseases. P209L BAG3 mutant and P182L HSPB1 mutant are known to cause large, insoluble protein aggregates that impair the proteasome

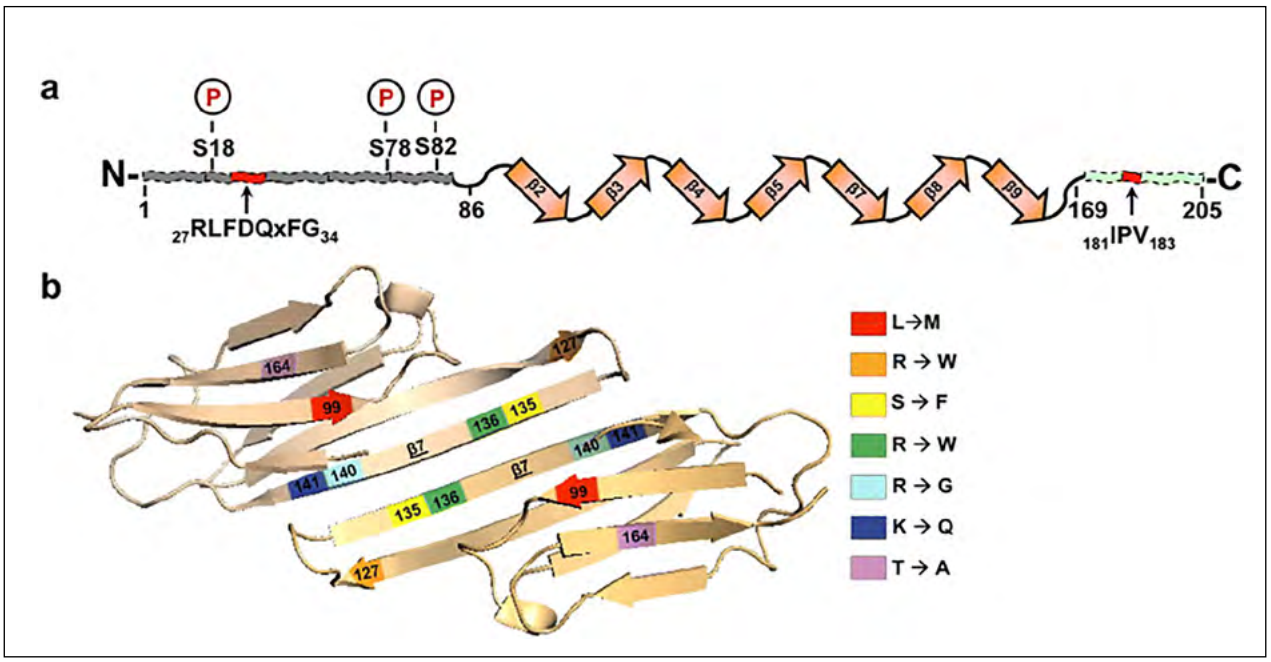

Figure 14. Representation of HSPB1 structure. (A) Primary structure: NTR (gray) has three phosphorylation sites and a conserved sequence of eight amino acids; ACD (orange) has seven β strands marked by arrows; CTR (green) has a conserved tripeptide (IPV) marked red. (B) Tertiary structure of HSPB1-ACD dimer. Marked with underlined β7 strands indicate the dimer interface with antiparallel alignment. Colored residues represent position of point mutations in HSPB1 associated with CMT (reproduced from the open source, from Muranova et al., 2020, mod.) [50].

stability of the cell [28,45,49,50]. These massive aggregates lead to the relocation of wild type BAG3 and HSPB1 proteins as well as other essential proteins, disrupting vital functions [38,45]. Additionally, the gain-of-function mutations reduce the solubility of each mutant, which may contribute to slower subunit exchange [38,49]. The importance of BAG3 and HSPB1 in regulating waste within cells makes them crucial for the chaperone network. The change in amino acid from proline to leucine in the IXI motif of both proteins causes diseases with severe clinical symptoms and aggregation-prone conditions [38,53]. Therefore, the significance of the IXI motif for BAG3 and HSPB1 and the similar pathological effects induced by the mutated motif hints at a potential association between DCM and CMT2. This calls for a closer understanding of the IXI motif and its role within the cell. Currently, specific details related to the role of the IXI motif in protein homeostasis within human cells are largely unknown.

The importance of the IPV motif in oligomerization has manifested itself in the self-interactions among sHSPs [34,49,54,55]. The IPV motif in the CTR binds to the ACD at an interdimeric level, facilitating the formation of larger oligomers and subunit exchange [6,49,55]. It outcompetes other protein partners of the ACD, promoting structurally stable oligomers [6,55]. It is known that sHSPs possessing this motif have the capability of forming larger oligomeric structures compared to sHSPs without this motif [9,55]. HSPB8 does indeed lack the IPV motif, but studies suggest that it can hetero-oligomerize with proteins containing the IPV motif, including other sHSPs and BAG3 [2,8,55-57]. Binding to BAG3 has been identified as essential for the chaperone capacity of HSPB8, which is reflected in its nature of predominantly forming a complex with BAG3 and Hsp70 as a dimer [2,55-57]. The dependency of HSPB8 on BAG3 may indicate the possibility of HSPB8 instability in the presence of a point mutation in the IPV motif of BAG3 [34,57]. The importance of the IPV motif in oligomerization further suggests the potential for a disruption in the oligomeric nature of HSPB8, other protein partners, and BAG3 itself [6,34]. Therefore, the role of the IPV motif in the sHSP family leaves implications for possible structural changes within HSPB8 when bound to P209L BAG3, which can harm the functioning of cardiomyocytes [34].

Due to its function and involvement in proteostasis, the IPV motif should be an area of interest for future studies that seek to discover the enigma behind protein aggregation in DCM and CMT2. A substantial portion of the existing evidence in this topic comes from in vitro, human models, and case studies, suggesting that, molecularly and clinically, DCM and CMT2 may be related to each other in humans. However, the abundance of transgenic mice models and in-vitro studies also point to some limitations in fully applying this association to the human field. We are hopeful that future research on the IPV motif may lead to uncovering the specific mechanism of DCM and CMT2 in humans and provide a complete picture of their relationship.

Acknowledgements

None.

Conflict of interest statement

The Authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

NY wrote the article; VLK and IFT suggested a topic and outline of the article; VLK made corrections in the article; SK supervised the medical part of the article.

Ethical consideration

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

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ACTA MYOLOGICA 2022; XLI: p. 76-83
doi:10.36185/2532-1900-074

Muscle quantitative MRI in adult SMA patients on nusinersen treatment: a longitudinal study

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The recent approval of disease-modifying therapies for spinal muscular atrophy (SMA) raised the need of alternative outcome measures to evaluate treatment efficacy. In this study, we investigated the potential of muscle quantitative MRI (qMRI) as a biomarker of disease progression in adult SMA3 patients during nusinersen treatment. Six adult SMA3 patients (age ranging from 19 to 65 years) underwent 2-point Dixon muscle qMRI at beginning of nusinersen treatment (T0) and after 14 months (T14) to evaluate the muscle fat fraction (FF) at thigh and leg levels; patients were clinically assessed at T0 and T14 with the Hammersmith Functional Rating Scale Expanded (HFMSE), the Revised Upper Limb Module (RULM) and the 6-minute walk test (6MWT). At T0, vastus lateralis muscle displayed the highest mean FF (67.5%), while tibialis anterior was the most preserved one (mean FF = 35.2%). At T0, a slightly significant correlation of FF with HFMSE ($p = 0.042$) and disease duration ($p = 0.042$) at thigh level and only with HFMSE ($p = 0.042$) at leg level was found. At T14, no significant change of mean FF values at thigh and leg muscles was found compared to T0. Conversely, a statistically significant ($p = 0.042$) improvement of HFMSE was reported at T14. We observed no significant change of FF in thigh and leg muscles after 14 months of nusinersen therapy despite a significant clinical improvement of HFMSE. Further studies with longer follow-up and larger cohorts are needed to better investigate the role of qMRI as marker of disease progression in SMA patients.

Key words: SMA, qMRI, fat fraction, outcome measures, biomarker

Received: April 13, 2022
Accepted: June 20, 2022

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How to cite this article: Gallone A, Mazzi F, Bonanno S, et al. Muscle quantitative MRI in adult SMA patients on nusinersen treatment: a longitudinal study. Acta Myol 2022;41:76-83. https://doi.org/10.36185/2532-1900-074

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by a homozygous deletion or smaller mutations of *SMN1* gene causing a reduction of survival motor neuron (SMN) protein, and leading to bulbar and spinal motor neuron degeneration. Four clinical SMA subgroups have been described according to age at onset and maximal motor function achieved, with SMA1 being the most severe phenotype and SMA4 presenting in adult age and characterised by non-progressive mild muscle weakness [1]. Recently, the introduction of new therapeutic approaches has changed the disease natural history [2-4]. The first dis-

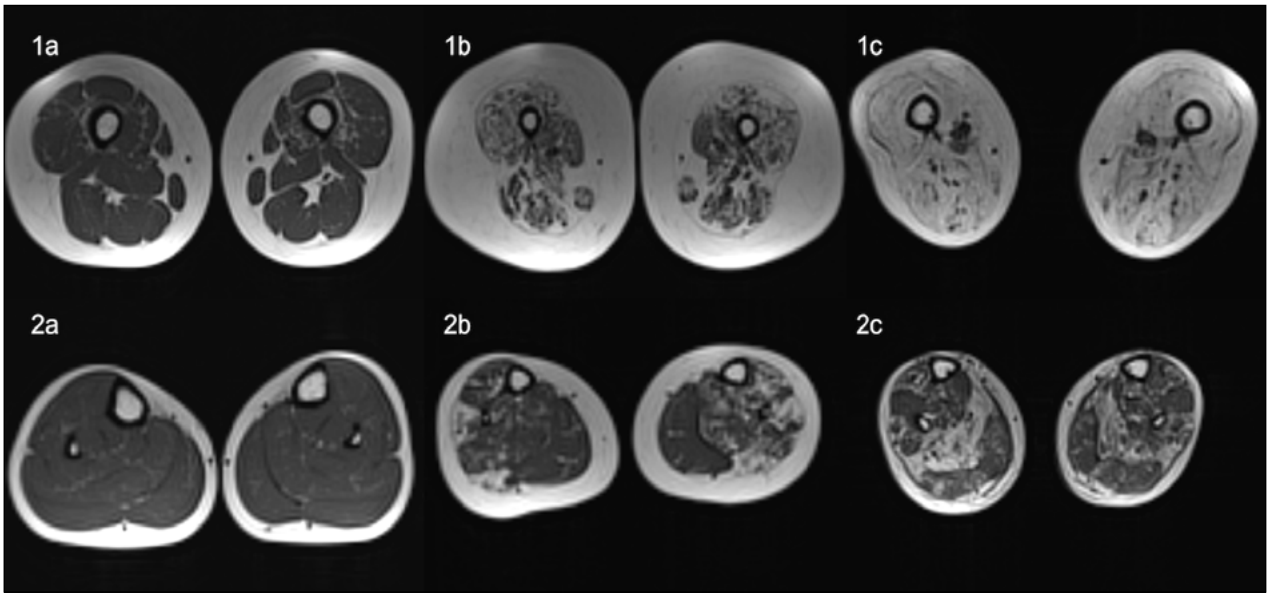

Figure 1. Examples of muscular involvement at thigh (1) and leg level (2) in SMA3 patient with mild (A), medium (b) or severe (c) muscular fat infiltration.

ease-modifying treatment approved in US and EU was the antisense oligonucleotide nusinersen, as reported by two randomized double-blind clinical trials in infantile and later-onset SMA [2,3]. Furthermore, nusinersen has been proved to be effective even in adult age according to observational studies [5,6].

The progression of SMA2 and SMA3 in untreated adult patients is typically slow over the years, regardless the age [7]. However, available clinical outcome measures to assess SMA progression have been validated in pediatric patients and mainly focused on motor function; in addition, they are not always able to catch clinical changes reported by patients [8]. Hence, alternative clinical and nonclinical outcome measures are needed to assess disease progression and evaluate treatment effects in adult SMA patients. In this regard, muscle quantitative magnetic resonance imaging (qMRI) represents a promising biomarker in neuromuscular disorders, being able to discriminate and quantify sub-clinical modifications of the fatty changes in the muscle tissue due to disease progression or treatment response. Indeed, muscle qMRI has been already included as outcome measures in pharmacological clinical trials or natural history studies, particularly in Duchenne muscular dystrophy [9-11]. To date, 4 studies investigating SMA disease progression through qMRI have been reported [12-15]; among them, only 2 focused on nusinersen treatment effect, including respectively 3 and 2 adult SMA3 patients [15,12].

Here, we aimed to study clinical and qMRI modifications in 6 adult SMA3 patients treated with nusinersen over a 14-month period.

Methods

Patients

In this longitudinal study inclusion criteria were the following: (1) clinical and molecular diagnosis of SMA3; (2) ongoing treatment with nusinersen. Patients with contraindications to MRI were excluded.

Standard protocol approvals, registrations and patient consents

This monocentric study has been approved by the Ethics Committee of Fondazione IRCCS Istituto Neurologico 'Carlo Besta', on 17 March 2021. Written informed consent was obtained from all the participants, according to the Helsinki declaration.

Nusinersen administration

All patients received nusinersen intrathecal loading doses of 12 mg at baseline (T0), day 14, day 28 and day 63, followed by maintenance doses every 4 months according to the standard protocol.

Intrathecal injections were performed with standard lumbar access or via X-ray-guided procedure.

qMRI protocol

Patients underwent 2 muscle qMRI (Fig. 1), at baseline (T0) and after 14 months of treatment (T14), respectively. The study focused on the proportion of fatty infil-

tration of the thigh and leg muscles, defined as fat fraction (FF) and measured using the 2-point Dixon imaging technique, as follows. The subject images were acquired with a 1.5T MRI scanner (Avanto, Siemens, Erlangen, Germany). The MRI protocol included the following sequences: 1) a standard axial T1-weighted sequence (TR/TE = 550/8.6 ms, matrix = 192 × 192 × 30, flip angle = 146°, voxel size = 1.98 × 1.98 × 5 mm); 2) a 2-point Dixon sequence for fat/water fraction quantification (TR = 11.1 ms, TE = 2.39/4.78 ms, matrix = 352 × 260 × 192, flip angle = 10°, voxel size = 1.13 × 1.13 × 1.1 mm). The mean duration of the muscle MRI protocol was around 30 minutes.

Post-processing

The fat fraction (FF), expressed as F/(F+ W) × 100 (F = signal of the fat-only image, W = signal of the water-only image), was estimated from the Dixon sequence using Matlab (www.mathworks.com).

Regions of interest (ROIs) were traced on water images by a neurologist (AG) and a neuroradiologist (MM) on two slices, at the midlevel of thighs and at the midlevel of calves, covering all the cross-sectional area of a muscle (Fig. 2). ROIs were traced on 11 muscles in the thighs: rectus femoris, vastus lateralis, vastus medialis, vastus intermedius, sartorius, gracilis, adductor magnus, adductor longus, semimembranosus, semitendinosus and biceps femoris. Six muscles were included for the calves: tibialis anterior, medial head of gastrocnemius, lateral head of gastrocnemius, soleus, tibialis posterior, peroneus longus. Then, using ROIs as binary masks and applying them to the maps, all the metrics were extracted. Mean FF in each muscle of both sides were averaged together (global mean) at thigh and calf levels.

Clinical assessments

The following clinical outcome measures were assessed by trained evaluators at T0 and T14: the Hammersmith Functional Rating Scale Expanded (HFMSE) [16]; the Revised Upper Limb Module (RULM) [17]; the 6 minute walk test (6MWT) [18]. HFMSE assesses the global motor performance and includes 33 items, each scored from 0 to 2, up to a maximum of 66 points. RULM is a scale focused on the upper limb motor function and consists of 20 items with a maximum score of 37. Higher scores correspond to a better motor performance for both scales. The 6MWT test measures the distance in meters walked by the patient in 6 minutes.

Clinically meaningful changes were considered as an improvement from T0 to T14 by at least 3 points with HFMSE, 2 points with RULM or 30 meters with 6MWT, as defined in previous studies [16,19,20].

The patients were considered as wheelchair-bound when not able to walk at least few steps without the aid of other people.

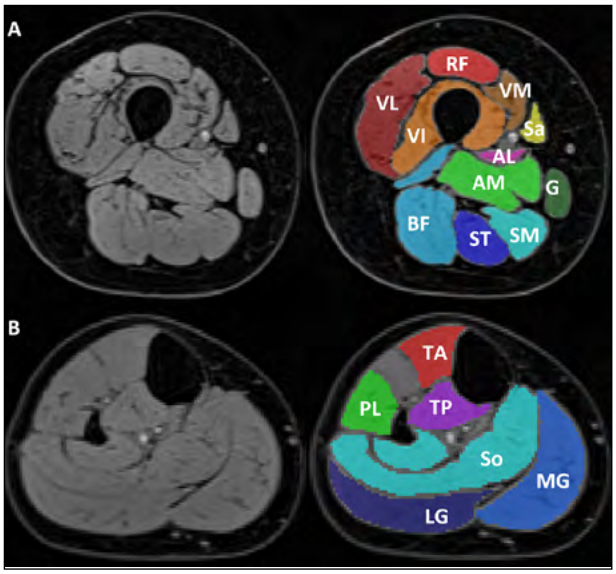

Figure 2. Examples of water images at middle thigh level (A) and middle calf level (B) in a SMA3 patient before and after drawing the muscle regions of interest (ROIs). SM: semimembranosus; ST: semitendinosus; BF: biceps femoris; VI: vastus intermedius; VL: vastus lateralis; RF: rectus femoris; VM: vastus medialis; Sa: sartorius; G: gracilis; AM: adductor magnus; AL: adductor longus; TA: tibialis anterior; PL: peroneus longus; TP: tibialis posterior; So: soleus; MG: medial head of gastrocnemius; LG: lateral head of gastrocnemius.

Statistical analysis

This study was exploratory, thus, a formal sample size was not provided. The number of patients recruited was based pragmatically on the number of patients known to have the disease of interest and eligible for enrolment.

Wilcoxon Signed Rank test was used to research possible significant modification of fat fraction and clinical measures between T0 and T14 assessments. The association between muscle FF and disease duration or clinical variables has been described with the use of Spearman correlation coefficients. Statistical significance was set at $p < 0.05$.

Results

All included patients (4 females and 2 males) were affected by SMA3 caused by a homozygous deletion of exons 7 and 8. Mean age at onset was 7 ± 6.6 years and mean age at T0 was 40.7 ± 18.8 years. At baseline, no patient had received ventilator support or spinal surgery for scoliosis. Clinical and molecular features of patients and their performance on clinical assessments at T0 and T14 are shown in Table I.

Table I. Clinical and molecular features of patients.

Patient/ gender	SMN2 copy number	Age at onset (y)	Ability to walk/age at loss of ambulation (y)	Comorbidities	Age at T0 (y)	Disease duration at T0 (y)	HFMSE		RULM		6MWT	
							T0	T14	T0	T14	T0	T14
1/F	4	16	yes	no	17	1	66	66	37	37	536 m	640 m
2/F	4	15	yes	bipolar disorder	65	50	45	49	36	37	375 m	400 m
3/M	4	3	no/16	no	32	29	12	18	19	22	wb	wb
4/F	3	2	no/45	no	55	53	14	23	21	25	wb	wb
5/F	4	3	yes	no	25	22	54	57	37	36	275 m	300 m
6/M	4	3	yes	no	50	47	44	47	29	27	172 m	182 m

n: number; y: years; HFMSE: Hammersmith Functional Motor Scale Expanded (score); 6MWT: six-minute walk test distance (m); RULM: Revised Upper Limb Module (score); wb: wheelchair-bound; T0: baseline; T14: 14 months of therapy. Clinically meaningful changes at T14 are underlined.

Clinical scores

HFMSE scores were significantly (p = 0.042) improved in our cohort at T14, with a median change of 3.5 (range = 0-9); except for patient 1, displaying normal HFMSE and RULM scores already at T0, HFMSE score improved in all patients by at least 3 points. RULM score did not significantly improved during the follow-up (median change = 1.5; range = 0-4); clinically meaningful improvement with RULM was found at T14 only in patients 3 and 4. Similarly, 6MWT did not significantly improve at T14 (median change = 25; range = 10-104); clinically meaningful improvement with 6MWT was observed only in patient 1.

qMRI

Pattern of muscle involvement at T0

FF for thigh and leg muscles at baseline was reported in Figure 3 and 4, respectively. At thigh level the anterior compartment (vastus intermedius, vastus lateralis, rectus femoris and vastus medialis) was the most involved at baseline, showing a mean FF of 65.4%, with vastus lateralis (mean FF = 67.5%; range = 11.0-87.4%) representing the most impaired muscle. The posterior (semimembranosus, semitendinosus, biceps femoris) and middle compartment (sartorius, gracilis, adductor magnus and adductor longus) displayed a comparable mean FF (respectively 56.8 and 55.5%). At the thigh level the adductor longus was the most preserved muscle (mean FF = 47.9%; range = 7.2-91.2%). At leg level the extensor compartment (tibialis anterior and peroneus longus) showed a mean FF of 40.4%, comparable to the mean FF (41.3%) of the flexor compartment (soleus, medial head of gastrocnemius, lateral head of gastrocnemius and tibialis posterior). Soleus was the most fat-replaced muscle in the leg (mean FF = 47.0%; range = 6.4-90.7%), while tibialis anterior showed the lowest mean FF value (35.2%) with a range of 4.5-74.5%.

Leg muscles were more preserved in ambulant patients compared to wheelchair-bound patients (patients 3 and 4),

pt/muscle	SM	ST	BF	VI	VL	RF	VM	Sa	G	AM	AL
1	6,2%	6,4%	7,7%	24,1%	11,0%	6,4%	11,5%	8,7%	6,8%	7,4%	7,2%
2	22,4%	75,8%	33,4%	78,7%	87,4%	85,0%	84,7%	87,3%	69,1%	29,8%	59,0%
3	83,3%	77,6%	84,6%	74,6%	78,3%	82,6%	69,0%	69,3%	80,7%	74,4%	55,4%
4	76,0%	77,5%	70,0%	87,9%	80,4%	85,5%	75,2%	88,1%	81,5%	81,7%	91,2%
5	48,0%	61,7%	52,8%	55,4%	64,5%	61,4%	37,5%	34,5%	55,9%	35,3%	34,0%
6	80,0%	78,5%	80,4%	84,3%	83,6%	83,0%	78,6%	83,7%	72,1%	78,3%	40,8%

Figure 3. Heatmap of muscle fat fractions at thigh level (baseline). Values are an average of right and left FF. Red colour corresponds to the highest FF levels, green colour to the lowest one; orange and yellow colours correspond to intermediate values of fat fraction. Pt: patient; Muscles: SM: semimembranosus; ST: semitendinosus; BF: biceps femoris; VI: vastus intermedius; VL: vastus lateralis; RF: rectus femoris; VM: vastus medialis; Sa: sartorius; G: gracilis; AM: adductor magnus; AL: adductor longus.

pt/muscle	TA	PL	TP	So	MG	LG
1	4,5%	7,5%	5,9%	6,4%	5,2%	4,0%
2	6,2%	11,3%	13,0%	18,0%	13,8%	13,2%
3	74,5%	83,2%	72,1%	90,7%	85,9%	89,5%
4	61,2%	73,7%	66,9%	80,3%	78,5%	84,1%
5	41,4%	55,8%	34,1%	24,4%	9,2%	71,7%
6	23,6%	42,3%	22,9%	62,3%	19,6%	18,6%

Figure 4. Heatmap of muscle fat fractions at leg level (baseline). Values are an average of right and left FF. Red colour corresponds to the highest FF levels, green colour to the lowest one; orange and yellow colours correspond to intermediate values of fat fraction. Pt, patient. Muscles: TA: tibialis anterior; PL: peroneus longus; TP: tibialis posterior; So: soleus; MG: medial head of gastrocnemius; LG: lateral head of gastrocnemius.

showing severe fatty changes in both proximal and distal muscles. Notably, patient 1 displayed overall a mild muscle fat replacement, with the greatest FF in the vastus intermedius (24.1%), in agreement with the short disease duration and the normal motor performance by HFMSE and RULM. Conversely, patients with the longest disease duration (patients 2, 4 and 6) exhibited the highest FF in sartorius (range: 83.7%-88.1%). In this subgroup patient 2 had a lower global FF at thigh (64.8%) and leg (12.6%) level compared to patients 4 and 6, mainly as a consequence of a lower fat infiltration of semimembranosus, biceps femoris, adductor magnus and of all the leg muscles. These data are probably related to a relatively mild disease severity, being this patient still able to walk after a 50-year disease duration.

FF at thigh level resulted slightly correlated with disease duration ($p = 0.044$) and HFMSE ($p = 0.042$) score; conversely, FF at leg level was slightly associated only with the HFMSE ($p = 0.042$) score (Tab. II).

FF changes at T14

Total mean FF at thigh and calf levels at T0 and T14 are shown for each patient in Table III. Considering the whole cohort, the mean thigh FF resulted unchanged from baseline (59.8%) to T14 (60.9%); similarly, no significant change of mean FF at leg level was detected between T0 (41.0%) and T14 (41.8%). Although not significant, the mean increase of FF across the 2 timepoints was higher in vastus intermedius (3.9%), vastus medialis (3.6%) and adductor longus (3.3%). All remaining muscle showed mean FF modifications below 3%.

Discussion

SMA natural history in adult age still needs to be completely elucidated. Moreover, better comprehension of factors predicting disease progression and response to new treatments is needed. In this regard, qMRI is increasingly recognised as a promising biomarker for disease severity and progression in different neuromuscular disorders. However, poor data on qMRI have been reported in SMA, especially in patients under treatment.

In our study, we did not find any significant change of FF values at thigh and leg levels after a 14-month period of treatment with nusinersen, regardless clinically meaningful changes detected by HFMSE and RULM. Lack of significant modification of FF and concordance with clinical improvement may be related to different factors, as the small sample size, the relatively short observational period and the high muscle fat fraction detected at the baseline in our cohort (thigh FF > 50% in 5/6 patients and leg FF > 30% in 3/6 patients), suggesting a relevant muscle fat replacement before the beginning of the treatment. However, we cannot exclude that unchanged FF values in our co-

Table II. Correlation between FF and clinical scores or disease duration at T0.

		Spearman p-values	Correlation coefficients
FF at thighs	**HFMSE**	**0.042**	- 0.829
	RULM	0.050	- 0.812
	6MWT	0.200	- 0.800
	DD	**0.044**	0.829
FF at legs	**HFMSE**	**0.042**	- 0.829
	RULM	0.084	- 0.754
	6MWT	0.200	- 0.800
	DD	0.544	0.314

Significant p values are highlighted in bold. FF, fat fraction; HFMSE, Hammersmith Functional Rating Scale Expanded; RULM, Revised Upper Limb Module; 6MWT, six-minute walk test; DD, disease duration

hort may be the result of the ongoing treatment with nusinersen. The discrepancy between qMRI data and the small clinical improvement could be related to the inability of FF to catch the positive treatment effect, which could instead be linked to other mechanisms acting on the spared muscle tissue. Savini and colleagues [15] reported a progression of FF in thigh muscles of 3 SMA3 adult patients during 21 months of treatment and a concurrent slight reduction of wT2 over time. This apparent mismatch with our data could be related to the longer follow-up period in the aforementioned study (21 months against 14 months in our study) and a smaller sample size. In this landscape, considering also literature data about nusinersen efficacy in adult SMA patients during the first 14 months of treatment [6], we cannot exclude that nusinersen could be more effective in preventing the muscle degeneration during the first year of therapy, followed by possible resumption of the muscle deterioration. Conversely, Barp and colleagues [12] showed a reduction of fractional anisotropy through diffusion tensor imaging MRI after 24 months of nusinersen therapy in 2 SMA3 adult patients, suggesting a disease stabilization during the treatment, in agreement with our data. However, the application of different qMRI techniques (DTI *vs* 2-point Dixon) do not allow a real comparison among the two studies.

Furthermore, a considerable limitation of all the aforementioned studies, including the present one, is the absence of a control group of untreated SMA patients. In this regard, 2 longitudinal studies investigated muscle deterioration through qMRI in SMA patients, providing contrasting data [13,14]. Bonati and colleagues [13] did not report any significant progression of muscle FF in 18 SMA3 patients over a period of 13 months, suggesting that a longer observation period could be necessary to detect possible FF modifications. Conversely, Otto and colleagues [14] showed a significant increase of FF and a significant decrease of T2 over a 13-month follow-up in a cohort of 10 (5 SMA3 and 5 SMA2) patients, despite any decline of muscle power and motor function scores. The discrepancies between these two studies could be partially explained by the inclusion of more severe patients in the study by Otto and colleagues. Indeed, a further limitation of data from literature and our study is represented by the heterogeneity of the investigated population in terms of age and disease severity.

In addition, considering that the pattern of muscle involvement in SMA could be the result of a degeneration more prominent in specific groups of motor neurons [21], the different study design in the aforementioned studies represent a further confounding factor. Barp and colleagues [12] focused their analyses on 4 leg muscles, without including thigh muscles; on the other side, the remaining 3 longitudinal studies [13-15] were limited to thigh muscles. To our knowledge, thigh and leg muscles were both investigated for the first time in the present study. Higher FF values were detected in leg muscles in wheelchair-bound than in ambulant SMA3 patients at T0, regardless the disease duration, without any apparent difference at the thigh level. With the limitations of the small sample size, these data may suggest that focusing qMRI on leg muscles could be more helpful to predict clinical decline and loss of walking ability; further studies are needed in this regard on large cohort of patients. Besides, longitudinal studies are needed to investigate the role of the upper limb muscle qMRI, particularly in patients with severe phenotypes characterized by residual upper limb motor function and loss of lower limb motor abilities.

Table III. Total mean fat fractions for each patient.

	Total mean FF THIGHS		**Total mean FF LEGS**	
Pt	**T0**	**T14**	**T0**	**T14**
1	9.4% ± 27.1%	10.4% ± 27.4%	5.6% ± 31.6%	6.1% ± 31.4%
2	64.8% ± 27.1%	64.4% ± 27.4%	12.6% ± 31.6%	11.8% ± 31.4%
3	75.4% ± 27.1%	80.8% ± 27.4%	82.6% ± 31.6%	82.8% ± 31.4%
4	81.4% ± 27.1%	81.6% ± 27.4%	74.1% ± 31.6%	73.7% ± 31.4%
5	49.2% ± 27.1%	51.1% ± 27.4%	39.4% ± 31.6%	43.3% ± 31.4%
6	76.7% ± 27.1%	77.2% ± 27.4%	31.5% ± 31.6%	33.4% ± 31.4%

FF, fat fraction; T0, baseline; T14, 14 months of therapy; pt, patient

A correlation at baseline between FF and HFMSE score and between FF and disease duration at thigh level and between FF and HFMSE at legs further strengthen the role of FF as a marker of disease severity, as already reported in SMA patients [22].

Our study revealed a pattern of muscle involvement in agreement with data already reported in literature [21,23-25], although in our cohort sartorius was severely involved as the gracilis and the adductor magnus was relatively preserved. However, utilization of semiquantitative scales in place of qMRI to assess the amount of fatty degeneration in most of these studies, may explain some discrepancies with our data. Notably, vastus intermedius displayed the highest FF in our patient with only a 1-year disease duration, suggesting that this muscle is early involved in the pathological process.

Conclusions

Our study showed stability of fatty infiltration values in thigh and leg muscles of SMA3 adult patients during

14 months of therapy with nusinersen, regardless the clinical improvement. Further studies with longer follow-up, larger cohorts of patients and including other techniques as T2 and DTI or upper limb muscles are needed to better investigate muscle qMRI value as marker of disease severity and progression in SMA patients.

Acknowledgments

LM, SV, RZ, AG are members of the ERN Muscle Working group.

Conflict of interest statement

LM has received honoraria for speaking and compensation for congress participations from: Sanofi Genzyme, Roche and Biogen; SV received honoraria for advisory board activities, and compensation for travel and congress participation from Sanofi Genzyme, Biogen and Roche; RZ received funds for travel and congress participation from Biogen.

Funding

This work was partially supported by the Italian Ministry of Health (RRC)

Authors' contributions

AG performed data analysis and their interpretation, drafted the manuscript; FM performed data analysis and their interpretation and revised the manuscript. SB collected data and revised the manuscript. RZ collected data; MM performed data analysis and their interpretation and revised the manuscript; DA performed data analysis and their interpretation and revised the manuscript; LM planned the study, performed data analysis and their interpretation, drafted and submitted the manuscript.

Ethical consideration

The study was approved by by the Ethics Committee of Fondazione IRCCS Istituto Neurologico 'Carlo Besta', on 17 March 2021 (No. 24/2022). This study was performed in line with the principles of the Declaration of Helsinki.

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ACTA MYOLOGICA 2022; XLI: p. 84-88
doi:10.36185/2532-1900-072

Peripheral circulation disturbances in two consecutive children with spinal muscular atrophy and literature review

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Spinal muscular atrophy is a progressive and severe hereditary (autosomal recessive) neuromuscular disease characterized by lower motor neuron degeneration in the spinal cord and brainstem causing a clinical picture of progressive muscle atrophy and weakness of skeletal and respiratory muscles. There is an ongoing discussion on the extent to which other tissues might be affected in patients with SMA. Several animal models and some case reports or small case series report involvement of other organ systems, such as peripheral nerve, brain, muscle, heart, vascular system, and pancreas. Recent literature reviews identified a number of cases with vascular abnormalities. We present two consecutive cases of patients diagnosed with SMA who developed peripheral circulation disturbances and combine the findings with a thorough review the literature.

Key words: spinal muscular atrophy, peripheral circulation disturbances, children

Received: April 5, 2022
Accepted: June 11, 2022

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How to cite this article: Cristofano G, Fucci M, Oliva MC, et al. Peripheral circulation disturbances in two consecutive children with spinal muscular atrophy and literature review. Acta Myol 2022;41:84-88. https://doi.org/10.36185/2532-1900-072

Introduction

Spinal muscular atrophy (SMA) is a progressive and severe hereditary (autosomal recessive) neuromuscular disease characterized by lower motor neuron degeneration in the spinal cord and brainstem causing a clinical picture of progressive muscle atrophy and weakness of skeletal and respiratory muscles. It is one of the most common causes of infantile mortality with an estimated incidence of 1:6000 - 1:11,000 newborns. It is caused by a homozygous mutation, deletion, or rearrangements in the survival motor neuron 1 (*SMN1*) gene on chromosome 5q13 [1,2]. These mutations are responsible for a dysfunctional SMN protein. SMN protein is ubiquitously expressed in all cells. The human genome also contains the *SMN2* gene, which is a SMN1 paralog and differs only in few nucleotides, the most crucial of which is a C to T transition in exon 7 causing the skipping of this exon in a large proportion of SMN2 transcripts. Consequently, SMN2 mainly produces a non-functional protein, which is rapidly degraded [3]. It is important to note that SMN2 expression accounts for only a small proportion of the full-length fully functional SMN protein and thus only partially compensates for the loss of SMN1. Even though the number of SMN2 copies is not essential to diagnose SMA, it is an important positive modulator of the severity of SMA phenotype: in fact, the disease severity appears to

be inversely proportional to the *SMN2* gene copy numbers and SMN protein levels despite the mechanisms of disease progression are not clear yet. SMA can be classified into five clinical types ranging from SMA 0 or 1 – the most severe and devastating types – to milder subtypes SMA 3 and SMA [4], based on age of onset and severity [4]. There is an ongoing discussion on the extent to which other tissues might be affected in patients with SMA [5,6]. Several animal models and some case reports or small case series report involvement of other organ systems, such as peripheral nerve, brain, muscle, heart, vascular system, and pancreas [7]. Recent literature reviews identified a number of cases with vascular abnormalities [8-10]. It is known that populations of neurons, astrocytes, and vascular endothelial cells constitute the so-called neurovascular unit (NVU), in which neuronal and synaptic metabolism is closely coupled to capillary blood flow by astrocyte-mediated vasodilator control. Various neurodegenerative disorders, such as Alzheimer's disease and amyotrophic lateral sclerosis, are characterized by a disruption in NVU [11]. The loss of motor neurons of anterior horn induces metabolic stress in neighboring astrocytes. These events lead to a reduction in the control of capillary blood flow [12]. *SMN1* gene provides instructions for making the SMN protein and plays a role as translational regulator [13]. Among the peripheral circulation disturbances, the Raynaud's phenomenon-like clinical pictures and more generally paroxysmal vasospasms of the extremities are relatively common, but often unrecognized clinical syndromes causing reversible color changes, from white (arterial spasm) to blue (resultant cyanosis) and red (reactive arteriolar dilation) as a result of vasospasm.

We present two consecutive cases of patients diagnosed with SMA who developed peripheral vascular abnormalities.

Patients

Patient 1

A 13-year-old girl with spinal muscular atrophy type 3 (homozygous deletion of exons 7 and 8 in the *SMN1* gene with 3 copies of *SMN2;* Revised Hammersmith Scale: 52/66; Revised Upper Limb Module scale: 34/37) treated with nusinersen with a good clinical response, presented with changed skin color on her feet. Acrocyanosis and sweating affected her feet only. No edema, arthritis, fever or other changes occurred. The patient did not complain pain or discomfort, and no apparent infection, previous traumas or cardiovascular event preceded these signs. Feet became suddenly and temporarily purple (Fig. 1A-B) for about 10 minutes, and then showed an almost spontaneous resolution (Fig. 1C), resembling a Raynaud's phenomenon-like clinical picture. The physical examination revealed trophic changes of nails indicating a probable chronic onset. This phenomenon occurred both in clinostatism, when the girl was in bed, and in orthostatism (for example, under the shower – Fig. 1D). Clinical evaluation, including venous and arterial Doppler scanning, coagulation studies, serological parameter for autoimmune diseases and echocardiography was unremarkable. The patient had no known family and personal history for vascular abnormalities. Our patient did not exhibit a certain trigger, but multiple risk factors: chronic immobility and inconsistent prolonged sitting on wheelchair, limb contractures, external compression (i.e. due to unsuitable orthoses or wheelchair cushions), neuromuscular disease itself, emotional stressdue to invasive therapies (lumbar punctures for intrathecal nusinersen administration). She is now clinically monitored for this. No symptomatic therapy was started.

Patient 2

An 8-year-old girl with spinal muscular atrophy type 2 - having a homozygous deletion of exons 7 and 8 in the SMN1 gene with 3 copies of SMN2; Revised Hammersmith Scale: 8/66; Revised Upper Limb Module scale: 24/37 – treated with nusinersen with a good clinical response, presented a first vascular episode characterized by changed skin color of her legs and feet bilaterally. Mild edema was reported before that. The patient did not report any associated pain or discomfort. Her legs and feet suddenly and temporarily turned purple but this gradually and spontaneously disappeared residing a mottled reticulated vascular pattern with a purplish lace-like discoloration of the skin (Fig. 2). This phenomenon occurred mostly in clinostatism. Clinical evaluation, including cardiological exam, coagulation studies, serological parameter for autoimmune diseases and echocardiography was unremarkable. Venous and arterial Doppler scanning showed reduced flow velocity in the arterial circulation as per peripheral vasoconstriction without acute vascular diseases. The patient had no known family and personal history for vascular abnormalities. Like patient 1, this patient neither exhibited specific triggers but multiple risk factors: chronic immobility and excessive supine position, limb contractures, external compression (i.e. due to unsuitable orthoses or wheelchair cushions), neuromuscular disease itself, emotional stress due to invasive therapies (lumbar punctures for intrathecal administration of nusinersen). We decided to closely follow-up the clinical picture without starting any therapy.

Discussion

We described two children with SMA (one case of type II and one case of type III) and peripheral vascular

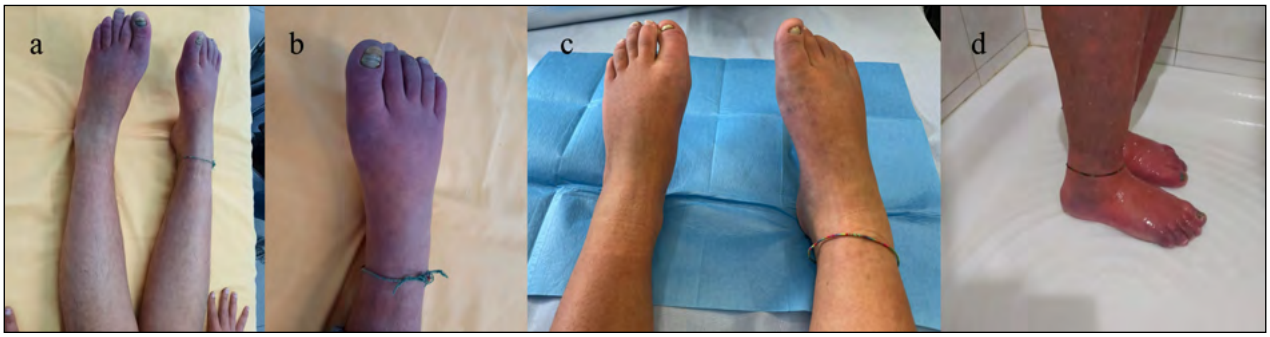

Figure 1. Feet temporarily purple (A,B). Spontaneous resolution (C). Feet under the shower (D).

abnormalities. There are very few data in the literature about this phenomenon: only four other cases of SMA associated to vascular diseases have so far been reported [9,10]. In particular, digital necrosis is reported in two patients and thrombotic occlusions of small vessels are described in other two cases [10]. Both patients (1 female, 1 male) with digital necrosis had the most severe subtype of SMA, SMA 1 with only one SMN2 copy. The male began to show progressive digit necrosis at 4 months without pain reaction; at 6 months, a skin biopsy showed necrosis of the epidermis and upper dermis and thrombotic occlusion of small vessels. Other causes for distal necrosis such as diabetes, autoimmune disorders, infections and coagulation defects were excluded. The girl developed skin necrosis on all digits and toes from the age of 3 months, which could not be accounted for by medical interventions, heart defect, or other conditions. In this case, skin biopsy revealed nonspecific vasculitis without structural defects of the dermis. With regards to two female patients with thrombotic occlusions of small vessels, one was found to have homozygous deletion of SMN and NAIP, the other one was diagnosed with SMA with 2 copies of SMN2. In the first case, at age 4 months a blue color was noted on the tip of the patient's first left foot digit which became purple and then black. In the following weeks, this spread to almost all digits in both feet and finger digits without causing pain or discomfort, and with no apparent infection. Diagnostic evaluations, including venous Doppler and coagulation studies, were all unremarkable with the exception of echocardiography which revealed atrial septal defect and asymmetric ventricular hypertrophy. Empiric treatment with aspirin, heparin, pentoxiphylline, diosmine, and local care with antiseptics was administered. Over the following weeks the lesions wax and waned and healed, and the following 10 months were event-free. In the second case, at age 5 months, the child's palms and fingers as well as nails turned bluish. In the following days, the color evolved to purple and black, then tissue necrosis started without apparent infection, medication exposure, or cardiovascular event. Again, the diagnostic evaluation was unremarkable. Treatment was started 2 weeks after the onset of symptoms including aspirin, heparin, pentoxiphylline, and diosmine, as well as local care with antiseptics. Symptoms improved over a period of 3 months, followed by normal nail growth.

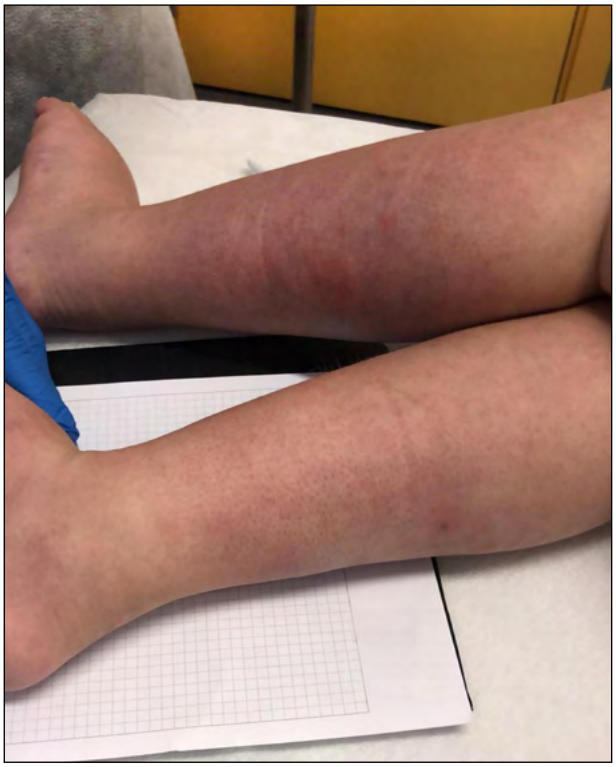

Figure 2. Mottled reticulated vascular pattern with a lace-like purplish discoloration of the skin.

These findings suggest a probable relationship between innervation and vascularization in motor neuron disease, yet there is limited evidence. One study carried in a mouse model for human SMA type I shows that in mice treated successfully with trichostatin A, long-living mice developed tissue ischemia with a black discoloration

of the tail and digits. Histological examination showed tissue necrosis and thrombosis of small vessels. The investigators thought that it could be an adverse effect of trichostatin A, however vascular dysfunction was also observed in non-treated SMA mice, suggesting that vascular alterations could be caused by SMN protein deficiency [14]. SMA type I has recently been reported to be causally related to congenital heart defects mostly in the presence of one *SMN2* gene copy, therefore it was assumed that there could be an association between heart defects and vascular alterations, but these perfusion abnormalities were also found in the subject without heart defect [15]. Araujo et al. believe that autonomic nervous system abnormalities, which are found in severely paralyzed infants surviving mechanical ventilation over a longer period of time, could influence perfusion and suggest that symptomatic treatment or passive movements or regular posture change could reduce vascular dysfunction [9]. In SMA I patients, chronic hypoperfusion associated with sympathetic hyperactivity was observed, which causes metabolic stress with accelerated loss of anterior horn motor neurons, triggering a vicious circle with further regression of capillaries and astroglial dysfunction [12,16,17]. The NVU is therefore a critical therapeutic target for treating SMA I [18].

Conclusions

Although there is limited data in the literature about the possible correlation of SMA and perfusion alterations, it can be hypothesized that several pathophysiological mechanisms are – directly and indirectly – linked to SMA. Furthermore, SMN protein would play a central role not only in the neuronal system but also in vascular and metabolic functions, while the number of *SMN2* gene copy – besides determining the clinical phenotype - could also influence the degree of involvement of other organs and systems. However, further studies are required to understand the function of SMN in the neurovascular unit and other observations will hopefully provide more significant data.

Acknowledgements

The Authors wish to thank the patients and their family for their cooperation and permission to publish these findings. This work was supported by the Italian Ministry of health RC 2019-2021.

Conflict of interest statement

The Authors declare no conflict of interest.

Authors' contributions

GF, MF and AT acquired the clinical data, reviewed the literature, and drafted the manuscript; AT designed the study, oversaw data acquisition, supervised the initial drafting, and critically revised the manuscript; MD and MCO contributed to manuscript writing analyzed the clinical data and critically revised the manuscript. All Authors contributed to the interpretation of results and reviewed the final manuscript.

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ACTA MYOLOGICA 2022; XLI: p. 89-94
doi:10.36185/2532-1900-075

Impact of the COVID-19 pandemic on neuromuscular rehabilitation setting. Part 2: patients and families' views on the received health care during the pandemic

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This study explored views of users with muscular dystrophies and their caregivers on staff-user relationships and the treatments provided by a Rehabilitation Centre during the pandemic. Patients and relatives were asked to anonymously complete an open-ended questionnaire exploring their views on these aspects. Fifty-four patients and 40 caregivers gave their informed consent and participated in the survey. Fifty-three patients were adults, 28% suffering from Duchenne/Becker muscular dystrophy. Patients reported 269 comments on health care services provided during the pandemic, 132 (49%) concerning positive aspects and 137 (51%) negative aspects. The prompt restart of the rehabilitation therapies and the staff closeness over the pandemic were the practical aspects most frequently appreciated (46.9%), while closer family contacts and the perception of being able to rely on the Centre's constant support were the most cited psychological aspects (53.1%). Architectural barriers, difficulties in accessing public health services, economic difficulties, and lack of support from welfare and other agencies were the practical critical points most frequently reported (89%). In addition, social isolation, and loneliness due to fear of contagion were the most negative psychological aspects (10.1%). As regard the caregivers' views, participants reported 151 comments. Of these, 86 (56.9%) were positive and 65 (43.1%) were negative. Among the positive aspects, the psychological ones – such as closer family contacts, not feeling abandoned and counting on the constant Centre's professional support prevailed (53.5%). As for the negative aspects, most caregivers (92.6%) believe that the pandemic exacerbated their financial and bureaucratic difficulties, particularly in poorer families.

Key words: COVID-19 pandemic, muscle diseases, benefits and difficulties, activities of daily living, rehabilitation setting

Received: May 25, 2022
Accepted: June 20, 2022

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How to cite this article: Magliano L, Citarelli G, Esposito MG, et al. Impact of the COVID-19 pandemic on neuromuscular rehabilitation setting. Part 2: patients and families' views on the received health care during the pandemic. Acta Myol 2022;41:89-94. https://doi.org/10.36185/2532-1900-075

Introduction

Since the beginning of 2020, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a worldwide pandemic that has had a significant impact on not only medical treatments, but also on society and economy.

In muscular dystrophies (MDs) – multisystem diseases that often affect heart, respiratory system, and other organs – many patients require ongoing assistance in their daily life. It follows a demanding burden [1,2] and an inevitable close contact with caregivers. Patients with MDs are at high risk of developing severe disease if they get COVID-19. Therefore, the prevention of the contagion is of greatest importance for their life [2-4]. In addition, for patients who require medical care, the number of people needed for care can be large, making it especially difficult to balance infection control, conducting activities of daily life and providing medical care. A number of studies have examined the impact of COVID-19 on the life of people with MDs and the care these patients received during the pandemic. Bertrand Recasens and Rubio [3] identified in 1) direct SARS-CoV-2 effect on different neuromuscular pathologies; 2) limitation of physical rehabilitation (one of the essential aspects of myopathies treatments) due to safety measures and 3) economic difficulties for patients and their families – due to the scarcity of resources in terms of public healthcare – the main effects of COVID-19 on neuromuscular diseases care. Another study by Matsumura et al. [5] reported the results of a web-based survey exploring the influence of COVID-19 on the care of 542 patients with MDs. Approximately 30% of patients had to postpone regular clinical follow-up, and 25% to use telephone consultations. A shortage of ventilator accessories and infection protection equipment, occurring during the pandemic, had a serious impact on medical care and infection prevention measures. Nishizara et al. [6] investigated changes in motor function in a group of 85 DMD patients after travel restriction due to COVID-19. They found that a more sedentary lifestyle and lack of regular physical therapy services most likely contributed to negatively affect the ankle dorsiflexion ROM, but not other motor functions. To this regard, Sobierajska-Rek et al. [7] investigated the rehabilitative situation of 69 young patients with DMD in the pandemic, to establish an online rehabilitation program and motor assessment and determine the usefulness of telerehabilitation in these patients. They concluded that patients, under the physiotherapist guidance and caregivers' help, can continue home based rehabilitation. Kenis-Coscun et al. [8] compared telerehabilitation or home-based video programs in patients with DMD who have lost their access to on-site rehabilitation, due to pandemic. They showed that telerehabilitation was better in improving muscle strength than a video-based home exercise, although none was able to improve functional outcomes.

Palazzo et al. [9] recently explored the usefulness of religious faith and assistance of associations on patients' capacities to deal with critical times. They found that closeness of family and activities promoted remotely by the associations played a crucial role, allowing the participants to feel united, to discover new aspects of themselves and to give more value to life.

In a study aiming to explore the staff views on the care provided by a Rehabilitation Centre during the pandemic and the impact on the professionals assistance, we recently reported [10] that participants, most of them physiotherapists, highlighted 169 aspects, 48.5% referring to the resources used to cope with critical issues, and 51.5% concerning the difficulties encountered. Emotional aspects prevailed on practical aspects, both in terms of resources (52.4 *vs* 47.6%) and difficulties (57.5 *vs* 42.5%).

As the second part of the study, here we present the results of a survey aimed to explore the views on the care provided by the same centre during the pandemic from the perspectives of patients with MDs and their caregivers.

Patients and methods

The survey was carried out at the "Gaetano Torre" Centre (G. Torre) for MDs, a rehabilitation centre, operating within the framework of a regular agreement with the Northern Health District of Naples, Italy. The Centre provides a range of outpatient and at-home clinical and rehabilitative care to persons with MDs over their life span. Patients assisted by G. Torre Centre (n = 105) and their caregivers were invited to complete an anonymous open-ended questionnaire of seven/eight items exploring the practical and psychological aspects emerged during the pandemic in relation to the healthcare services provided by the Centre (see Tables I and II for the items list). The questionnaire was given to participants during the routine follow-up visits between September and December 2021 by a social worker, who was available to clarify questions upon request. Participants were free to fill in the questionnaire immediately or later, depositing it in a special box. The study protocol was approved by the Ethics Committee of the Naples 1 Local Health Authority, (Prot. 362/2021).

Results

Patients' clinical and social demographic variables

Fifty-four patients (participation rate: 51.4%) and 40 caregivers gave their informed consent and participated in the study. Fifty-three out of 54 participating patients were adults and affected by several types of MDs. In particular, 28% had Duchenne/Becker MDs, 17.5% Limb-girdle MDs, 17.5% Myotonic Dystrophy type 1, 17.5% Facio-Scapulo-Humeral-Dystrophy, 5% Spinal Muscular Atrophy type 3, and 14.5% had other neuromuscular dis-

Table I. Patient version of the Questionnaire.

As a person with a neuromuscular disease, we invite you to answer the following questions about the services you received at the Gaetano Torre Centre and your experience during the COVID-19 pandemic.
Your answers will be useful for: a. Improve the quality of services offered. b. Obtain more guidance in dealing with similar experiences
Please note that the questions refer to situations that occurred during the pandemic.
1) Based on your experience as a person with a neuromuscular disease, how did the pandemic affect and influence the service and performance received at the G. Torre Centre?
2) Based on your experience as a person with a neuromuscular disease, what are the positive aspects of the services received from the G. Torre Centre?
3) Based on your experience as a person with a neuromuscular disease, what are the critical aspects of the services provided by the G. Torre Centre?
4) Based on your experience, what are the positive aspects in the daily life of people with neuromuscular diseases?
5) In your experience, what are the problematic aspects in the daily life of people with neuromuscular diseases?
6) In your experience, what are the positive aspects in the families of people with neuromuscular diseases?
7) Based on your experience, what are the main difficulties faced by families of people with neuromuscular diseases?
8) In your opinion, what could be the changes to improve the service offered by the G. Torre Centre?

Table II. Caregiver version of the Questionnaire.

As the caregiver of a person in the care of the G. Torre Centre, we invite you to answer the following short series of questions on the services (practical work and assistance) provided by the Centre during the COVID-19 pandemic, with the aim of: a. Obtain more elements for dealing with similar experiences in the future and at the same time, b. Improve the quality of services offered to patients and their families.
Remember that the questions refer to situations that occurred during the pandemic.
1) Thinking about the current situation, how do you think the pandemic has affected the service and performance provided by the G. Torre Centre?
2) What strengths and weaknesses did you find in the services provided by Centro G. Torre during the pandemic period?
3) What aspects, in your opinion, were or are most problematic for patients and their families during this phase of the pandemic? Why?
4) Were there, or are there anyway, positive aspects in the daily lives of patients and their families during the pandemic period?
5) What are the main difficulties, from your point of view, that the patients' families have faced and are facing?
6) Based on your experience, what positive aspects could there have been for patients' families during this phase of the pandemic?
7) In your opinion, what could be the changes to improve the service offered to patients at the G. Torre Centre?

orders. Males were 33 (61.1%) and females 21 (38.9%). The mean age of patients was 46.8 ± 14.4 years. No socio-demographic data on caregivers was collected due to privacy regulations.

Patients' views on health care services provided during the pandemic

Question n. 1 investigated the *impact of the pandemic on the service and the assistance provided* by the G. Torre Centre. Of the 53 patients who completed the item, 46 (86.7%) did not report any difference compared to pre-pandemic time, one participant stated that the pandemic has had a mild impact on the services and six (11.3%) complained about the discontinuation of the physiotherapy (FKT) during the lockdown (first pandemic wave).

Question n. 2 invited patients to *identify positive aspects – if any – in the services received*. Patients reported 67 positive comments, including 44 (65.7%) practical and 23 (34.3%) psychological. Among the practical aspects highlighted by participants, best practice and professional skills prevailed, while among the psychological aspects the relationship with the health professionals (i.e., the psychological support and the perception of staff full

availability despite objective difficulties due to pandemic) was the most appreciated one.

Question n. 3 regarded the *perception of critical aspects in the services received.* Fifty-two (96.3%) patients completed the item. Of these, 37 patients (71.1%) did not identify any negative aspect, while 15 (28.8%) identified in the bureaucratic difficulties in the renewal of the approvals for treatments and the geographical distance from the G. Torre Centre the most critical points.

Question n. 4 addressed the potential *positive aspects of the pandemic on daily life activities*. Fifty-one (94.4%) patients completed the item. Twenty-seven (52.9%) reported 29 positive comments, 11 of which (37.9%) were psychological and 18 (62.1%) were practical. Among the psychological aspects, family support and resilience skills were the most appreciated, while rehabilitation and their positive health effects and the readiness of health care professionals prevailed among the practical aspects. Twenty-one patients (44.4%) did not report any positive comment.

Question n. 5 concerned the *critical aspects of the pandemic on daily life activities*. Forty-seven (87%) patients mentioned 66 general and specific negative practical aspects, mainly related to functional autonomy limitations due to the disease and architectural barriers.

Question n. 6 concerned the *positive aspects present in the families*. Forty-two (77.8%) patients answered to this item. Of these, six patients (14.3%) did not find any positive aspect, while 36 reported 36 statements on positive aspects, mainly including feeling of being loved, perception of family cohesion and family sharing of problems.

Question n. 7 addressed the *negative aspects present in the families*. Fifty patients (92.6%) indicated 56 critical issues, 15 (26.8%) concerning psychological aspects and 41 (73.2%) practical aspects. Among the psychological aspects, feeling of worry, psychological consequences of the disease on personal wellbeing, and a sense of isolation/loneliness were the most frequently mentioned points. Among the practical aspects, need for daily help in performing one's activities and the poor interest/attention to own problems by the Authorities and Institutions emerged as critical points.

Question n. 8 invited patients to suggest what changes the G. Torre Centre should implement to improve the health services offered. Thirty-two (68%) patients did not suggest any change, underlining their full satisfaction with the health assistance received, while 15 (32%) reported 17 suggestions, the most frequent being a higher psychological support in presence and/or remotely.

Caregivers' views on health care services provided during the pandemic

The caregivers' version of the questionnaire included the same questions covered in the patient's version of the tool, except for questions n.2 and n.3, which merged.

Question n. 1 investigated the *perceived impact of the pandemic on the care and assistance offered* by G. Torre Centre. Thirty-nine (97.5%) caregivers responded. Of these, 20 (51.3%) did not mentioned any perceived impact on the health care provision, 10 (25.6%) pointed out a positive impact and 9 (23.1%) reported a negative impact limited to the lockdown period in which FKT was interrupted.

Question n. 2 concerned the *strengths and weaknesses in the health services received*. Thirty-two (80%) caregivers highlighted 43 positive aspects, 32 (74.4%) practical and 11 (25.6%) psychological. Among the practical aspects, the continuity of heath care provision, even at home, the use of the personal protective equipment (PPE) provided for, the monitoring of the patient' health conditions – even at a distance – were the most appreciated ones. Among the psychological aspects, the continuous support provided to the patients by the health professionals even remotely, was perceived as a main strength. Looking at the weaknesses, 32 caregivers completed the item. Of them, 21 (65.6%) did not find any negative element, while 11 (34.4%) identified as negative aspects the suspension of the rehabilitation during the lockdown, and the bureaucratic delays.

Question n. 3 concerned the presence of potential *positive aspects of the pandemic on daily life activities*. The caregivers identified 19 positive aspects, mainly psychological. Among them, the continuous presence of family members at home and the closer family contacts were the most cited ones. Four caregivers did not report any further change in family daily life due to pandemic.

Question n. 4 focused on the *critical aspects on daily life activities.* The caregivers identified 26 negative aspects, 17 (65.4%) of which were practical and 9 (34.6%) were psychological. Among the practical aspects, reduction of social contacts, difficulties in doing daily shopping and in travelling for medical visits were the most frequently reported ones. Among the psychological aspects, the fear of contagion prevailed.

Question n. 5 concerned *positive aspects present in the families*. Twenty-four positive aspects were underlined, of which 8 were practical and 16 were psychological. Among the practical aspects, home assistance and support for the disabled people were the most frequently mentioned, while among the psychological aspects to be together at home, not feeling abandoned, counting on the staff professional support, were those most appreciated. One caregiver stressed the importance of religious faith in dealing with daily troubles.

Question n. 6 concerned the *negative aspects present in the families*. Caregivers identified 28 negative aspects,

both practical (19, 67.8%) and psychological (9; 32.2%). Architectural barriers, difficulties in accessing health services, economic difficulties, and lack of support from the Institutions were the most frequently mentioned practical aspects. Loneliness, isolation due to fear of contagion, lack of psychological support and increased burden due to patient's management were among the most negative psychological aspects.

Question n. 7 invited caregivers to *report any change that G. Torre Centre should implement to improve the health service offered*. On this question, the caregivers were equally divided, as 20 (50%) did not suggest any changes and underlined their full satisfaction with the received health assistance, while 20 (50%) reported 13 suggestions. The most frequent suggestions included the reinforcement of the multidisciplinary team and of the psychological support for patients and their families. From a practical point of view, caregivers strongly recommended strategies to guarantee greater help in dealing with the bureaucratic procedures (access to economic benefits, renewal for physiotherapy prescription, etc.) as a priority.

Discussion

The COVID-19 pandemic had a deep impact on individuals' life, particularly on that of patients with MDs and their families due to the clinical characteristics of these disorders and the need of continuous rehabilitative treatments [3,6-9]. As underlined by Bertrand Recasens and Rubio [3], "COVID-19 pandemic has pushed health systems to their limit and forced readjustment of standards of care for different pathologies. Management of neuromuscular diseases becomes a challenge since most of them are chronic, disabling, progressive, and/or require immunosuppressive drugs."

We have previously shown [10] that, despite the critical issues emerging in the pandemic period, professionals of the G. Torre Centre highlighted 82 aspects (48.5%) referring to the resources used to cope with critical issues, and that emotional aspects prevailed on practical aspects both in resources (52.4 *vs* 47.6%) and in difficulties (57.5 *vs* 42.5%) categories. In particular, regarding patients' resources, the staff indicated that psychological benefits were greater than practical ones (87 *vs* 13%), in the form of improved intra-family relationships, feeling more cared for, and satisfaction for the received care. As for the patients' relatives, the staff indicated again more resources than difficulties (72.8 *vs* 17.2%), mainly concerning the emotional sphere, such as the perception of having a point of reference even in such a challenging time.

In this second part of our study, addressing the impact of COVID-19 pandemic on the rehabilitation care received from the perspective of patients and their caregivers, we found that patients highlighted both positive (prevailing) and negative aspects. Among the psychological positive aspects, closer family contacts, not feeling abandoned and counting on the constant professional Centre's support were the most cited. The prompt restart of rehabilitation services, the compliance of the professionals with the anti-COVID-19 rules, and their closeness always, were the most appreciated positive practical aspects. On the other hand, architectural barriers, difficult access to health services, economic difficulties, and lack of support from the Institutions were the most frequently mentioned negative practical points, while social isolation and isolation due to fear of contagion were the most signalled negative psychological aspects.

A prevalence of positive aspects (56.9% *vs* 43.1%) during the pandemic was reported also by caregivers who underlined the same psychological aspects reported by the patients. As for the negative aspects, most caregivers (92.6%) complained about increased bureaucratic and economic difficulties. This was particularly evident in poorer families.

Limitation of the study

Although this study may have methodological weaknesses, it is nevertheless the first study exploring the impact of the COVID-19 pandemic on a routine rehabilitation setting from the point of view of patients and their caregivers. In particular, the findings of this study highlight the resilience of patients and caregivers, and their ability to activate psychological resources even in such a difficult pandemic time.

Acknowledgements

The Authors thank the 54 patients and the 40 caregivers who have given their time.

Conflict of interest statement

The Authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

LM and LP conceived the project, wrote and revised the paper; GC contributed to the drafting of the questionnaires and analyzed the data; MGE and VT distributed and collected the questionnaires.

Ethical consideration

This study was approved by the Ethics Committee of the Naples 1 Local Health Authority, (Prot. 362/2021).

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

Written informed consent was obtained from each participant/patient for study participation and data publication.

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ACTA MYOLOGICA 2022; XLI: p. 95-98
doi:10.36185/2532-1900-073

CASE REPORT

Autosomal dominant Ullrich congenital muscular dystrophy due to a *de novo* mutation in *COL6A3* gene. A case report

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Mutations in the genes encoding collagen VI cause Bethlem myopathy (MIM 158810), Ullrich congenital muscular dystrophy (MIM 254090), and myosclerosis myopathy (MIM #255600). BM is a dominantly inherited disorder, characterised by proximal muscle weakness and joint contractures mainly involving the elbows, ankles, and fingers, which usually follows a relatively mild course. By contrast, UCMD is a severe muscular dystrophy characterized by early onset, rapidly progressive muscle wasting and weakness, proximal joint contractures and distal joint hyperlaxity. Rapid progression usually leads to early death due to respiratory failure. UCMD is usually inherited as an autosomal recessive trait though dominant *de novo* heterozygous variants have recently been reported. We describe a further patient with UCMD classical presentation who showed, at the NGS analysis, the *de novo* variant c.6210+1G > A in the intron 16 of the gene *COL6A3*, known in the literature as pathogenic (VCV0000949S6.5).

Key words: collagen VI disorders, Ullrich congenital muscular dystrophy, UCMD, COL6A3

Received: May 25, 2022
Accepted: June 11, 2022

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How to cite this article: Picillo E, Torella A, Passamano L, et al. Autosomal dominant Ullrich congenital muscular dystrophy due to a *de novo* mutation in *COL6A3* gene. A case report. Acta Myol 2022;41:95-98. https://doi.org/10.36185/2532-1900-073

Introduction

Collagen VI-related dystrophies (COL6-RDs) represent a continuum of overlapping clinical phenotypes with Bethlem myopathy (BM) at the milder end, Ullrich congenital muscular dystrophy (UCMD) at the more severe end, and a phenotype between UCMD and BM, referred to as intermediate COL6-RD [1-3].

Bethlem myopathy (OMIM #158810), is characterized by a combination of proximal muscle weakness and joint contractures. Hypotonia and delayed motor milestones occur in early childhood; mild hypotonia and weakness may be present congenitally. By adulthood, there is evidence of proximal weakness and contractures of the elbows, Achilles tendons, and long finger flexors. The progression of weakness is slow, and more than two thirds of affected individuals older than age 50 years remain independently ambulatory indoors. Respiratory involvement is not a consistent feature [2]. Ullrich congenital muscular dystrophy (UCMD, OMIM #254090) was originally described by Otto Ullrich in 1930 as ''congen-

ital scleroatonic muscular dystrophy'' characterized by generalized muscle weakness and striking hypermobility of distal joints in conjunction with contractures of more proximal joints and normal intellectual development. Additional findings may include kyphoscoliosis, torticollis, prominent calcanei, follicular hyperkeratosis, excessive scar formation following skin trauma. Respiratory insufficiency develops progressively, and ultimately patients almost invariably need ventilation support[3]. Decreased fetal movements are frequently reported. Some affected children acquire the ability to walk independently; however, progression of the disease results in a loss of ambulation around the age of 10 [3].

Intermediate COL6-RD phenotype is characterized by independent ambulation after the age of 11 and respiratory insufficiency that is later in onset than in UCMD (early 20s). In contrast to individuals with Bethlem muscular dystrophy, those with intermediate COL6-RD typically do not achieve the ability to run, jump, or climb stairs without use of a railing [1-3].

UCMD is considered to be a recessive condition and homozygous or compound heterozygous mutations have been defined in *COL6A2* and *COL6A3* genes. In contrast, the milder disorder Bethlem myopathy shows clear dominant inheritance, caused by heterozygous mutations in *COL6A1, COL6A2* and *COL6A3*. However, there is phenotypic as well as genetic overlap between these two disorders, as patients with Bethlem myopathy not uncommonly may have first symptoms at birth[2], and dominant mutations in COL6A1 were recently identified in a patient with a severe UCMD phenotype [4].

We report a further case of AD-UCMD, confirming that dominant mutations are not as rare as previously believed in patients with UCMD.

Case report

An 11.5-year-boy came to our observation for a clinical picture characterized by early tendon retractions, kyphoscoliosis, respiratory insufficiency (FVC 52%). The symptoms began at the age of 7-9 months, with delay in the acquisition of the motor skills: the child was unable to maintain the sitting position and never acquired autonomous walking. Serum CK values were normal. A muscle biopsy, previously performed at another hospital, showed a myopathic picture with variation in fiber size, increased interstitial connective tissue, and occasional necrotic and regenerating fibers. There was no cardiac involvement after ECG and echocardiogram, nor mental retardation or other intellectual impairments. After the consent of the parents a blood draw was taken for DNA analysis.

Over the years, muscle condition remained stable, while vital capacity progressively deteriorated (Fig. 1).

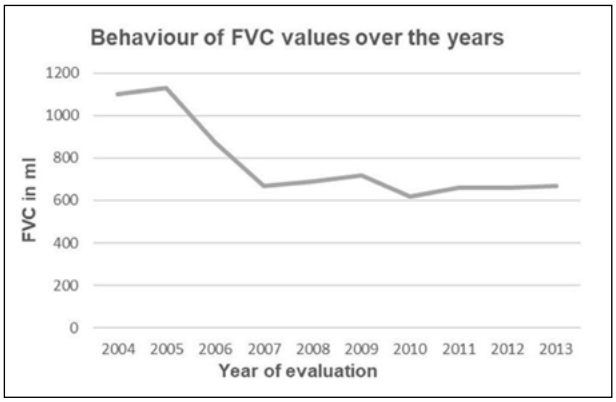

Figure 1. Changes in FVC values over the years.

Although he and his family had been advised several times to have a pulmonological consultation aimed at starting non-invasive assisted ventilation, the patient always refused it. He died at 25.9 years of acute respiratory failure.

Genotyping results

Genomic DNA was extracted from peripheral lymphocytes, by standard procedures. The analysis of *COL6A1* and *COL6A2* genes was negative for mutations. The trio whole exome sequencing, later performed, identified a heterozygous IVS16+1 G > A mutation in the mandatory 5' consensus splice site of intron 16 that accounts for the observed exon skipping. Importantly, the mutation was not present in either of the unaffected parents, indicating that it was a *de novo* mutation in the patient (Fig. 2). Because the patient's cDNA was not available and he refused to perform a skin biopsy, it was impossible to perform RNA RT-PCR from muscle or dermal fibroblasts.

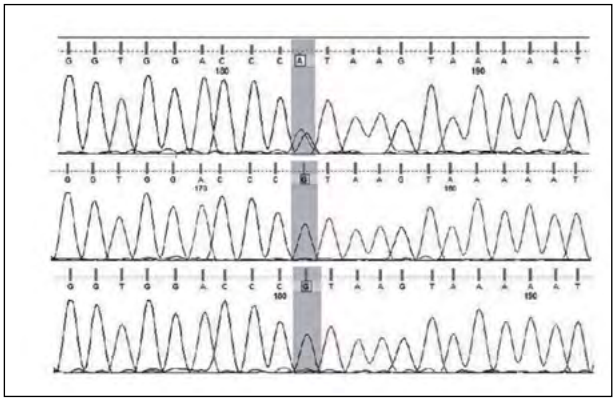

Figure 2. Sequencing of COL6A3 gene in the patient (Top), mother (Center) and father (Bottom). The variation G > A is present only in the patient.

Discussion

The genetics of the collagen VI related disorders is complex. Collagen VI is composed of three different peptide chains encoded by three large genes; the assembly of collagen VI involves a number of different stages. Different mutations may have variable effects on protein assembly, secretion, and its ability to form a functioning extracellular network [5]. In general, heterozygous mutations in the three collagen VI genes *COL6A1, COL6A2* and *COL6A3* cause BM, while homozygous or compound heterozygous mutations in *COL6A2* and *COL6A3* cause UCMD [2,3]. Accordingly, UCMD is considered to be a recessive condition, whilst the milder disorder BM a dominant condition. However, the model was recently questioned as *de novo* dominant collagen VI gene mutations have been found in more than half of severely affected UCMD patients [4-7]. The mutations that often affects the amino acid sequence in the N-terminal region of the triple helical domain before the single cysteine, are either splice site mutations that cause small in frame deletions in the triple-helical domains, or missense changes that alter the obligatory glycine residues of the repetitive Gly-X-Y sequences [7]. In contrast to the total absence or severe deficiency of collagen VI in recessive UCMD, abnormal collagen VI protein is abundantly present in the interstitial connective tissue between muscle fibres in the dominant UCMD patients [8], suggesting that the pathological mechanisms for the dominant and recessive patients are not identical. For instance, the presence of mutant collagen VI in the endomysium seems to alter the muscle extracellular microenvironment, which in turn may influence the cellular activities of the adjacent muscle cells in a manner that differs from the total absence of collagen VI protein in recessive UCMD [8].

The first *de novo* heterozygous deletion of the *COL6A1* gene resulting in the severe phenotype of classical UCMD precluding ambulation, was reported by Pan et al. [4] in 2003. Soon after Lampe et al. [5] reported that 10/26 patients with UCMD they studied, showed a single variation despite a severe presentation, and that 3 of them had the heterozygous variation c.6210+1 G > A splice donor in the intron 16 of the gene *COL6A3*.

Baker et al. [6] found heterozygous in-frame deletions in the N-terminal region of the triple helical domain, chain in 3/5 patients with a clinical diagnosis of UCMD, one of them in alpha3 (VI). The mutations were all located towards the N-terminal end of the 335–336 amino acid triple helical domain. Studies on protein biosynthesis and assembly of Collagen VI showed that these mutations act in a dominant negative fashion and result in severe collagen VI matrix deficiencies [6].

Briñas et al. reported that *de novo* mutations in *COL6A3* are usually located within the TH domains of the chains and that *COL6A3* intron 16 is mutated preferentially, making up 18% of the mutated alleles [7]. Furthermore they detected several exon skipping events due to dominant de novo splice-site mutations in 14/49 patients (28.5%), representing 21% of all mutations. Notably, they observed that different genomic mutations lead to identical consequences both at the mRNA and protein levels. For example, the skipping of *COL6A3* exon 16 can be caused by three different changes affecting the same donor site (c.6210 + 1 G > A, c.6210 +1 G > T, and c.6210 + 5 G > A). Lampe et al. [9] found that > 50% of the *de novo* mutations lead to heterozygous skipping of exon 16 in the alpha3 (VI) chain making this the single most common UCMD mutation mechanism. This was also the most common single exon skipping mutation in a recent series of collagen VI mutations from Japan [10]. In these cases, the severity has been explained as probably due to the proximity of the only cysteine residue encoded by exon 17 of *COL6A3*, which is thought to be involved in the disulfide bonds that assemble dimers and tetramers prior to secretion [11]. Taken together (Leiden Muscular Dystrophy Database; www.dmd.nl; references 5-11) these data indicate that at least as many as 50% of UCMD patients will have dominant mutations.

In conclusion, the patient described here confirms that collagen VI disorders represent a continuous clinical and genetic spectrum from severe to mild phenotypes, and that autosomal dominant UCMD caused by heterozygous *de novo* mutations are not as rare as previously believed.

However, the different patterns of inheritance are of great importance for the impact on genetic counselling of patients and their families.

Acknowledgements

None.

Conflict of interest statement

The Authors declare no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

LP: Conceptualization, methodology, data curation, original draft preparation, writing, review and editing and supervision; VN: formal analysis of genetic data, investigation and data collection; EP, AT: validation.

Ethical consideration

The study was conducted in accordance with the Declaration of Helsinki. The approval of the Ethics Committee was not necessary as no particular procedures other than those routinely performed were employed.

Written informed consent was obtained from the parents of the patient for genetic analysis and data publication.

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NEWS FROM AROUND THE WORLD

AIM

The XXII National Congress of the Italian Association of Myology will be held at the Auditorium R. Gervasio in Matera, from 19 to 22 October 2022.

The deadline for submitting abstracts is July 24. Similarly, it is possible to proceed with the registration for the event, by 17 July with a reduced fee.

To learn more, please visit the congress website *https://congressoaim2022.it*

MSM

Due to pandemics, the 14th Meeting of the Mediterranean Society of Myology (MSM) is moved to the 2023. Proposals to organize and host the event are welcome.

WMS

The 27th International Annual Congress of the World Muscle Society will take place 11th-15th October 2022, in Halifax, Nova Scotia, Canada. The congress venue is the Halifax Convention Centre at 1650 Argyle Street, in the heart of this Atlantic seaport. Electronic presentations and posters will be made available via the Society's platform as well as apps for delegates wishing to participate virtually. Hard work is being done to ensure that the registration process is as flexible as possible in these ever-changing and uncertain times.

To learn more, please visit the congress website: *https://www.wms2021.com*

FORTHCOMING MEETINGS

2022

June 10-12

257th ENMC Workshop: The 3rd ENMC workshop on Dystroglycan and the Dystroglycanopathies. Information: website: *https://www.enmc.org*

June 15-17

TREAT-NMD Conference 2022. Vancouver Convention Centre 1055 Canada Pl, Vancouver, BC V6C 0C3, Canada. Information: website: *https://treat-nmd-conference.org/*

June 17-19

261st ENMC Workshop. Management of safety issues arising following AAV gene therapy. Information: website: *https://www.enmc.org*

June 24-26

253rd ENMC workshop. Skeletal muscle laminopathies – natural history and clinical trial readiness. Information: website: *https://www.enmc.org*

June 25-28

8th EAN Congress. Vienna, Austria. Information: website: *https://www.ean.org*

July 5-9

17th International Congress on NeuroMuscular Diseases (ICNMD). Brussel, Belgio. Information: website: *www.icnmd.org*

July 15-16

AAN Summer Conference. Autoimmune Neurology and Neurology Year in Review. San Francisco, CA. USA. Information: website: *https://www.aan.com/events/summer-conference*

August 26-29

ESC Congress 2022. Barcelona, Spain. Information: website: *https://www.escardio.org*

September 13-15

7th Congress of Myology. Nice Acropolis, France. Information: website: *https://www.institut-myologie.org*

September 15-17

Mitochondrial Medicine Meeting. Nice Acropolis, France. Information: website: *https://www.institut-myologie.org*

October 11-15

27th Congress of World Muscle Society. Halifax, Canada. Information: website: *https://worldmusclesociety.org*

October 19-22

22nd Congress of the Italian Association of Myology. Matera, Italy. Information: website: *www.miologia.org; segreteria.aim@fclassevents.com*

2023

January 9-11

12th World Gene Convention. Sapporo, Japan. Information: website: *https://www.bitcongress.com/WGC2023*

April 22-28

75th AAN Annual Meeting. Boston, MA. USA. Information: website: *https://www.aan.com*

May 20-23

Heart Failure 2023 and World Congress on Acute Heart Failure. Prague, Czech Republic. Information: website: *https://www.escardio.org/Congresses-&-Events/Heart-Failure*

July 1-4

9th EAN Congress. Budapest, Hungary. Information: website: *https://www.ean.org*

October 3-7

28th Congress of World Muscle Society. Charleston, USA. Information: website: *https://worldmusclesociety.org*

2024

April 13-19

76th AAN Annual Meeting. Denver, CO. USA. Information: website: *https://www.aan.com*

June 29 - July 2

10th EAN Congress. Helsinki, Finland. Information: website: *https://www.ean.org*

October 8-12

29th Congress of World Muscle Society. Prague, Czech Republic. Information: website: *https://worldmusclesociety.org*

2025

April 5-11

77th AAN Annual Meeting. San Diego, CA. USA. Information: website: *https://www.aan.com*

October 7-11

30th Congress of World Muscle Society. Vienna, Austria. Information: website: *https://worldmusclesociety.org*

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INSTRUCTIONS FOR AUTHORS

Acta Myologica publishes articles related to research in and the practice of primary myopathies, cardiomyopathies and neuromyopathies, including observational studies, clinical trials, epidemiology, health services and outcomes studies, case report, and advances in applied (translational) and basic research.

Manuscripts are examined by the editorial staff and usually evaluated by expert reviewers assigned by the editors. Both clinical and basic articles will also be subject to statistical review, when appropriate. Provisional or final acceptance is based on originality, scientific content, and topical balance of the journal. Decisions are communicated by email, generally within eight weeks. All rebuttals must be submitted in writing to the editorial office.

Starting from 2020, a publication fee of 200 Euros is required. The Corresponding Author must fill in the appropriate form and send it with the corrected proofs. 50% off is offered for members of Associazione Italiana di Miologia (AIM) and/or Mediterranean Society of Myology (MSM) in good standing with dues. A copy of the payment receipt for the current year is mandatory to prove the membership).

On-line submission

Manuscript submission must be effected on line: www.actamyologica.it according to the following categories:

Original articles (maximum 5000 words, 8 figures or tables). A structured abstract of no more than 250 words should be included.

Reviews, Editorials (maximum 4000 words for Reviews and 1600 words for Editorials). These are usually commissioned by the Editors. Before spontaneously writing an Editorial or Review, it is advisable to contact the Editor to make sure that an article on the same or similar topic is not already in preparation.

Case Reports, Scientific Letters (maximum 1500 words, 10 references, 3 figures or tables, maximum 5 authors). A summary of 150 words may be included.

Letters to the Editor (maximum 700 words, 5 references). Letters commenting upon papers published in the journal during the previous year or concerning news in the myologic, cardio-myologic or neuro-myologic field, will be welcome. All Authors must sign the letter.

Rapid Reports (maximum 400 words, 5 references, 2 figures or tables). A letter should be included explaining why the author considers the paper justifies rapid processing.

Lectura. Invited formal discourse as a method of instruction. The structure will be suggested by the Editor.

Congress Proceedings either in the form of Selected Abstracts or Proceedings will be taken into consideration.

Information concerning new books, congresses and symposia, will be published if conforming to the policy of the Journal.

The manuscripts should be arranged as follows: 1) Title, authors, address institution, address for correspondence; 2) Repeat title, abstract, key words; 3) Text; 4) References; 5) Legends; 6) Figures or tables. Pages should be numbered (title page as page 1).

Title page. The AA are invited to check it represents the content of the paper and is not misleading. A short running title is also suggested.

Key words. Supply up to six key words. Wherever possible, use terms from Index Medicus – Medical Subject Headings.

Text. Only international SI units and symbols must be used in the text. Tables and figures should be cited in numerical order as first mentioned in the text. Patients must be identified by numbers not initials.

Illustrations. Figures should be sent in .jpeg or .tiff format. Legends should be typed double-spaced and numbered with Arabic numerals corresponding to the illustrations. When symbols, arrows, numbers, or letters are used to identify parts of the illustrations, each should be explained clearly in the legend. For photomicrographs, the internal scale markers should be defined and the methods of staining should be given.

If the figure has been previously published a credit line should be included and permission in writing to reproduce should be supplied. Color photographs can be accepted for publication, the cost to be covered by the authors.

Patients in photographs are not to be recognisable

Tables. Tables should be self-explanatory, double spaced on separate sheets with the table number and title above the table and explanatory notes below. Arabic numbers should be used for tables and correspond with the order in which the table is first mentioned in the text.

References. Indicate all Authors, from 1 to 3. If their number is greater than 3, indicate only the first 3, followed by “et al.” . Arabic numbers in the text must be superscript. References in the list must be numbered as they appear in the text, with the reference number superscript. **DOI name must be included with each reference** (when available). If not available, indicate the PMID number.

Examples of the correct format for citation of references:

Journal articles: Shapiro AMJ, Lakey JRT, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230-238. doi.org/10.14639/0392-100X-1583

Books and other monographs: Dubowitz V. Muscle disorders in childhood. London: WB Saunders Company Ltd; 1978.

Please check each item of the following checklist before mailing:

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- Summary (maximum 250 words).
- References, tables and figures cited consecutively as they appear in the text.
- Figures submitted actual size for publication (i.e., 1 column wide or 2 columns wide).
- Copyright assignment and authorship responsibility signed (with date) by all Authors.
- References prepared according to instructions.
- English style.
- Patients in photographs not recognisable.