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(Myopathies, Cardiomyopathies and Neuromyopathies)

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Mediterranean Society of Myology
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Founders: Giovanni Nigro and Lucia Ines Comi

Three-monthly

G. CONTE ACADEMY - NAPOLI

PACINI EDITORE MEDICINA

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Special issue dedicated to the memory of Professor Giovanni Nigro, on the 25th anniversary of the World Muscle Society constitution

Guest Editors

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Corrado Angelini
Giovanni Meola
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Issue presentation

This issue is dedicated to the memory of Professor Giovanni Nigro, in the occasion of the 25th anniversary of the World Muscle Society (WMS), he contributed to constitute in 1995 in Bologna (Italy).

The suggestion came from Luciano Merlini, who invited us to make it happen with the contribution of those who knew, appreciated, and loved him.

The project immediately found us favorable. Therefore, we made a tentative list of "Myology friends" to whom we sent the request for a contribution. The response was immediate, and the request accepted enthusiastically by many friends from Europe, USA, Brazil, Australia.

We are very happy to pay this little tribute to Prof. Nigro who dedicated his life to the social and scientific progress of Myology.

Vincenzo Nigro
Assistant Editor

Luisa Politano
Editor in Chief

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25th Anniversary of the Founding of the World Muscle Society (1995-2020). The Contribution of Prof. Giovanni Nigro (1931-2017)

Luciano Merlini

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For the 25th Anniversary of the World Muscle Society (WMS), I would like to commemorate Giovanni Nigro's contribution to the founding and prosperity of this organization, which he served as treasurer for 12 years after its foundation.

The conception and birth of the WMS occurred very quickly in over the course of a five-month period marked by two moments: a meeting in Bologna on 6 March 1995 by Victor Dubowitz, Giovanni, and myself, which was followed by a memorable celebration dinner, and the meeting of the organization's 15 founding members in London on 4 June 1995, which was followed by an equally memorable dinner. From the beginning, we instinctively adopted two attitudes (excitement and enjoyment) in our activities. Then, in 2001, Victor dubbed us the Triple E Society (Education, Enjoyment, and Excitement) to reflect the three important themes of WMS congresses [1].

In early February 1995, Victor and I began to reflect on the opportunity to create a new scientific society specifically dedicated to muscle diseases. We gave this idea the provisional name of "International Muscle Society".

Our reasons were as follows:

1. A specific society for muscle disorders did not exist, which was a bit surprising given the widespread interest and multidisciplinary nature of the field.
2. There was indeed a subdivision of the World Federation of Neurology related to neuromuscular disorders. However, it organized international congresses only every four years, which is too long to keep up with progress in the field. Above all, they lacked multidisciplinary breadth and focused mainly on neurological issues.
3. The workshops of the European Neuromuscular Center (ENMC) represented a frequent meeting point, but by its nature, it was confined to a single disease and limited to a single group of experts.
4. Two examples of what we had in mind, even if they had a regional basis, were i) the Mediterranean Society of Myology (MSM) that Giovanni Nigro established in 1993 in Ischia, which brought together members from 22 countries, especially in the Mediterranean region, and which organized biennial meetings in Naples that attracted a good international participation, and ii) Georges Serratrice, who organized similar meetings in Marseilles at approximately two-year intervals. Nigro and Serratrice collaborated in 1995 for a combined meeting in Marseille.

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5. By 1995, the journal *Neuromuscular Disorders* (NMD), which was founded in 1991, was well established as a high-quality journal and the recognized leader in the field, but it suffered from reduced impact due to poor circulation.

We therefore decided to hear Giovanni Nigro's opinion. On February 20, I called Giovanni, who was enthusiastic about the idea of the new society, and he declared himself available for a meeting. The meeting took place on the afternoon of Monday, 6 March 1995, in my office at the Rizzoli Orthopedic Institute in Bologna. Giovanni was quite enthusiastic about the concepts expressed above, and he gave us some useful hints, thoughts, and ideas in this context, including the concept of a "world" society rather than an "international" one. As he pointed out, any meeting that involves more than one or two countries is in fact international. We immediately liked the change, and the society has been called the World Muscle Society – WMS ever since. Our first priority was to get a core executive committee going to organize the society's basic charter. Giovanni suggested the three of us evaluate the MSM's charter as a template. We then decided that the next important step was to call a meeting of the new society's founding members, who would be internationally representative of the neuromuscular field's multidisciplinary nature and geographic spread. We drew up a tentative list of 15 eminent individuals worldwide, along with five reserves in case some of our initial choices had difficulty attending. Victor offered to host a meeting of the founding members in London, and we set the date for Sunday, 4 June 1995.

Finally, Victor came up with the idea of making NMD our official journal and Giovanni agreed. Giovanni explained that Acta Cardiomyologica, of which he was the Editor, was not constant in the publication of the issues that often concerned the proceedings of the congresses he organized. So, he felt it was more appropriate for NMD to be the Society's official journal.

As usual, in the best Bolognese tradition, which Victor had been able to appreciate for several years, we ended the day by going to dinner at a famous restaurant to celebrate the conception of the new society and toast its health and prosperity. Both of my guests enjoyed the typical Bolognese specialties and the sparkling Lambrusco wine. Of course, we asked the waiter to photograph us after the memorable day (Fig. 1).

Communications between us at the time took place by fax, so, of course, things proceeded as fax as possible (AFAP). A list of 70 potential foundation members was prepared, and all but two responded positively to the need for the new society. In addition, 15 agreed to attend the meeting in London.

The foundation meeting was held at the Ciba Foundation, 41 Portland Place, London W1, on Sunday, 4 June 1995, from 9:30 a.m. to 6:00 p.m. The agenda was as follows:

Figure 1. Victor Dubowitz, Giovanni Nigro and Luciano Merlini at celebratory dinner after conception of the World Muscle Society in Bologna, on Monday, 6 June 1995.

1. Formation of new society / founding members;
2. Charter (draft to be tabled at meeting);
3. Proposed structure of:
   - Executive committee;
   - Advisory committee.
4. Election of executive committee;
5. The society's official journal;
6. The society's first congress;
7. Other business.

The founding members in attendance were Corrado Angelini (Italy), Victor Dubowitz (United Kingdom), Laszlo Dux (Hungary), Lars Edstrom (Sweden), Robert Griggs (United States), Hyam Isaacs (South Africa), Jean-Claude Kaplan (France), Luciano Merlini (Italy), Giovanni Nigro (Italy), Eijiro Ozawa (Japan), Georges Serratrice (France), Hideo Sugita (Japan), Michael Swash (United Kingdom), Fernando Tomé (France), and Gerta Vrbova (United Kingdom) (Fig. 2).

Everything happened in the best and most productive way. The name of the society, World Muscle Society (WMS), was approved, as was its charter, which included the following as its main aim: "To provide a multidisciplinary scientific forum to advance and disseminate knowledge in the neuromuscular field for the benefit of patients".

An executive board was then elected. It consisted of a president (Dubowitz), a secretary (Merlini), a treasurer (Nigro), and eight members: Kiichi Arahata (Japan), Laszlo Dux (Hungary), Robert Griggs (United States), Eric Hoffman (United States), Francesco Muntoni (United Kingdom), Georges Serratrice (France), Fernando Tomé (France), and Thomas Voit (Germany).

The executive board agreed to adopt NMD as the official journal of the WMS. In addition, it elected an inter-

Figure 2. Foundation meeting of the World Muscle Society, held at the Ciba Foundation in London, on Sunday, 4 June 1995. From left back row Corrado Angelini, Laszlo Dux, Gerta Vrbova, Robert Griggs, Michael Swash, Georges Serratrice, Giovanni Nigro, Jean-Claude Kaplan, Eijiro Ozawa, Luciano Merlini; front row Hyam Isaacs, Lars Edstrom, Hideo Sugita, Fernando Tomé, Victor Dubowitz.

national advisory board comprising 68 clinicians and scientists from various specialties and parts of the world who had expressed interest in forming the society. Finally, the executive board approved Dubowitz's proposal to organize the first WMS congress in London in September 1996.

Thus, WMS was born. It was celebrated the same evening during a dinner offered by Victor (Fig. 3). In the editorial that Victor wrote for NMD in 2005 to celebrate the WMS's 10th anniversary, he included a description of that memorable dinner, which I share below [1]:

"We then set off for a celebratory dinner at Chez Gerard, a French restaurant nearby in Charlotte Street. It was reassuring that our French colleagues were reasonably happy with the quality of food as well as the wine. One of our foundation members who had a passion for whiskey and a relative aversion for wine, was able to achieve level pegging with the wine drinkers on a glass for glass basis, and considerably enhanced our bill for the evening".

Since then, the WMS has thrived by organizing annual conferences in various parts of the world, thus contributing to the advancement and dissemination of knowledge in the neuromuscular field for the benefit of patients.

Figure 3. Michael Swash and Giovanni Nigro at celebratory dinner after foundation of the World Muscle Society in London, on Monday, 6 June 1995.

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[1] Dubowitz V. A short history of the World Muscle Society. Neuromuscul Disord 2005;15:642-7. https://doi.org/10.1016/j.nmd.2005.07.003

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Giovanni Nigro
A note of remembrance

Lefkos T Middleton, MD, FRCP

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Acta Myologica is proud to celebrate, with this special issue, the 25th anniversary of the World Muscle Society (WMS). An important landmark for the field of neuromuscular diseases, made possible through the vision and energy of its three founders, Victor Dubovitz, Luciano Merlini and Giovanni Nigro.

This is a unique opportunity for me to reflect on and share my thoughts of my dear friend Giovanni Nigro, who left us on October 13th, 2017. His legacy and memory will remain vivid with us, his friends, colleagues and patients, as well with future clinicians and researchers in neuromuscular diseases.

Giovanni first became Professor of Medical Pathology at the early age of 29 and then Professor of Therapy, at the Federico II University of Naples (now known as the University of Campania "Luigi Vanvitelli"). Over the years, he developed a keen research interest in muscle diseases and their cardiac complications. He created, in 1971, the first rehabilitation centre for muscular dystrophy patients, in the region of Campania and, in 1976, the first centre of Cardiomyology and Myology at his University Hospital. In 1981, he organized the first international conference of myology, in Naples. He went on to establish the journal of Cardiomyology, in 1982.

I had the privilege to meet, and soon develop strong ties of friendship with Giovanni in the late 80s. From our very first meeting, I was impressed by his dedication and enthusiasm in advancing the science and our understanding of the biology and genetic basis of inherited neuromuscular diseases. Giovanni and I had several long discussions, often over a glass of wine and a nice dinner, on the high prevalence of these diseases, mainly of autosomal recessive inheritance, in the Eastern and Southern Mediterranean countries, of known high consanguinity rate. Giovanni was passionate about developing a Mediterranean network, aimed at facilitating the creation of specialized neuromuscular centres in countries of the Mediterranean basin. His dream was to improve the quality of patients' care and to foster scientific collaboration and links, connecting existing regional centres of excellence with the medical community and patients of the wider region. This was the basis for his vision to create and launch the Mediterranean Society of Myology (MSM) at the first MSM Conference that he organized that year on the beautiful island of Ischia. In the following years, Giovanni and I were in regular contact, both at the MSM Conferences, organized (every second year) in different Mediterranean countries and at other conferences and committee meetings, such as those of the European Neuromuscular Centre, in the Netherlands. The impact of MSM, in the 90s, was truly impressive, as several regional Hospitals from Middle Eastern and North African countries became involved and strong links of collaboration were developed within the Mediterranean region,

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under the auspices of MSM. Over time, he also formulated the vision of transitioning the Cardiomyology journal into a dedicated journal in neuromuscular diseases. Acta Myologica was established in 1997, aimed at providing a publication platform of novel and impactful research but, also importantly, to become an educational tool in disseminating state-of-art scientific and medical knowledge to young physicians and scientists in the region and other developing countries. In those pre-pubmed years, they would not normally have access to such scientific information. Giovanni was adamant that there should be no barriers to accessing the journal and, therefore, that Acta should be free of subscription or other fees. This was a precursor of the *open access* model, which has now become the norm, within the medical and scientific literature. He undertook (quite successfully) the massive task of securing independent funding and sponsorship to ensure the journal's sustainability.

Giovanni's infectious enthusiasm and energy have remained unaffected over the years, as he continued working hard in his role of Editor-in-chief of Acta Myologica and in organizing the MSM conferences in 2011, 2013 and 2015 and, notably, the World Federation of Neurology Congress, in 2010, well beyond his formal retirement. Throughout the years, I have always been impressed by Giovanni's unique personality, optimism and passion for the neuromuscular field, as well as his contributions to improving the care of patients with neuromuscular diseases and facilitating and fostering educational programmes to developing countries. Parallel to science, Giovanni has always been patient-centric, both *vis-à-vis* his individual patients, to whom he was a trusted friend, as well as being their treating physician; and all patients with muscular dystrophy and their families, around the globe.

Throughout his illustrious medical and academic career, Giovanni has remained a true gentleman, a generous host to all colleagues attending MSM and other conferences and a dear friend to many of us. As Luisa and Vincenzo emphasized in their 2018 Obituary in Neuromuscular Disorders, he will also be remembered for his quick wit, his ability to make light of difficult situations (with a smile) and capacity to come back stronger after each setback. Above all, he was a family man, devoted to his beloved wife, Maria Rosaria, from whose loss he could never recover; and his three children, Vincenzo, Angela Marina, and Gerardo. Also, his wider family of close associates; among several colleagues trained by and having worked with Giovanni, a special note for Luisa Politano and the late Lucia Comi, whose optimistic personality and inimitable smiling face shall remain in our hearts.

Vincenzo and Luisa are now carrying Giovanni Nigro's torch. What a task!

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ORIGINAL ARTICLES

Causes of clinical variability in Duchenne and Becker muscular dystrophies and implications for exon skipping therapies

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Becker muscular dystrophy is caused by mutations in the *DMD* gene that permit significant residual dystrophin protein expression in patient muscle. This is in contrast to *DMD* gene mutations in Duchenne muscular dystrophy where little or no dystrophin is produced (typically < 3% normal levels). Clinically, Becker muscular dystrophy is extremely variable, from slightly milder than DMD, to asymptomatic hyperCKemia at old age. The factors driving clinical variability in Becker muscular dystrophy have now been studied in some depth, and the findings are likely highly relevant to anticipated clinical findings in exon skipping therapy in DMD. The specific mutations in Becker dystrophy play an important role, and clinical variability is less with high frequency mutations (deletions exons 45-47, 45-48). The percentage of dystrophin content in patient muscle is not well-correlated with clinical findings. Muscle MRI findings (degree of fibrofatty replacement) are very well-correlated with the degree of patient disability, regardless of mutation or muscle dystrophin content. Taken together, data to date suggest that the main determinant driving clinical disability in Becker dystrophy patients is the degree of fibrofatty replacement in muscle. Thus, as with DMD, *DMD* gene mutations and resulting dystrophin protein abnormalities initiate the disease process, but downstream tissue pathophysiology plays a dominant role in disease progression. Factors influencing the age-dependent rate of fibrofatty replacement of muscles are responsible for much of the clinical variability seen in Becker dystrophy , as well as Duchenne dystrophy. These fibrosis-related factors include genetic modifiers, degree of muscle inflammation, and induction of microRNAs in muscle that bind to dystrophin mRNA and down-regulate dystrophin protein content in patient muscle. Studies to date regarding clinical variability in Becker dystrophy suggest that exon skipping therapy in DMD may show variable efficacy from patient to patient.

Key words: Duchenne muscular dystrophy, Becker muscular dystrophy, dystrophin

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Conflict of interest
The author is a stock holder and holds management roles in ReveraGen BioPharma, AGADA BioSciences, and TRiNDS LLC.

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Genotype/phenotype correlations in Becker dystrophy (or lack thereof)

Often genotype/phenotype correlations are discussed as 'good' or 'bad' – there is a strong correlation of specific genotypes with phenotypes, or not strong. The *DMD* gene is the largest in the human genome, and also complex with multiple transcriptional initiation points (alternative promoters), over 80 exons, all spread out over 2 megabases of the X chromosome (Fig. 1). With such complexity, and the highest spontaneous mutation rate of any gene (1 in 10,000 sperm and eggs), genotype/phenotype correlations might be expected to be more nuanced than "good or bad".

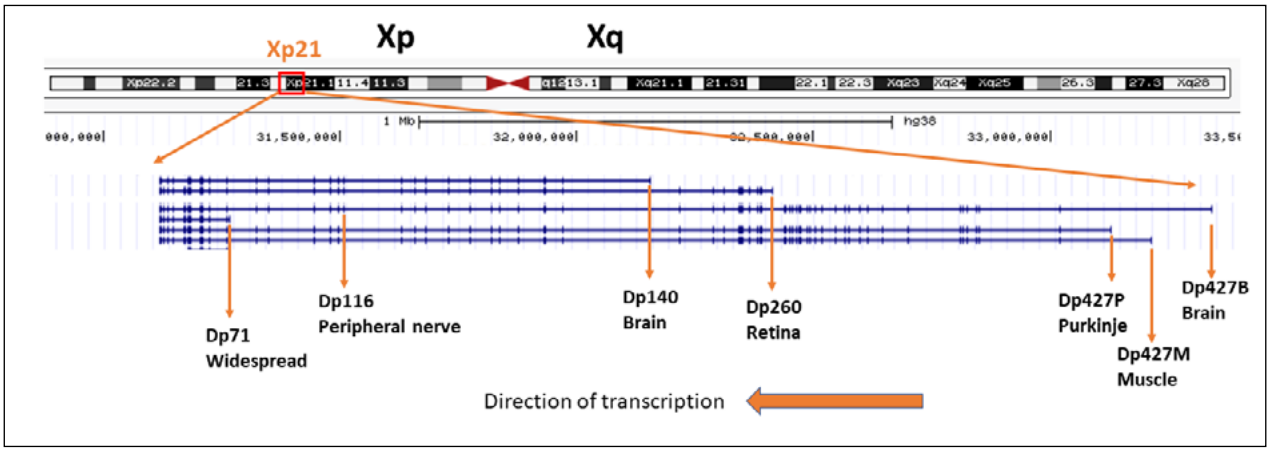

Figure 1. Schematic of the *DMD* gene. Shown is a genome browser visualization (www.genome.ucsc.edu), with labels added for the 6 different gene promoters, resulting mRNAs, and encoded proteins. Gene mutations in the first half of the gene may affect dystrophin expression driven from the first 3 promoters driving 'full length' dystrophin (Dp427B, Dp427M, Dp427P), but leave intact expression of downstream promoters (Dp71, Dp116, Dp140). Mutations located in the second half of the gene may disrupt expression or most or all dystrophin mRNAs and proteins driven from all promoters.

Excellent reviews of genotype/phenotype correlations in the dystrophinopathies have been published for both skeletal muscle and cardiac disease [1-5]. Dystrophin isoforms and pathologies in non-muscle tissues have also been well-described, such as retina [6], peripheral nerve [7], vascular smooth muscle [8-9], intestinal smooth muscle [10-11]. Assessments of dystrophin mRNA transcript levels in human tissues show relatively high levels in most tissues with smooth muscle layers (visceral and vascular), skeletal muscle, cardiac muscle, and peripheral nerve (Fig. 2). Specific mutations should affect different dystrophin mRNAs, and cell-specific dystrophin function in variable ways. Indeed, the genotype/phenotype correlations of *DMD* gene mutations with the latter half of the gene showing greater cognitive involvement, and correlation with 3' mRNAs expression in brain, are quite compelling [12,13].

With DMD and BMD mutations distributed throughout the *DMD* gene, affecting changes to the dystrophin mRNA and protein in different ways, sometimes isoform-specific, in multiple organ systems (skeletal muscle, cardiac muscle, smooth muscle, peripheral nerves and neurons), things are 'quite complicated'. Thus, the response to the question of the reliability of genotype/phenotype correlations in the dystrophinopathies is not so much 'good or bad', but instead, "Well, it's complicated".

The definition of a syndromic disorder is that multiple organ systems are affected. To date, the dystrophinopathies have been defined as a disease restricted to skeletal muscle, and hence called a non-syndromic 'muscular dystrophy'. It is probably time to re-evaluate this and acknowledge that both DMD and BMD are syndromic disorders, with involvement of multiple organ systems. This is likely an important distinction; the clinical phenotypes of syndromic disorders are acknowledged to be the interactive summation of perturbations of multiple organ systems. It is increasingly likely that the DMD and BMD phenotypes are interactive summation of perturbations of skeletal muscle, vascular smooth muscle, visceral smooth muscle, heart, peripheral nerve and central neurons. A corollary of this logic is that the *DMD* gene mutations initiate a process, but the downstream events leading to a phenotype are quite complex and variable; typical of syndromic disorders. Or stated in another way, if one views DMD and BMD as non-syndromic muscular dystrophies, then one could view the genotype/phenotype correlations as 'poor'. On the other hand, if one views DMD and BMD as syndromic disorders, the genotype/phenotype correlations could be considered 'quite good'.

What are known of drivers of clinical variability?

Becker muscular dystrophy is extremely clinically variable. Certainly, the gene mutation and resulting perturbations of the dystrophin protein are a major component of this variability (e.g. the extent to which dystrophin function is retained). However, it is possible to hold the BMD mutation constant with the most common mutations (deletion exons 45-47, and exons 45-48). Excellent correlative studies of genotype/phenotype, dystrophin content of muscle, MRI findings, and clinical symptoms

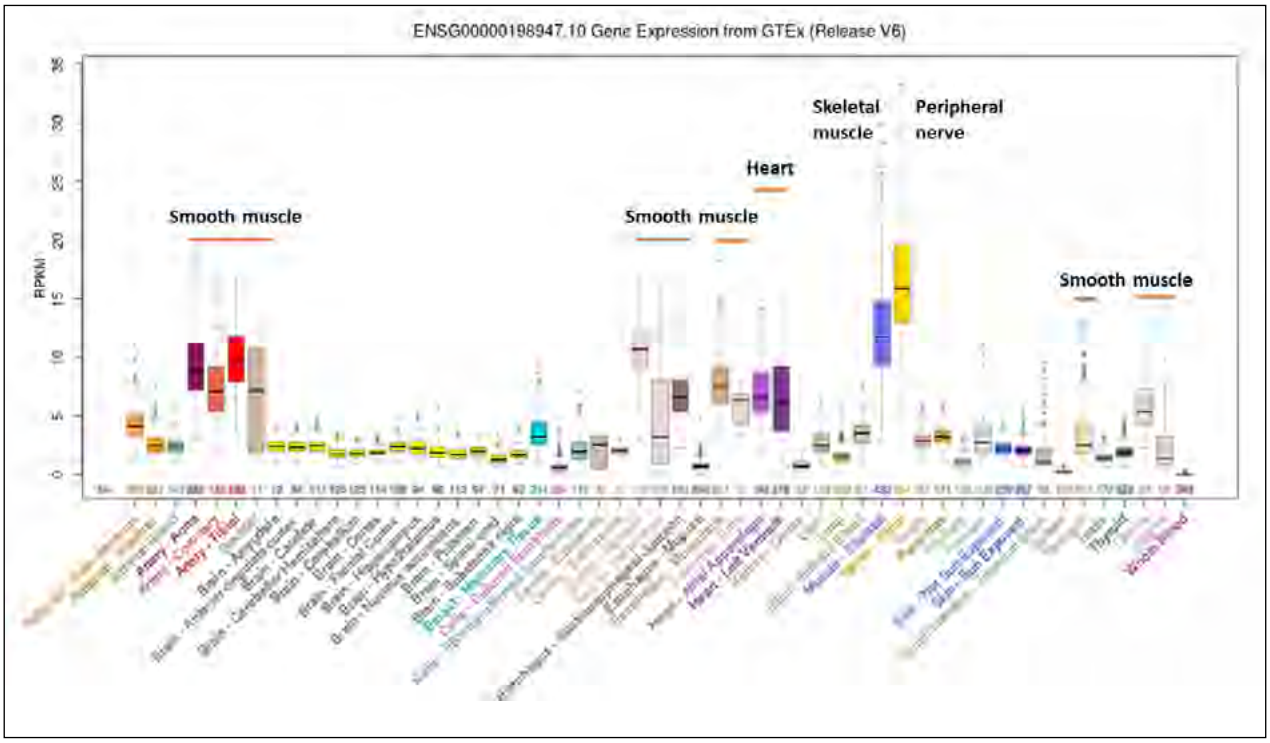

Figure 2. Dystrophin mRNA expression in human tissues. Shown is RNAseq data from multiple human tissues from the genome browser (genome.ucsc.edu). Dystrophin is highly expressed in all types of muscle (skeletal muscle, cardiac muscle, visceral smooth muscle, vascular smooth muscle), peripheral nerve, and some neurons. Dystrophin deficiency leads to pathology in most or all these tissues, but depending on the relative position of the gene promoter relative to the mutation involved.

have now been published in large series of Becker dystrophy patients [14,15]. These have shown that Becker patients do become more homogeneous when holding the mutation constant, but there remains extensive clinical variability both between patients with the same mutation, as well as within families with the same mutation. Thus, there are clearly variables downstream of the specific abnormal dystrophin that contribute to clinical variability. These studies also show that MRI findings in skeletal muscle (degree of fibrofatty replacement) is much more predictive of clinical disability (stage of disease) than is dystrophin mutation or dystrophin protein content (% of normal).

The fact that MRI measures of fibrofatty replacement are more predictive of clinical phenotype in Becker dystrophy than genotype (mutation) or biochemistry (dystrophin protein), points to the importance of cellular and tissue events downstream of the biochemical defect. Again, in Becker dystrophy, dystrophin abnormalities initiate a process, but clinical disability results from events downstream (fibrofatty replacement of muscle). The prominent (if not dominant) importance of cellular and tissue pathophysiology far downstream of the initiating dystrophin perturbations is clearly evident in Duchenne muscular dystrophy as well (where the biochemistry – dystrophin null – is held constant). In DMD, different muscle groups show dramatically different MRI findings, and the MRI findings correlate well with histopathology [16-17]. DMD is also a quite variable disease given a homogeneous biochemical defect, with marked clinical variability in young boys studied in a highly controlled clinical trial study (4 to < 7 years, steroid naïve) (Fig. 3). Genetic modifiers of DMD (common polymorphisms in other non-*DMD* genes) have generally been found to involve TGFβ fibrosis and inflammation cascades, consistent with the importance of the downstream progressive histopathology sensitively seen by MRI [18-21]. Genetic modifiers affecting disease severity through fibrosis pathways have been successfully replicated in the mouse [22].

The importance of pathophysiological processes, specifically early inflammation, later TGFβ, and end-stage fibrofatty replacement as critical drivers of clinical phenotype and disability cannot be disputed. Further, it is now well-established that genetic modifiers of dystrophinopathy in both human and mouse are centered on these downstream pathways. That said, *DMD* gene mutations

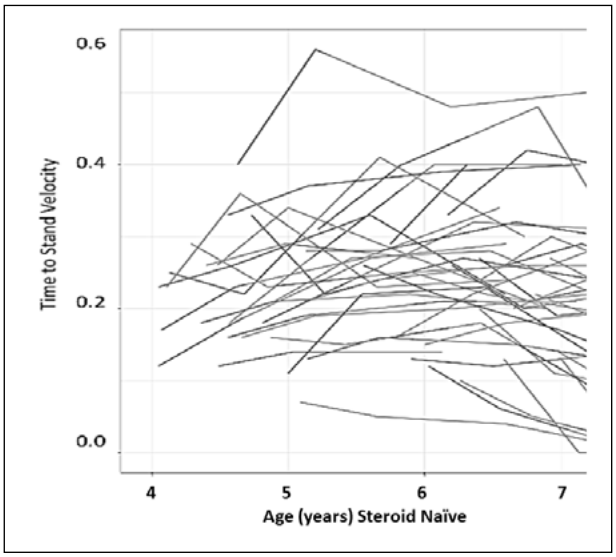

Figure 3. Clinical variability in 48 steroid-naïve Duchenne muscular dystrophy boys, age 4 to < 7 years. Shown is Time to Stand velocity (1/event) in DMD boys enrolled into vamorolone clinical trials. Extensive variability in disease severity is seen, with a 6-fold difference in velocity from the most severe to the mildest patient (from Dang et al., 2020 and Smith et al., 2020, mod.) [38],[39].

and resulting dystrophin production is not irrelevant, as it clearly initiates the process. Critical here is relatively low levels of dystrophin seen with certain out-of-frame 'leaky' mutations can mitigate the severity to some degree (~3-4%), although generally not to the point where the patient would be clearly characterized as a mild/moderate Becker patient. For example, an intermediate DMD/BMD phenotype was seen in a patient with an out-of-frame deletion that expressed low levels of dystrophin (~4%), and also had the LTPB4 rare genotype associated with milder disease [23]. Also, certain deletions seem generally 'leaky' permitting some low-level dystrophin, and many of these patients show later loss of ambulation. For example, exon 44 skippable mutations have about 3-5 year later loss of ambulation [24-27], whereas exon 51 skippable about a 2-year earlier loss of ambulation [26-27]. Mutations involving the initial exons of the dystrophin gene (e.g. del exons 3-7) are known to show mRNA translation of dystrophin at alternative AUG initiation codons, and thus result in dystrophin production despite an out-of-frame mutation.

The multiple studies of genetic modifiers of loss of ambulation in DMD have been increasingly robust and relatively corroborative, considering that such genetic studies are challenging in rare diseases (lack of statistical power). Indeed, the ability to identify and replicate genetic modifiers in DMD suggests that the 'effect size' of the modifiers is surprisingly large (hence easy to detect). It also speaks to the importance of the TGFβ fibrosis pathways, as the majority of genetic modifiers modulate this pathway. It can be concluded that genetic modifiers lead to differences in the speed of the transition of skeletal muscle from successful regeneration to failure of regeneration (and fibrofatty replacement).

Effect size of genetic modifiers can be quantitated in DMD by the number of years change in mean age at loss of ambulation (Fig. 4). Genetic modifiers to date all show about 1-2 years change in mean age of loss of ambulation (where mean age is LOA at around 11-12 years). One must consider the genotype frequencies, the inheritance model (dominant [SPP1, CD40], or recessive [LTBP4]), and the calculated percentage of DMD boys that have the "at-risk" genotype (Fig. 4). These allele frequencies also vary in different ethnicities and world populations. For example, in China the SPP1 genotype associated with earlier loss of ambulation in Europeans is at a very low allele frequency, and thus does not show significant association with LOA in Chinese DMD boys. However, a different SPP1 promoter at high allele frequency in Chinese DMD boys shows highly significant association with LOA. In effect, one might consider this an independent validation of the importance of SPP1 (osteopontin) in the progression of DMD [21].

Data emerging in genetic modifiers of heart involvement DMD and BMD are quite interesting. Dystrophin-deficient cardiac tissue shows distinct pathology and functional deficits compared to skeletal muscle. Heart does not show the repeated bouts of degeneration and regeneration, and fibrotic replacement is slowly progressive and limited initially to basolateral free wall of the left ventricle (likely due to inflammation and death of myocardiocytes where functional load on the heart tissue is highest) [28]. Also, dystrophin driven from the brain promoter can compensate for a deleted muscle gene promoter in skeletal muscle but not cardiac muscle [29]. One might expect some genetic modifiers to be consistent between dystrophin-deficient heart and skeletal muscle, and some distinct. Consistent with this, genetic modifiers can be found that are specific to heart[30], and also shared with skeletal muscle [31].

Pulling variables together into models of DMD and BMD disease progression

For DMD, the initiating event is loss of dystrophin in multiple tissues that likely interact to lead to gradual fibrotic replacement of skeletal muscle, and, later, heart. The initiating event is relatively homogenous, and generally leads to loss of ambulation about 11-12 years of age. Genetic modifiers, low level dystrophin 'leakiness' of the

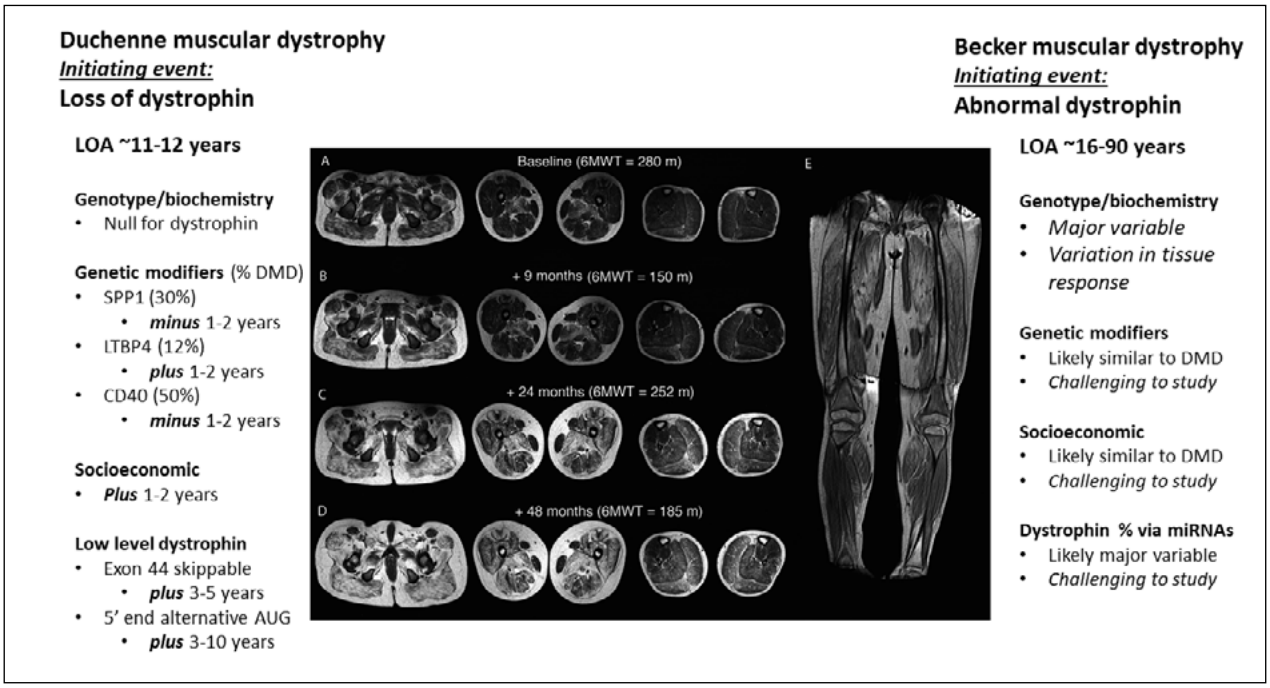

Figure 4. Factors contributing to clinical variability in Duchenne and Becker muscular dystrophies. Shown is a schematic of progressive MRI findings, with gradual fibrofatty replacement over time (from Godi et al., 2016, mod.) [17]. The progressive clinical phenotypes of both Duchenne and Becker muscular dystrophies is driven predominantly by the extent of fibrofatty replacement of muscle. The factors driving the fibrofatty replacement are listed for both Duchenne and Becker dystrophy, as discussed in the text. *Plus* and *minus* refer to later or earlier age at loss of ambulation.

mutation, and socioeconomic status[32] all contribute to increased severity or decreased severity relative to this 'average' (Fig. 4). For each genetic modifier, the inheritance pattern, and the allele frequency can be calculated from the published papers, and define whether the 'rare allele' genotype causes earlier loss of ambulation (e.g. minus 1-2 years), or later loss of ambulation (e.g. plus 1-2 years) (Fig. 4). Indeed, all genetic modifiers have been found to change the age of loss of ambulation by 1-2 years, with SPP1 and CD40 rare allele causing a more severe progression (minus 1-2 years) (both dominant inheritance patterns), and LTBP4 rare allele a milder disease progression (plus 1-2 years; recessive inheritance pattern). Likewise, the effect size of low level dystrophin expression due to exon 44 skippable mutations can be quantified (3-5 years later LOA), the effects of alternative AUG use and dystrophin expression in 5' mutations (plus 3-10 years), and the effects of socioeconomic factors (minus 1-2 years for low socioeconomic status [32] (Fig. 4). This begins to paint a more complete picture of factors influencing clinical severity in DMD, and also points to the multi-variate and complex nature of these factors.

For BMD, the initiating event of abnormal dystrophin is a much greater 'driver' of disease variability in onset and progression than it is in DMD where lack of dystrophin is held constant. The great heterogeneity of gene mutations resulting in many different abnormal dystrophin proteins with variable residual function leads to variability in the initiation and progression of disease. Moreover, it could be expected that different dystrophin-expressing tissues and cells may respond differentially to specific abnormal dystrophins. For example, the abnormal dystrophin resulting from a deletion of exons 45-47 may lead to some cellular and physiological abnormalities in skeletal muscle, but different abnormalities in smooth muscle. Thus, the 'syndromic' nature of dystrophinopathies may be accentuated in Becker dystrophy due to differential perturbations of different tissues (Fig. 4).

It is highly likely that the same genetic modifiers and effects of socioeconomic status observed in DMD are relevant to BMD as well. That said, it is nearly impossible to study and prove effects of genetic modifiers and socioeconomic status in Becker dystrophy. This is because the major effects of genotype and biochemistry (abnormal dystrophin) on clinical symptoms in Becker dystrophy creates extensive population stratification, leading to precipitous loss of statistical power. To detect the effects of genetic modifiers and socioeconomic status on phenotype in Becker dystrophy would require the study of cohorts of a single

Becker mutation (e.g. only those with either of the common BMD mutations; e.g. del 45-47 or del 45-48). One would not want to mix the del 45-47 and del 45-48 patients as they have different dystrophin proteins (and this is too much of a variable). As each of these two genotype groups is only about 15% of all Becker patients, it will be challenging to assemble such cohorts. Until these studies can be done, it is probably safe to assume that genetic and socioeconomic modifiers of DMD are shared in BMD (Fig. 4).

A variable that may drive clinical severity in Becker dystrophy that is not relevant to DMD is effects of microRNAs. The dystrophin mRNA has a very large number (~80) putative microRNA binding sites that have the potential to individually and/or collectively decrease protein translation from the dystrophin mRNA, and thus in turn lead to decreased amounts of dystrophin (in muscle, heart, smooth muscle or nerve) [33-35]. It has been well-established that inflammation in muscle leads to induction of inflammation-associated microRNAs, and these in turn bind to the dystrophin mRNA and decrease dystrophin protein content in muscle.

This inflammation/microRNA/dystrophin pathway seems to explain the highly variable dystrophin levels seen in Becker dystrophy patients, even when controlling for the same causative exon 45-47 mutation [33]. Further, inflammatory disease of muscle unrelated to dystrophinopathy appears to have the same pathway active, leading to reductions of dystrophin in skeletal muscle secondary to inflammation[35]. It is intuitively attractive to consider that the microRNA pathway has effects on Becker patient disease severity, assuming that 'more dystrophin is better' (Fig. 4). However, there is no evidence for this. Indeed, in contrast, there is little evidence that dystrophin levels between 20-100% normal are in any way correlated with clinical severity in Becker dystrophy [14,36]. Muscle biopsy studies include a high degree of 'sampling error', where only a small area of a single muscle, in a single stage of disease is studied for dystrophin protein amounts. Thus, a single biopsy is not representative of dystrophin levels in the patient as a whole. The lack of correlations of dystrophin content in Becker patient muscle biopsies with clinical phenotypes does not rule out the potential importance of the microRNA/dystrophin pathway. As with genetic modifiers, carrying out studies to prove the contribution of microRNA-mediated reductions in dystrophin in Becker patients would be important, but studies will be challenged to control for the mutation/protein stratification problem noted above.

Relevance of BMD models of clinical severity to exon skipping therapy in DMD

There are now 3 exon skipping drugs approved by the FDA in the USA, targeted towards exon 51 (Etiplersen, Sarepta), and exon 53 (Golodirsen, Sarepta; Viltepso, NS Pharma). The Sarepta drugs led to a mean ~1% normal dystrophin levels in treated DMD patient muscle (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/211970Orig1s000SumR.pdf; https://www.accessdata.fda.gov/drugsatfda_docs/nda/2016/206488Orig1s000SumR.pdf).

The NS Pharma drug showed induction of a mean ~6% (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2020/212154Orig1s000SumR.pdf). The NS Pharma drug has shown preliminary evidence of clinical benefit in 16 boys treated with viltolarsen, with drug-related improvements in multiple clinical motor outcomes [37]. All 3 drugs were approved under an "accelerated" pathway dependent on surrogate outcome measures (dystrophin protein by immunoblot).

In the context of the above discussion regarding the variables contributing to clinical variability in both Duchenne and Becker dystrophies, it may be instructive to interpret the status and promise of exon skipping in DMD. First, one can interpret existing exon skipping data in the context of clinically meaningful dystrophin levels. Data to date suggests that dystrophin levels ~3 – 10% of normal levels typically lead to an intermediate phenotype between Duchenne and Becker muscular dystrophies (sometimes called "severe Becker dystrophy") [14,36]. This is consistent with the ~3% dystrophin content of a leaky exon 44 mutation [23]. Given this data, the two Sarepta drugs with ~1% dystrophin would might not be expected to show significant clinical benefit. The report of a mean ~6% dystrophin induced from the NS Pharma drug is consistent with preliminary evidence of clinical benefit [37].

Assuming that exon skipping drugs were able to produce 3% or more of Becker-like dystrophin, one expects the extensive variability in patient clinical response given all the variables influencing clinical variability in Becker muscular dystrophy (Fig. 4). Functionalities of specific abnormal dystrophins, levels of dystrophin, microRNA/dystrophin pathways, genetic modifiers, and socioeconomic status are all likely to factor relatively heavily into the clinical response to exon skipping.

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Giovanni Nigro and the Naples's school: historical contribution to the knowledge of heart involvement in Duchenne/Becker muscular dystrophies

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It is now accepted worldwide that cardiac involvement in Duchenne and Becker muscular Dystrophies, is a constant feature. The concurrent impairment of the heart as a muscle in dystrophic process was an inspired idea by Prof. Giovanni Nigro ten years before the discovery of the dystrophin gene, occurred in 1987. This article is intended to be a recognition to him and to the Neapolitan School he directed for the contribution in the knowledge of cardiac involvement in the course of Duchenne (DMD) and Becker (BMD) Muscular Dystrophies and in DMD/BMD carriers.

Key words: dystrophinopathic cardiomyology, Duchenne muscular dystrophy, Becker muscular dystrophy, Duchenne/Becker carriers

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Giving honour to the memory of Professor Giovanni Nigro is not an easy task, considering the versatility of his character and his ability to be always 20 years ahead.

I had the chance to meet him at the 4th year of my medical degree at the Federico II Naples University, as he was my teacher in the two-year *Special Medical Pathology* course. I was immediately impressed and fascinated by his passionate lessons, especially those regarding the heart and muscular dystrophies. After passing the exam, I decided to carry out my thesis on muscular dystrophies. I asked him to attend his laboratory as an internal student and I was accepted.

I could never imagine how large could be the field that I was approaching, but from the first moment, muscle diseases – in the plurality of their aspects, myological, cardiological and genetic – came into my life as the most important subject.

Professor Nigro used to entrust internal students to his collaborators and to involve them in the research. I took part in the first census of muscular dystrophies he was carrying out in Campania. It was the second half of the 70s: I still remember the trips made from province to province in our region, consulting the lists of disabled people in the provincial offices,

and going house-to-house visiting patients to confirm or exclude the diagnosis of muscular dystrophy. The results were published in Muscle & Nerve in 1983 [1].

Giovanni Nigro was basically a cardiologist, heart was his main interest, hence his studies on cardiac involvement in muscular dystrophies. At that time there were no available statistical programs, so I remember the long days we students spent together reporting on large cardboards the data of hundreds and hundreds ECG traces, which had to be manually processed for statistical purposes. This huge work let him hypothesize for first that in muscular dystrophies the heart is primarily affected as a "muscle" [2]. However, his pioneering vision was accepted by the scientific community only after the discovery of the dystrophin gene [3] and the demonstration that the protein is expressed in the heart, in the same quantity as in muscles [4].

The cornerstone of his work remains the paper on the *Incidence and evolution of cardiomyopathy in Duchenne Muscular Dystrophy*, published in the International Journal of Cardiology in 1990 [5] and worldwide cited. In that paper, thanks to the three-four decades of experience with patients affected by Duchenne/Becker muscular dystrophy, he showed that cardiomyopathy associated with muscular dystrophies is constantly progressive and evolves passing from a pre-symptomatic condition (P type) [5] to dilated cardiomyopathy and intractable heart failure [6] (Fig. 1).

The discovery of dystrophin strengthened his hypothesis that dystrophinopathic cardiomyopathy (DCM) can present with different clinical pictures based on the dystrophin alterations. He speculated that DCM is caused by a complete absence of dystrophin at the myocardium as in skeletal muscles in Duchenne patients, while in Becker (BMD) patients it is caused by a reduced/abnormal amount of dystrophin [7]. He pointed out in the latter how cardiomyopathy is frequently observed even before the age of 30 years; dilation can be the first manifestation of heart involvement and underlying myopathy [7]; the onset of arrhythmias can cause sudden cardiac death [8-10]; life expectancy is severely conditioned by the presence of cardiomyopathy [8,9] and finally how the onset and severity of cardiomyopathy is closely related to the type of dystrophin gene mutation [11].

The presence of a puzzling dilated cardiomyopathy in the mother of a Duchenne patient he followed for many

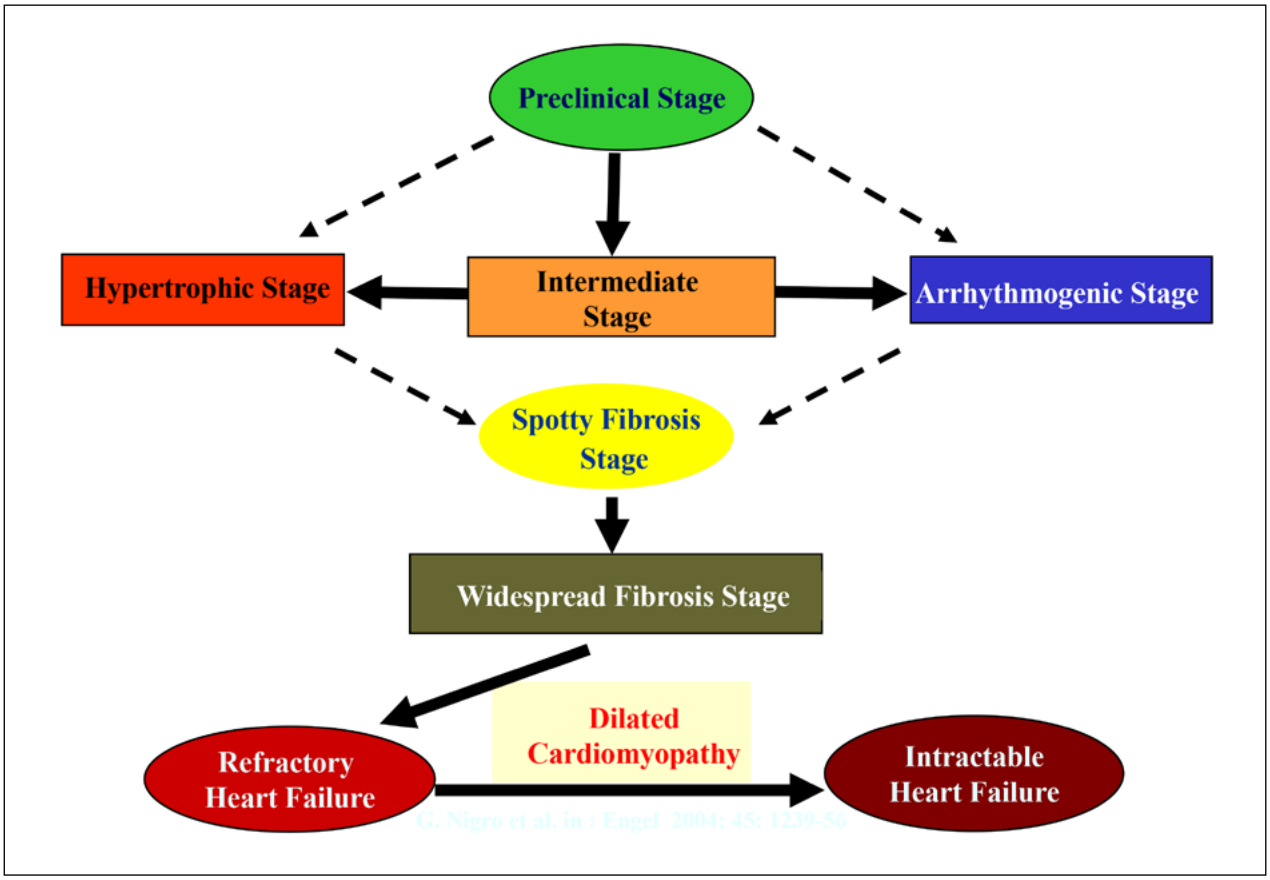

Figure 1. Evolution of Dystrophinopathic Cardiomyopathy according to Giovanni Nigro (1977).

years, led him to analyze the ECG traces and the echocardiograms of about 200 women obliged carriers of DMD/BMD, showing that cardiomyopathy is a common feature in this group of individuals and often the only marker of their carrier status [12,13].

Therapy was very important to him. He didn't used to passively follow the new "emerging" guidelines, but he strongly believed in the importance of a personalized therapy according to the patient clinical condition. For this reason, in the last 5 years of his career, after 45 years of teaching *Special Medical Pathology,* he asked and obtained to teach *Medical Therapy* to the 6th year students in the same University.

He used to teach that in all pathologies, but especially in muscular dystrophies, it is necessary to have a holistic view of the patient, hence the need for a multidisciplinary approach with the participation of experts from various disciplines, myologists, cardiologists, pneumologists, orthopedists etc. This model was immediately implemented by his staff, and "rediscovered" after 30 years as the ideal approach for muscular dystrophies [14].

Professor Nigro used to involve patients on the results of his own and others' research as well as any pharmacological new treatment [15-17], once excluded the possible side effects. He was open minded to any therapeutic possibilities, from the minimal orthopedic surgery suggested by Rideau in France in the early 80s [18-20] to the steroid therapy proposed at the Munich Congress in 1990, to the aminoglycosides perspectives.

The collaboration between Poitiers and Naples has always been intense and over time more than 200 DMD children underwent the same surgery also in Naples, getting an average extension of walking about 3 years, at a time when there were no other therapeutic options.

He immediately believed in the power of steroids to improve muscle strength in Duchenne boys, and indeed he was convinced that minimal surgery and steroids would act synergistically to improve the outcome. Likewise he was the first to believe and to practice the prophylactic use of ACE inhibitors, to delay the onset of overt cardiomyopathy in Duchenne and Becker patients [15-17] as far as the first in Europe to treat with gentamicin four patients with DMD caused by a stop codon dystrophin gene mutation, with promising results [21].

After about 20 years, during which more than 250 Duchenne patients had been treated with steroids (deflazacort [22] rather than prednisone), ACE-inhibitors and anti-oxidants, he was able to affirm that this treatment,

Figure 2. Prof Giovanni Nigro together with his collaborators and some of his internal students.

associated with physical rehabilitation, was effective not only for the motor function, but also on the onset and severity of cardiomyopathy and on the decline of the vital capacity curves [15-17,23].

All his thought is included in the chapter on cardiomyopathies associated with muscular dystrophies, in the textbook "Myology" published by Andrew Engel in 2004 [23], in which he was happy and honored to collaborate.

It was a privilege and an honour for me to have met and worked for so many years with such a *special* person. He has always put the patients and their families at the center of his interests, and it's thanks to him that I had the chance to meet so many special people.

I'd like to conclude this brief and certainly lacking tribute to Professor Nigro, with one of his sentences which summarizes his close relationship with muscle diseases: "Myology? The passion of a lifetime!".

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This contribution is a special thank in memory of Prof Giovanni Nigro from his coworkers and students (Fig. 2) who had the privilege of having him as a "Master" and sharing a life path with him.

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Impaired myocardial strain in early stage of Duchenne muscular dystrophy: its relation with age and motor performance

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Duchenne muscular dystrophy (DMD) is complicated by an early and progressive left ventricular (LV) dysfunction. Despite the reduction of ejection fraction (EF) usually manifests in the second decade, subtle alterations in LV mechanics can be detected earlier. Longitudinal and circumferential LV deformation, evaluated by speckle tracking echocardiography (STE), are considered sensitive markers of early dysfunction. We retrospectively examined clinical and echocardiographic data of 32 DMD children with preserved LV function. According to the median age, patients were then divided into younger and older than 9 years, and compared to 24 age-matched healthy subjects. Six-minute-walk test (6MWT), North Star Ambulatory Assessment (NSAA), and a comprehensive cardiac evaluation were performed. Although EF was within the normal range, DMD patients had significantly lower values than healthy controls, and the same occurred for the remaining conventional systolic and diastolic indices. Global longitudinal strain (GLS) was reduced in all patients (older and younger, both $p < 0.001$). Global circumferential strain (GCS) was reduced only in older patients (< 0.001). Both GLS and GCS worsened with age in DMD patients (GLS $p = 0.005$; GCS $p = 0.024$). GLS was significantly worse in the apical segments and in the postero-lateral wall. GCS in the antero-septal, anterior and antero-lateral segments was significantly reduced in older patients, with a prevalent involvement of the sole septal wall in the younger boys. 6MWT appeared to be correlated inversely to GLS and directly to EF. A longitudinal evaluation should be scheduled in DMD boys to assess the global cardiac performance over time and to evaluate the impact of therapies.

Key words: Duchenne muscular dystrophy, cardiomyopathy, speckle tracking echocardiography, strain, motor performance

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by a mutation in the gene encoding the dystrophin protein. It affects 1 in every 5000 live male births (20,000 new cases worldwide each year) [1]. The lack of dystrophin results in a cascade of events leading to progressive loss of muscle function and to a multisystemic involvement [2]. Myocardial dysfunction is part of the natural history of DMD, although timing of onset, progression and severity of myocardial involvement can vary [3]. Respiratory failure has been in the past the most common cause of morbidity and mortality in DMD patients. However, with the improvement of the respiratory support and the use of non-invasive ventilation, cardiomyopathy represents nowadays one of the main sources of morbidity and mortality in this clinical setting [4].

The prevalence of cardiomyopathy increases inexorably over the years, and therefore age is a significant predictor of cardiomyopathy in DMD patients. If 5% is the estimated prevalence of myocardial dysfunction in patients aged 2 to 5 years, it is expected to rise over 60% beyond 18 years [4,5]. Unfortunately, the progression of cardiac damage is concealed most of the times, due to the severe functional limitations of DMD patients. Clinical diagnosis of cardiac failure is mostly unreliable, since patients with very limited activity do not develop symptoms until severe ventricular dysfunction occurs. For these reasons, DMD multidisciplinary care recommends that cardiovascular assessment, including electrocardiogram (ECG) and echocardiography, has to be performed at diagnosis and then annually [2]. The presence of abnormal ventricular function suggests the need for increased surveillance and should prompt initiation of drug therapy, following general recommendations for heart failure treatment [2,6,7].

How can we better define "abnormalities of ventricular function"? Historically, the officially accepted parameter that represents left ventricular (LV) function is the ejection fraction (EF). However, as widely demonstrated, EF as well as other conventional systolic and diastolic parameters fail to reveal subclinical LV dysfunction. A significant reduction of EF below the normal limits occurs when the disease progression has already reached an advanced stage. Therefore, early markers of LV dysfunction have been extensively evaluated, and longitudinal deformation or strain is now considered as one of the most sensitive parameters of LV function, along with circumferential deformation. Myocardial strain represents the change in myocardial fiber length compared with its original length in the plane in which it is measured [8]. LV longitudinal function is expression of the contraction of subendocardial fibers, which are known to be particularly vulnerable to damage. Speckle tracking echocardiography (STE) is the most widely used method for the evaluation of longitudinal and circumferential strain of the left ventricle, and nowadays it represents a validated and reproducible technique [8-10].

The aim of our study has been to identify and define the onset of LV abnormalities in DMD children, not reporting cardiological symptoms and with preserved EF, in order to answer the following questions: (1) which parameters should we follow to better define LV dysfunction in the earliest stage? and (2) when are these parameters expected to become altered in the natural history of the disease? We also attempted, for the first time, to evaluate a correlation among cardiological parameters and functional motor performances, assessed with the most used outcome measures in DMD clinical trials.

Methods

We retrospectively examined clinical and echocardiographic data of 45 children with DMD, undergoing routine cardiovascular follow up evaluation in our Institution. We excluded from our analysis 10 patients with manifest LV dysfunction, expressed by a LV EF below 55%. We also excluded 3 cases with unsuitable echocardiographic recordings due to the low quality of patients' acoustic window. Therefore, we considered a comprehensive assessment, including neuromuscular and cardiovascular evaluation, of 32 DMD patients with preserved LV function (median age 9 years, IQR 6) and not referring cardiological symptoms. The study was approved by the local Ethical Committee (Prot. Number 105/16).

6-minute-walk test (6MWT)

The 6MWT has been chosen as the primary outcome measure in international multicentre investigational drug clinical trials as well as in longitudinal natural history studies in DMD ambulant patients. 6MWT was performed in all DMD ambulant boys older than 5 years using a modified version of the American Thoracic Society guidelines [11,12]. Modifications include the addition of continuous encouragement from the testing staff, and a "safety chaser" to walk along behind the subject during testing. The test is generally completed within 15 to 20 minutes. Suitability and inter-rater and intra-rater reliability in DMD for the 6MWT have already been reported [12,13].

North Star Ambulatory Assessment (NSAA)

The functional scale NSAA represents an ideal additional tool to the 6MWT, as it provides information on a wider spectrum of functions that reflect everyday life

activities. The scale consists of 17 items, ranging from standing (item 1) to running (item 17) and includes several items assessing abilities that are necessary to remain functionally ambulant. Each item can be scored on a 3-point scale using simple criteria: 2 - Normal, achieves goal without any assistance; 1 - Modified method, but achieves goal independently of physical assistance from another person; 0 - Unable to achieve independently. The score can range from 0, if all the activities are failed, to 34, if all the activities are achieved. The scale is generally completed in a maximum of 15 minutes [12].

Cardiac evaluation

Cardiovascular assessment was based on clinical evaluation, physical examination, 12-leads ECG and bi-dimensional (2D) echocardiography, particularly aimed to study myocardial mechanics. A conventional 2D-echocardiogram was performed with a Vivid 7 echocardiography equipment (GE Vingmed Ultrasound AS, Horten, Norway) in each case, and records were collected in a private local archive. Clear loops of the LV in apical 4-chamber (4-ch), 2-chamber (2-ch) and 3-chamber (3-ch) views, and also parasternal short axis views at the level of mitral valve (MV-Sax), papillary muscles (PM-Sax) and apex (AP-Sax) were available for the majority of patients. Three consecutive end-expiratory cycles, in gray scale (frame rate > 70 frames/s), have been stored twice for each view and were analyzed by two experienced independent operators, unaware of the clinical conditions of the patients. All measures and functional evaluations were processed off-line on the Echopac GE, Vivid 7 workstation. Left ventricular diameters, volumes and mass were calculated. Diastolic function was studied by mitral early inflow Doppler velocity (E wave), early to late (A wave) inflow Doppler velocity ratio (E/A), medial and lateral mitral annular tissue Doppler imaging (TDI), early inflow velocity (E') and the E/E' ratio. Systolic function was expressed as global LV function (EF), fractional shortening (FS), longitudinal systolic function by M-Mode echocardiography and TDI, global longitudinal and circumferential strain (GLS, GCS) by STE. EF was obtained with biplane Simpson's method. FS was calculated on M-mode modality. Longitudinal systolic function was assessed by mitral and tricuspid annular plane systolic excursion by M-mode echocardiography (MAPSE, TAPSE), and by TDI mitral annular systolic velocity (S'). Since lateral MAPSE and S' were not accurately sampled for the majority of patients, only medial values were considered. Furthermore, systolic longitudinal deformation of the left ventricle was studied by STE, recording both global, partial (3-ch, 4-ch, 2-ch views) and segmental strain values, expressed as percentage. Also, global, partial (at mitral valve [MV]-, papillary muscles [PM]- and apical [AP]-short axis [SAX] views) and segmental circumferential deformation were studied at basal, medial and apical LV level. For longitudinal strain calculation a semi-automated method was used, Automated Function Imaging – AFI (GE Vingmed Ultrasound), in which the operator is required to track manually only three points, medial mitral annulus, lateral mitral annulus and LV apex; then, the software provides automatically the entire endocardial and epicardial border of the left ventricle, that can still be modified by the operator, if necessary. For circumferential strain, manual tracking of the whole endocardial border was performed by the operators.

The 6MWT and the same comprehensive echocardiographic study were performed also in a control group of 24 age-matched healthy children, sent to our hospital for evaluation of sport eligibility.

The intraobserver/interobserver variability was 6.8/8.8% (GLS) and 8.5/10.5% (GCS), respectively.

Statistics

A classic non-parametric approach was used since some numerical variables were not normally distributed, as verified by Kolmogorov Smirnov test, also considering the small sample size. Numerical data are consistently expressed as median with interquartile range (IQR) in brackets, categorical variables as number and percentage. Comparisons between patients and controls were carried out by the Mann-Whitney test. In order to address the aims of the present study, also considering that several parameters may be age-dependent, the DMD population has been then divided into two groups basing on the median age value (9 years), as were also control subjects, thus having four groups for statistical analysis: patients and controls younger than 9 years (DMD 1 and Controls 1, respectively); patients and controls older than 9 years (DMD 2 and Controls 2, respectively). Consistently, the Mann-Whitney test was applied in order to perform between-groups comparisons, including segmental strain analysis. Correlations among the variables were tested by Spearman's test. Moreover, dependence analysis was carried out by multiple regression models, in order to assess the contribution of any potential predictor on the study response variable(s) (GLS, GCS, EF%, E/A ratio, 6MWT, NSAA). Statistical analyses were performed using SPSS 17.0 for Window package. A two-tailed alpha of 0.05 was used to denote statistical significance.

Results

Motor assessment

The distance covered at 6MWT was significantly lower in DMD patients vs controls (median value 362 m, IQR 220 m, *vs* a median value of 585 m, IQR 118 m,

$p < 0.001$). NSAA median score was 30 (9.8) in patients, but not comparable with controls, since such a test is not usually administered to healthy individuals.

Cardiological assessment

All patients denied symptoms of cardiovascular involvement, like palpitations, shortness of breath or chest pain. Cardiovascular physical examination was unremarkable for all patients and controls. ECG was nearly normal, except for a mild degree of right bundle branch block in 5 patients and 3 controls. No significant arrhythmias were observed on ECG.

2D-echocardiography

Indexed LV diameters, volumes and mass were similar between patients and healthy children. Table I reports motor performance and conventional echocardiographic parameters in patients and controls, without age stratification. Although EF was within the normal range in both groups, a significant reduction was seen in patients and the same occurred for the remaining conventional systolic (FS, MAPSE, TAPSE, S') and diastolic (E, E') indices. E/A ratio also was reduced with respect to healthy controls.

The same parameters are summarized in Table II, based on age stratification: patients younger and older than 9 years are compared to controls younger and older than 9 years, respectively. All parameters were significantly reduced in older patients with respect to older controls (2 *vs* 2), although E/E' ratio has not reached the alpha level, whereas no significant difference was observed between younger patients and younger controls (1 *vs* 1), except for the E wave velocity that appeared reduced in patients.

Myocardial deformation analysis

GLS was significantly reduced in patients vs controls ($p < 0.001$), without age stratification (a less negative value reflects a more impaired GLS) (Tab. III). The same behavior was maintained if GLS was considered for each partial analysis of 4-ch, 2-ch and 3-ch view. Moreover, also GCS was significantly reduced in patients vs controls ($p = 0.013$), with preserved difference when considering separated MV-SAX, PM-SAX and AP-SAX views (a less negative value reflects a more impaired GCS).

The effect of age stratification on GLS and GCS is illustrated in Table IV. Either global or partial 4-ch, 2-ch and 3-ch GLS were reduced to the same extent in older and in younger patients, compared to their matched controls ($p < 0.001$ and $p < 0.001$ respectively). A different behavior was observed for GCS, that was clearly reduced in older patients respect to older controls ($p < 0.001$), but not equally altered in younger. Although a trend towards reduction was evident also in younger patients, a significant decrease was observed only at the midventricular level (PM-SAX GCS), whereas the global value of GCS, as well as basal and apical partial GCS, were not significantly different as compared to matched controls (GCS $p = 0.217$). Figure 1 illustrates areas with impaired GLS and GCS in DMD patients compared to age-matched controls. In younger patients, apical and

Table I. Motor performance and conventional echocardiographic parameters. Median (IQR)

	Controls	DMD patients	P-value
Age (years)	8.75 (5)	9 (6)	0.256
6MWT (m)	585 (118)	362 (220)	**< 0.001**
NSAA	--	30 (9.8)	--
BSA (m^2)	1.04 (0.64)	0.96 (1.04)	0.956
LV EDD mm/m^2	59.7 (11.3)	61.3 (11.3)	0.315
LV ESD mm/m^2	37.8 (8)	38.1 (9.3)	0.471
LV EDV ml/m^2	88.7 (41.9)	83.9 (41.9)	0.826
LV ESV ml/m^2	30.6 (16.1)	29.1 (13.7)	0.427
LV M g/m^2	54 (15)	55 (12)	0.723
FS%	37 (4)	35 (22)	**< 0.001**
EF%	69 (5)	64 (8)	**< 0.001**
MAPSE mm	12 (2)	10 (2)	**< 0.001**
TAPSE mm	20.5 (5)	18 (3)	**< 0.001**
E cm/s	105 (20)	90.5 (18)	**< 0.001**
E' cm/s	15 (2)	13.5 (3)	**< 0.001**
S' cm/s	8 (2)	7 (2)	**0.007**
E/A	2.1 (0)	1.86 (1)	**0.025**
E/E'	7.3 (2)	7.6 (1)	0.122

Table II. Motor performance and conventional echocardiographic parameters based on age stratification. Median (IQR)

	Controls 1	**Controls 2**	**DMD 1**	**DMD 2**	**P-value 1 *vs* 1**	**P-value 2 *vs* 2**
Age (years)	6 (2)	12 (3.2)	6.25 (3.5)	12.2 (3.5)	0.760	0.488
6MWT (meters)	519.5 (60)	630 (44)	385 (488)	160 (398)	**0.002**	**< 0.001**
NSAA	--	--	30 (9.5)	24 (7)	--	--
FS%	38.5 (7)	36 (3)	36 (3)	34 (6)	0.294	**< 0.001**
EF% BP Simpson	70 (9)	67 (4)	67 (4)	64 (8)	0.213	**< 0.001**
MAPSE mm	11 (2)	12 (3)	10.5 (2)	10 (1)	0.121	**< 0.001**
TAPSE mm	20 (5)	21 (4)	18 (3)	16 (2.0)	0.100	**< 0.001**
E cm/s	113 (25)	105 (10)	99 (14)	93 (20)	**0.029**	**0.007**
E' cm/s	15 (4)	16 (3)	15 (4.0)	12 (3.0)	0.545	**< 0.001**
S' cm/s	8 (1)	9 (1)	7 (1)	7 (1)	0.286	**0.001**
E/A	2.1 (1)	2.0 (0)	1.9 (1)	1.9 (1)	**0.038**	**0.041**
E/E'	7.6 (1)	6.1 (2)	8.7 (3)	7.3 (1)	0.320	0.211

Controls 1 and DMD 1 are younger than 9 years; Controls 2 and DMD 2 are older than 9 years.

Table III. Global longitudinal and circumferential strain. Median (IQR)

	Controls	**DMD Patients**	**P-value**
3ch GLS	-24.6 (4.1)	-19.7 (3.5)	**< 0.001**
4ch GLS	-22.8 (3)	-19.8 (4)	**< 0.001**
2ch GLS	-24.8 (4)	-21.5 (4)	**0.006**
GLS	-24.2 (3)	-20.6 (3)	**< 0.001**
MV-sax GCS	-18.8 (2.4)	-16.6 (3.9)	**< 0.001**
PM-sax GCS	-18.3 (3.4)	-15.5 (5.5)	**0.011**
AP-sax GCS	-25.3 (7.8)	-20.3 (11.4)	**0.040**
GCS	-21.4 (4.4)	-17.6 (4.6)	**0.013**

Table IV. Global longitudinal and circumferential strain based on age stratification. Median (IQR)

	Controls 1	**Controls 2**	**DMD 1**	**DMD 2**	**P-value 1 *vs* 1**	**P-value 2 *vs* 2**
3ch GLS	-25.2 (3.8)	-23.4 (6)	-21.2 (4.6)	-18.9 (3.2)	**0.004**	**< 0.001**
4ch GLS	-23.6 (3)	-20.7 (3.2)	-20.1 (3)	-18.0 (6.0)	**< 0.001**	**0.005**
2ch GLS	-24.9 (3)	-24.4 (8)	-22.1 (5)	-19.5 (6.0)	**0.003**	**0.007**
GLS	-24.9 (3)	-24 (6)	-21.2 (2)	-19.3 (4.0)	**< 0.001**	**< 0.001**
MV-sax GCS	-19.9 (3.5)	-18.0 (4.8)	-17.7 (5.2)	-15.5 (3.8)	0.103	**< 0.001**
PM-sax GCS	-17.7 (5.6)	-18.3 (3.9)	-14.0 (7.4)	-13.8 (4)	**0.032**	**< 0.001**
AP-sax GCS	-27.9 (6.3)	-24.3 (5.6)	-24.9 (9.5)	-17.5 (9.3)	0.210	**0.035**
GCS	-22.1 (5.3)	-20.4 (2.6)	-17.9 (3.9)	-15.9 (4.9)	0.217	**< 0.001**

Controls 1 and DMD 1 are younger than 9 years; Controls 2 and DMD 2 are older than 9 years.

infero-lateral segments show impaired GLS, while in older patients the damage is extended also to mid-septal and basal antero-lateral areas. GCS in younger patients appears to be impaired in the septal region, whereas in older patients reduced GCS involves also the apex, and the anterior and antero-lateral segments, but not the inferior septum.

Concerning age stratification, we also found that both GLS and GCS worsened with age in DMD patients (GLS $p < 0.005$; GCS $p = 0.024$. DMD 1 *vs* DMD 2), while no change was observed between control subjects (GLS $p = 0.247$; GCS $p = 0.456$). The regressive analyses further confirmed these findings, suggesting the increasing age as the variable most strongly associated with the subtle alterations in LV mechanics found in this population (GLS: Beta = 0.466; T = 3.078; $p = 0.009$; GCS: Beta = 0.477; T = 2.878; $p = 0.008$), and also with

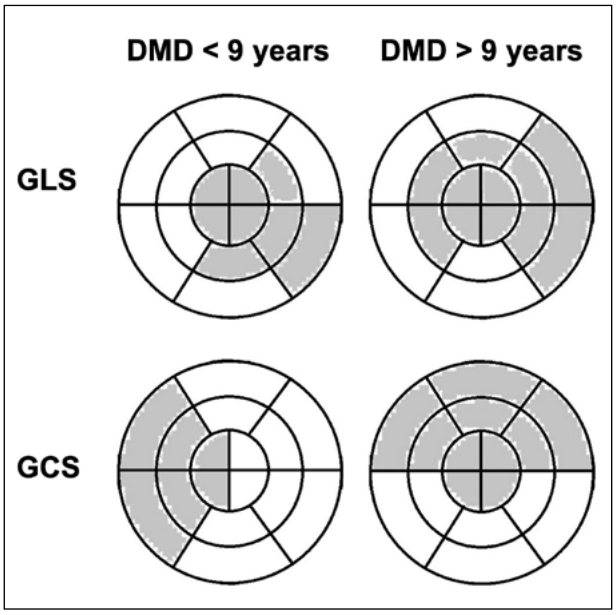

Figure 1. Bulls-eye diagrams illustrating the distribution of abnormal strain values in DMD patients according to age. A 16-segment model of the left ventricle is used. Grey areas depict impaired GLS and GCS segments, compared to age-matched normal controls.

EF% (Beta = -0.567; T = -3.363; p < 0.001). Moreover, also the physical performance, as assessed by 6MWT, is confirmed to decrease by increasing age (Beta = -0.490; T = -2.978; p = 0.006).

Correlation between motor and cardiological assessment

6MWT appeared to be inversely correlated to GLS (rho: -0.434, p = 0.021) and directly to EF (rho: 0.410, p = 0.035) and S' (rho: 0.584, p < 0.001). No significant correlations were found with NSAA.

Discussion

Cardiovascular complications are a leading cause of disease-related morbidity and mortality among individuals with DMD. Historically, individuals with DMD have not been referred to a cardiac specialist until late in the disease, contributing to poor clinical outcomes. Furthermore, cardiac management has been challenging because the New York Heart Association classification of heart failure relies on reduced exercise tolerance, a feature that in DMD arises from skeletal muscle and cardiac disease combined. The signs and symptoms of heart failure in the non-ambulatory patients are frequently subtle and overlooked. A proactive strategy of early diagnosis and treatment is essential to maximize quality of life and survival. Involvement of a cardiologist who is integrated into a multidisciplinary care team is recommended, given the complex decision making involved in managing DMD cardiomyopathy. Nowadays, however, LV dysfunction may be still often underdiagnosed and consequently undertreated [2,6-7].

The analysis of our results showed that in children with DMD, although EF is preserved (i.e. > 55%), LV function is definitely not normal. In younger patients (< 9 years), conventional parameters accounting for systolic and diastolic function are still comparable to age-matched control subjects. However, longitudinal function is already impaired, as demonstrated by significantly reduced GLS. At this stage global circumferential deformation appears to be preserved. In older patients (> 9 years), not only longitudinal and circumferential function are reduced, but also conventional parameters accounting for systolic and diastolic function have subtle alterations, that could not be defined "abnormal" if considered by themselves, but still appear significantly reduced when compared to normal subjects. Therefore, there might be a period, that we can define between 3 and 9 years of age, during which longitudinal function begins to decrease, while all the remaining compensatory mechanisms act to maintain a preserved LV systolic and diastolic performance. Beyond 9 years of age, it is expected that the worsening of the disease may involve progressively LV mechanics until the eventual decrease of the EF. Since circumferential strain appears to be globally preserved in younger and reduced in older patients, our hypothesis is that it might be considered a "transitional" parameter, which may help for a better characterization of patients with intermediate age, as those of 7 to 11 years of age. Nonetheless, a progressive trend can be identified for both GLS and GCS, which clearly worsened with age in DMD patients.

At the bivariate analysis we observed that the lower the distance covered at the 6MWT, the worse the EF, the S' and the GLS. In other words, motor performance and myocardial function are related to each other and both worsen with age, even in young children with DMD. As concerns segmental strain analysis, we noticed that longitudinal deformation of the apical segments and the infero-lateral wall was particularly affected, regardless of the age of patients, since it was equally impaired in either younger or older patients. However, the distribution of circumferential strain impairment was different from GLS, and also different between age groups. In fact, septal wall was the most affected in younger, while antero-septal, anterior and lateral walls showed the worst performance in older patients.

In the past years there has been increasing interest for circumferential strain in children with DMD. Several papers reported that circumferential strain is reduced in young children with normal ejection fraction [14-18]. How-

ever, in these studies strain analysis was performed with cardiac magnetic resonance imaging or with STE at the sole midventricular level. With respect to this, we also observed that in our population circumferential strain was particularly reduced at the level of the papillary muscles, but the global value was still preserved in patients younger than 9 years. Concerning the regional distribution of circumferential strain alterations, the lateral LV free wall has been reported with the earliest decline in young patients and the greatest involvement in older patients with DMD. Moreover, the lateral wall is also the area in which fibrosis is first recognized by cardiac magnetic resonance, in the advanced stage of the disease [15]. Ryan et al. described a different regional pattern of circumferential strain impairment in children with DMD, since the most involved appeared to be antero-septal, inferior and infero-lateral segments [14]. Furthermore, Taqatqa et al. reported that different segments could be affected, so that the reduction of circumferential strain cannot be localized to one specific region [19]. Since there is a great variability in the results reported by different authors, concerning the distribution of circumferential strain anomalies, it is likely that these might not be confined to one specific area, but are rather the expression of a diffuse myocardial damage. Recently, also longitudinal strain has been described as an early marker of ventricular dysfunction in young DMD patients [5,19]. Particularly, the reduction of GLS has been reported to be more pronounced in the apical area, as occurred in our population. It is worth noting that longitudinal strain values are usually higher at the apex in normal subjects, with a base-to-apex gradient [20]. We can speculate that in DMD longitudinal dysfunction might involve at first the region with the higher performance.

The awareness about the early natural history of ventricular mechanics in DMD boys is crucial to support the opportunity of cardioprotective therapy, as already attempted by several studies in human and animals [21,22]. This is particularly relevant as opinion differs on the use of angiotensin-converting enzyme (ACE) inhibitors in very young (< 10 years) asymptomatic individuals without evidence of abnormality on cardiac magnetic resonance or echocardiogram [2]. A reduced GLS could allow clinicians to start ACE inhibitors or beta-blockers even below 10 years of age and to monitor the therapeutic effect, aiming to preserve systolic and diastolic parameters within the normal range as long as possible.

Several drugs have been employed with the aim to contrast the evolution of cardiomyopathy toward stages of severe congestive heart failure, before an invasive approach should be considered, e.g. through the implantation of cardioverter defibrillator or cardiac resynchronization therapy defibrillator [23]. It is widely accepted that ACE inhibition could reduce mortality and hospitalization in DMD patients, delaying the onset and progression of cardiac dysfunction and reinforcing the usefulness of an early therapeutic approach [24-28]. Also, the adoption of a therapy with beta-blockers, alone or combined with ACE inhibitors, showed results for delaying progression of heart failure in these patients [28-30]. On that framework, STE-derived myocardial strain could be considered an additional outcome measure to test the efficacy of new therapeutic approaches, particularly in trials focusing on younger ambulant DMD patients.

Our study is limited by its retrospective nature and by the relatively small sample size. Also, deformation of the chest profile, which is often present in older patients with DMD, may have affected the accuracy of strain analysis, due to suboptimal ultrasound image quality.

Conclusions

Progressive LV dysfunction is a part of the natural history of DMD and begins very early. Despite the reduction of EF usually manifests in the second decade of life, subtle alterations in LV mechanics can be clearly seen as early as before 9 years of age. STE provides a reproducible and reliable evaluation of longitudinal and circumferential LV deformation, which represent sensitive markers of early dysfunction. Our results confirm that cardiac performances could be impaired already in very young DMD patients, below 9 years, apparently with a preferential involvement of the apical segments and the postero-lateral wall. Also, GCS begins to alter at this stage, even if the impairment becomes significant after 9 years of age. Both GLS and GCS show a progressive worsening with age in children with DMD. A longitudinal evaluation should be scheduled to assess the global cardiac performance over time and to evaluate the impact of therapies on the cardiovascular and overall outcome.

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Quadriceps muscle strength in Duchenne muscular dystrophy and effect of corticosteroid treatment

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Objectives. In Duchenne muscular dystrophy, quadriceps weakness is recognized as a key factor in gait deterioration. The objective of this work was three-fold: first, to document the strength of the quadriceps in corticosteroid-naïve DMD boys; second, to measure the effect of corticosteroids on quadriceps strength; and third, to evaluate the correlation between baseline quadriceps strength and the age when starting corticosteroids with the loss of ambulation.

Methods. Quadriceps muscle strength using hand-held dynamometry was measured in 12 ambulant DMD boys who had never taken corticosteroids and during corticosteroid treatment until the loss of ambulation.

Results. Baseline quadriceps muscle strength at 6 years of age was 28% that of normal children of the same age; it decreased to 15% at 8 years and to 6% at 10 years. The increase in quadriceps muscle strength obtained after 1 year of corticosteroid treatment had a strong direct correlation with the baseline strength (R = 0.96). With corticosteroid treatment, the age of ambulation loss showed a very strong direct relationship (R = 0.92) with baseline quadriceps muscle strength but only a very weak inverse relationship (R = -0.73) with the age of starting treatment. Age of loss of ambulation was 10.3 ± 0.5 *vs* 19.1 ± 4.7 (P < 0.05) in children with baseline quadriceps muscle strength less than or greater than 40 N, respectively.

Conclusions. Corticosteroid-naïve DMD boys have a quantifiable severe progressive quadriceps weakness. This long-term study, for the first time, shows that both of the positive effects obtained with CS treatment, i.e. increasing quadriceps strength and delaying the loss of ambulation, have a strong and direct correlation with baseline quadriceps muscle strength. As such, hand-held dynamometry may be a useful tool in the routine physical examination and during clinical trial assessment.

Key words: Duchenne muscular dystrophy, quadriceps muscle strength, hand-held dynamometry, corticosteroid treatment, prolongation of walking

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Conflict of interest
The Authors declare no conflict of interest

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Introduction

Sir William Richard Gowers (1845-1915) described and illustrated the peculiar maneuver that boys affected by the "pseudohypertrophic muscular paralysis", now known as Duchenne Muscular Dystrophy (DMD), use

to get up from the floor [1]. Gowers observed that the boy's "greatest defect is in the power of rising from the floor … he commonly has not sufficient power to extend the knees when the weight of the trunk is on the upper extremity of the femur … he therefore places his hands on his knees … when the knees are extended, the power of the extensors of the hip may be sufficient to raise the body into the upright position …" [1]. This maneuver, known as Gowers' sign, is adopted by the Duchenne boy to compensate for the quadriceps muscle weakness [2]. Gowers also noticed "the difficulty in going upstairs is especially due to the weakness of the extensors of the knee" [1].

All four quadriceps are powerful extensors of the knee, and are therefore crucial in walking, running, jumping and squatting [3]. When the quadriceps is weak, the patient will be unable to run and may have difficulty with stairs, because full extension is not attained in these cases and the knee tends to buckle into flexion [4].

A seminal work used hand-held dynamometry to quantify the peculiar weakness of knee extensors in corticosteroid-naïve Duchenne boys and its relationship with motor ability and time of loss of independent ambulation [5]. During a 3-year sequential study, 61 DMD boys, aged 4.3 to 11.8 years, were reviewed every 3 to 4 months, and underwent a total of 360 assessments [5]. The muscle strength of the knee extensors was very weak compared to that of normal peers, did not grow with age, and instead showed a progressive continual deterioration [5]. Loss of independent ambulation occurred when knee extensors exerted less than 2.0 kg (19.6 Newton) [5]. Another group subsequently confirmed both the knee extensor weakness and the declining trend with age in 27 corticosteroid-naïve DMD boys [6].

The age of loss of independent ambulation in DMD boys varies in a wide range (7 to 13 years) with a mean value of 9.5 [7,8]. The effect of corticosteroid (CS) treatment in term of prolongation of ambulation is also variable and could be related to dosage [9,10], age of administration [11-13], or other variables like residual muscle strength [14].

Although it is now recognized that the treatment goal in children with DMD is to keep them ambulant as long as possible, aiming to preserve clinically important function and postpone spinal deformities and muscle contractures [10], and that quadriceps insufficiency is the key factor in gait deterioration [15], no study has yet specifically evaluated the effect of corticosteroid treatment on knee extensors.

The objective of this work was three-fold: first, to document the strength of the quadriceps in corticosteroid-naïve DMD boys; second, to measure the effect of corticosteroids on quadriceps strength; and third, to evaluate the correlation between baseline quadriceps strength and the age when starting corticosteroids with the loss of ambulation.

Materials and methods

Patients

We included in the study the Duchenne boys who were able to walk and had never received corticosteroid treatment and who subsequently began it and were followed until the loss of ambulation.

All patients had a clinical diagnosis confirmed by genetic investigation and in many of them also by the absence of dystrophin in the muscle biopsy.

Corticosteroid treatment

The corticosteroid treatment was approved by the Ethical Committee of the Istituto Ortopedico Rizzoli [11]. In our study regimens, dosing and corticosteroids varied with time [11,12]. At the start of the treatment, and for the first 2-4 weeks, the regimen was daily (prednisone 0.75 mg/kg or deflazacort 0.90 mg/kg), and then on alternate days. The alternate day dose was prednisone 1.25 mg/kg (50 mg maximum) or deflazacort 1.5 mg/kg (60 mg maximum). During periods of stability corticosteroid dosage was not increased with weight. However, after the age of 12-14 years, if the patient showed more weakness or fatigue, prednisone/deflazacort was given for 1-3 months at 0.75/0.90 mg/kg daily with a ceiling dose of 50/60 mg.

Hand-held dynamometry

To test knee extension, the subject was seated with the hip and knee flexed at 90°, and the foot dorsiflexed at 90°. The examiner sat in front of the subject and the dynamometer was placed on the anterior surface of the distal tibia just proximal to the ankle joint. The patient performed each movement three times with a 30-s pause between each. The highest score obtained on the dominant side was used for further analysis. If a patient complained of discomfort, additional padding was available to place on the applicator. Maximum voluntary isometric contraction of quadriceps was measured until 1997 using the Hammersmith myometer (Myometer, Penny and Giles Transducers Ltd, Dorset, U.K.) [5], and then with the Citec dynamometer (CT 3001, Citec, C.I.T. Technics BV, Groningen, The Netherlands) [16]. The reliability and validity of both has been proven earlier [17,18].

Statistical analysis

To measure the strength of the linear association between two variables, we used linear regression with 95% confidence intervals and Wilcoxon two-tailed grade tests for paired samples, while the differences between the groups were evaluated using two-tailed Student's t-tests. To test the differences between regression lines, we used two-tailed tests. Parametric variables are shown as mean ± SD. P val-

Table I. Patients dystrophin gene mutations.

Patient #	DMD mutation
1	dup ex 65-79
2	del ex 10-44
3	del ex 8-44
4	del ex 20-25
5	del ex 44
6	del ex 51-62
7	del ex 48-52
8	c.10108C > T; p. Arg3370*
9	del ex 51-54
10	del ex 3-17
11	del ex 42-43
12	c.1264G > T; p. Glu422*

ues < 0.05 were considered statistically significant. Analyses were performed using IBM SPSS statistics [25].

Results

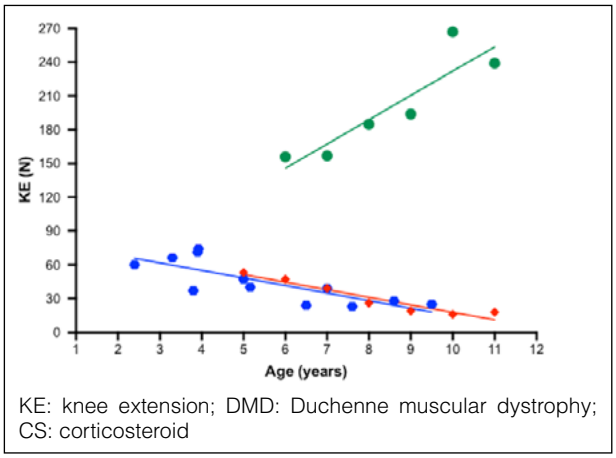
We assessed for eligibility 50 consecutive DMD boys evaluated from January 1994 to December 2018. Twenty-six were excluded: 19 were wheelchair-bound and 7 on CS were still ambulant. Corticosteroid treatment was proposed to the parents of 24 children: the parents of 20 children accepted and 4 refused the intervention. The remaining 20 children were allocated to intervention. Eight were excluded from the analysis: 3 were lost to follow-up, and 5 were on CS but still ambulant. The 12 patients who were corticosteroid-naïve and whose parents allowed corticosteroid treatment and were followed up to the time of loss of ambulation were therefore included in the study (see Table I for the genotype). The first 5 patients started corticosteroids treatment at a young age (< 4 years), between March 1996 and January 1997 [11,12].

Baseline quadriceps muscle strength

The quadriceps strength measured in the 12 DMD children (Fig. 1) between the ages of 2 to 10 exactly reflected the range of values and the declining trend observed in the previous studies [5,6]. DMD children were already much weaker than normal children at the age of 6, and their strength, unlike that of normal children [19], continued to decrease with age. In particular, the mean quadriceps strength of DMD children at 6 years was 28% that of normal children of the same age: it decreased to 15% at 8 years and to 6% at 10 years.

Corticosteroids effect on quadriceps strength

Corticosteroid treatment increased quadriceps strength (Figs.2-3A-B) in all but one patient (P6) in whom it was stabilized. Quadriceps muscle strength increased during the first months to a year of CS treatment (Figs. 2-3A) while the maximum increase in quadriceps strength (peak KE) was achieved at variable times between 1 and 7 years of treatment (Figs. 2-3B). There was a strong direct correlation (Fig. 3A-B) between the baseline KE and both the 1-year KE (R = 0.96) and the peak KE (R = 0.95). In the 12 boys, the increase in strength between baseline KE (44.5 ± 18 N) and peak KE (74.7 ± 48 N) was significant (p < 0.01).

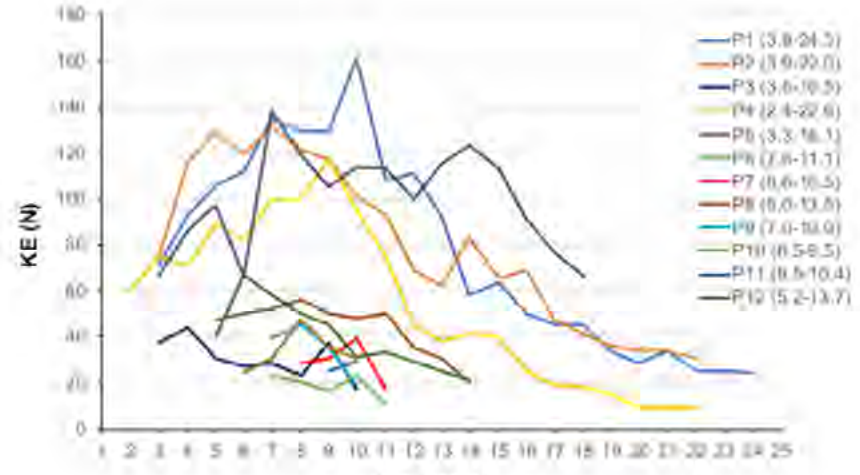


KE: knee extension; DMD: Duchenne muscular dystrophy; CS: corticosteroid

Figure 1. Regression lines between age and quadriceps/knee extension (KE) muscle strength in normal boys (aged 6-11 years, green line) [19], in 61 corticosteroid-naïve DMD boys (aged 5-11 years, red line) [5], and in 12 DMD boys before starting CS (aged 2-10 years, blue line). The differences between the regression lines y = -6.679x + 84.57 by Scott et al. [5] and y = -6.686x + 81.65 of the 12 DMD boys were not significant (p = 0.65). The linear equation for normal boys[19] aged 6-11 years was y = 21.543x + 16.55.

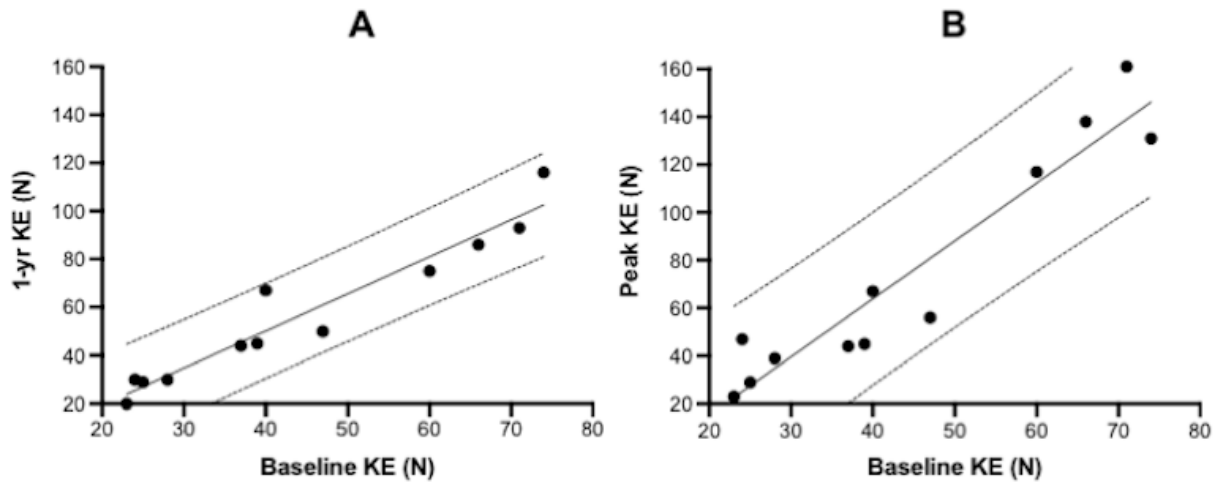
Age of starting CS treatment, quadriceps strength, and loss of ambulation

In these 12 boys, the correlation between the age of loss ambulation and the age of starting CS treatment (Fig. 4A) was weak and inverse (R = -0.73), while with the baseline quadriceps muscle strength (Fig. 4B) it was very strong and direct (R = 0.92). Note that the 6 children who lost ambulation before 12 years of age (Figure 2 and in Figure 4A from the left P3, P10, P9, P6, P7, P11) had started CS treatment between 3.8 and 9.5 years of age when their baseline KE (Fig. 4B) was below 40 N (23-39 N). In contrast, the 6 children who lost ambulation after 13 years of age (Figs. 2,4A) had started CS treatment between 2.4 and 5.2 years of age when their baseline KE (Fig. 4B) was 40 N or more (40-74 N). The mean age and IC95% of loss of ambulation was 10.3 (9.8-10.9) *vs* 19.1



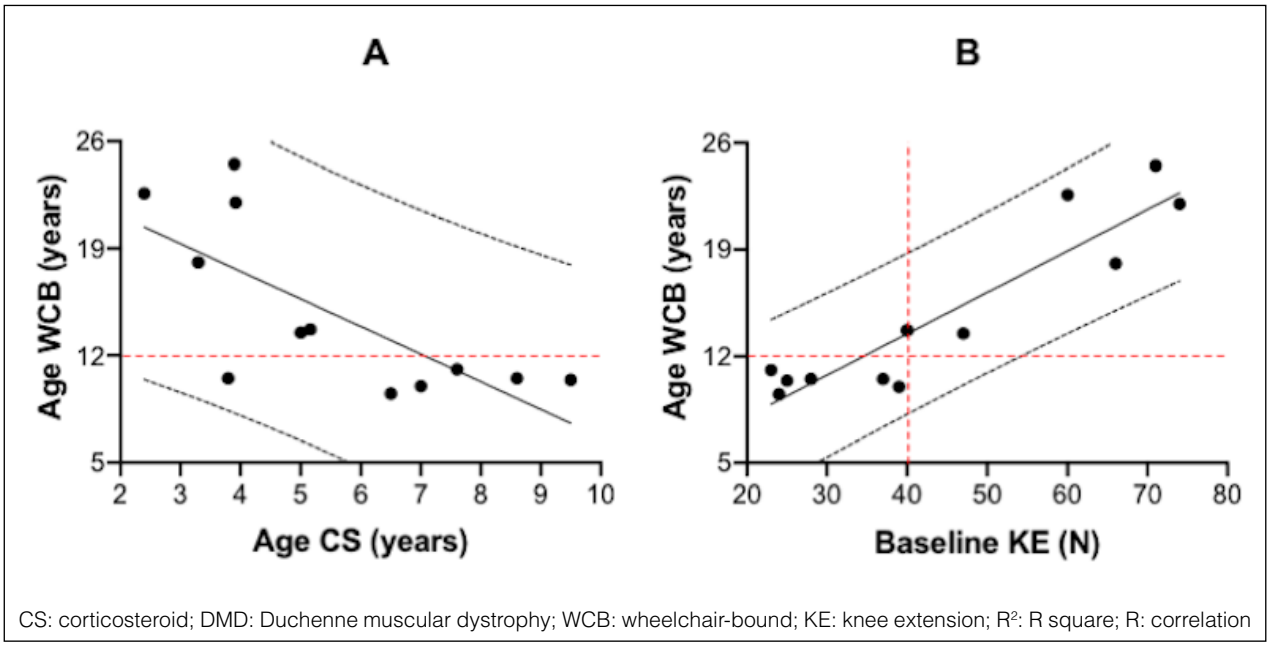
DMD: Duchenne muscular dystrophy; N: Newton; KE: knee extension; CS: corticosteroid

Figure 2. Linear trend of quadriceps strength for each of the 12 DMD boys from the age of initiation of corticosteroid treatment until the age of ambulation loss. The patients had 1-4 strength measurements each year and each line shows the maximum force value expressed during each year. The increase in knee extension muscle strength started in the first year of treatment in most patients and continued for 4-7 years in patients who at the beginning of the treatment had a force greater than 60 N (P1, P2, P4, P5). The 6 patients with baseline knee extension strength below 40 N (P3, P7, P9-P11) had a limited increase or only stabilization (P6) in KE muscle strength. For each of the 12 patients, the age of onset of CS and the age of loss of ambulation are shown in parentheses.



KE: knee extension; CS: corticosteroid; DMD: Duchenne muscular dystrophy; R^2: R square; R: correlation

Figure 3. Increase in KE muscle strength with CS treatment in 12 DMD boys. (A) Regression line between baseline KE (X) and 1-year KE (Y): R^2 = 0.9281. This means that 92.8% of the variability in Y is explained by X. R = 0.9634. This means that there is a very strong direct relationship between X and Y. P-value = 4.879e-7. Y = -11.413 + 1.53X. (B) Regression line between baseline KE (X) and peak KE (Y): R^2 = 0.9050. This means that 90.5% of the variability in Y is explained by X. R = 0.9513. This means that there is a very strong direct relationship between X and Y. P-value = 0.000001987. Y = -33.0743 + 2.4230X.



CS: corticosteroid; DMD: Duchenne muscular dystrophy; WCB: wheelchair-bound; KE: knee extension; R^2: R square; R: correlation

Figure 4. Regression lines in 12 CS treated DMD boys between (A) age of CS initiation (X) and age WCB (Y) and (B) baseline KE (X) and age wheelchair-bound (WCB) (Y). (A) $R^2 = 0.5347$. This means that 53.5% of the variability in Y is explained by X. R = -0.7313. This means that there is a very weak inverse relationship between X and Y. P-value = 0.006884. Y = 24.736 - 1.80X. (B) $R^2 = 0.8489$. This means that 84.9% of the variability in Y is explained by X. R = 0.9214. This means that there is a very strong direct relationship between X and Y. P-value = 0.00002072. Y = 2.6012 + 0.2719X.

(14.1-24.0) in children with baseline quadriceps muscle strength less than or greater than 40 N, respectively ($p < 0.05$).

The boy P3 who started CS at the age of 3.8 and lost ambulation at 10.5 years had a very low quadriceps strength value (37 N) at baseline and only a transient increase (44 N) after 2 months of corticosteroid treatment (Fig. 2). His cousin, with the same mutation and who was not treated with corticosteroids, ceased walking at 7.5 years [12]. The boy P5 who started CS at the age of 3.3 when his baseline KE was 66 N reached a peak of 138 N at age 7 and lost ambulation at age 18.1 when his KE was still high (66 N) because a leg fracture with long immobilization.

Discussion

Our study documents the loss of quadriceps strength in 12 corticosteroid-naïve DMD children, confirming both the marked quadriceps weakness with respect to the controls [5,6,20,21] and its characteristic linear rate of decline with age [5,6,22].

For the first time, this very-long-term follow-up study documented a very strong direct relationship between quadriceps muscle strength at baseline and its increase after 1 year of CS treatment (R = 0.96); moreover, the correlation between baseline quadriceps muscle strength and the age of loss of ambulation was very strong and direct (R = 0.92), but only weak and inverse (R = -0.73) with the age of starting CS treatment.

Previously, a better effect of early CS treatment [11,12,23] had been shown compared to a later start, i.e. after 6 years of age [24]. To reconcile the different positions, it is necessary to keep in mind two points:

- DMD boys lose ambulation at the average age of 9.5 years, but with a large range (7-13 years) [7,8];
- the mean strength of the quadriceps decreases with age, but the strength range per year is wide [5,6].

Since quadriceps strength is crucial for walking [3], reflects the overall strength of the child [5], and tends to decrease rapidly with age, it is to be expected that earlier treatment is better. However, this study demonstrates for the first time that the greater the basal strength of the quadriceps, the greater the increase in strength that is obtained following CS treatment. Above all, it is the basal strength of the quadriceps that best correlates with prolonging ambulation rather than the age of treatment initiation since DMD children of the same age have different quadriceps strength and the efficacy will be better in those with greater baseline quadriceps strength.

It should be noted that strength, measured on MRC-based scores of 34 muscle groups, showed a significant improvement in corticosteroid-treated boys compared with placebo [25,27]. In these studies, strength was already significantly greater at 10 days [26], reached a maximum by 3 months, and was maintained at 6 [26,27] and 18 months [28]. Knee extensor muscle strength, measured with an isokinetic dynamometer, was found higher in 9 DMD boys on corticosteroid compared to 6 corticosteroid-naive boys [29]. So far, the only other study that has measured force using a hand-held myometer showed that high dose weekly oral prednisone improved bilateral knee extension and flexion in all 17 boys with antigravity quadriceps strength compared to untreated boys, even after 6 months [30].

The fact that corticosteroid treatment has shown evidence of clinical efficacy with an early effect on muscle strength supported by a subsequent effect on motor function should be taken into consideration in the design of clinical trials. Instead, most of the Duchenne trials have had ordinal scales of muscle strength (MRC) or motor function (Vignos' lower limb score, the Brooke upper limb score) or the 6-minute-walk as the primary clinical endpoints of efficacy. However, it has been shown that the manual muscle test (MMT) and functional scales may take longer to demonstrate a trend than quantitative measures [31]. Therefore, it is expected that any effective treatment in muscular dystrophy would first increase muscle strength and subsequently improve motor function [31]. In addition, the MMT is known to be less reliable and sensitive compared to quantitative measurements; for example, by the time strength declined to MMT grade 4, isometrically measured strength was 40-50% of normal control [22], suggesting the use of a quantitative muscle test as an outcome measure in clinical trials in DMD to obtain maximum power and the greatest sensitivity [32]. The six-minute walk has failed to show improvements in recent trials and its validity for DMD children was questioned on several aspects [33].

Conclusions

Corticosteroid-naïve DMD children have very weak quadriceps muscles that do not increase in strength but rather rapidly become weaker with age, causing the loss of ambulation. Corticosteroid treatment is effective in increasing quadriceps muscle strength and in prolonging ambulation. For the first time, this very long-term follow-up study showed that the increase in the strength of the quadriceps after one year of CS treatment is directly proportional to the initial strength of the muscle itself, and above all the best estimate of the age of ambulation loss is based on the strength of the quadriceps at the start of CS treatment and not on the age at which it starts. The quantitative measurement of quadriceps muscle strength is an easy-to-apply, non-invasive and inexpensive method and should be part of the clinical evaluation of the myopathic patient and included between clinical trial endpoints.

There may be some possible limitations in this long-term single center study. The first is the limited sample size particularly at certain age. The second limitation is that the results may be specific to the corticosteroid regimen utilized in this study. However, the fact that the best estimate of the age of ambulation loss is based on the strength of the quadriceps at the start of corticosteroid treatment is a new exciting finding that deserves to be confirmed in future larger studies.

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LGMD. Identification, description and classification

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The term 'limb girdle muscular dystrophy' (LGMD) was first used in the seminal paper by Walton and Nattrass in 1954, were they identified LGMD as a separate clinical entity In LGMD description it is pointed out that the category of LGMD most likely comprises a heterogeneous group of disorders. After that the clinical entity was discussed but the LMGD nosography reached a permanent classification during two ENMC workshops held in 1995 and 2017, in the last one an operating definition of LGMD was agreed. This last classification included dystrophies with proximal or distal-proximal presentation with evidence at biopsy of fibre degeneration and splitting, high CK, MRI imaging consistent with degenerative changes, fibro-fatty infiltration present in individuals that reached independent walking ability. To be considered in this group at least two unrelated families should be identified.

A review is done of the first genetic characterisation of a number of LGMDs during the late twentieth century and a historical summary is given regarding how these conditions were clinically described and identified, the progresses done from identification of genetic loci, to protein and gene discoveries are reported. The LGMD described on which such historical progresses were done are the recessive calpainopathy (LGMD 2A/R1), dysferlinopathy (LGMD 2B/R2), sarcoglycanopathy (LGMD 2C-2F/R3-R6) types and the dominant type due to TPNO3 variants named transportinopathy (LGMD 1F/D2). Because of new diagnostic techniques such as exome and genome sequencing, it is likely that many other subtypes of LGMD might be identified in the future, however the lesson from the past discoveries can be useful for scientists and clinicians.

Key words: limb girdle dystrophy, calpain-3, dysferlin, sarcoglycans, transportin-3

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Introduction

At the end of 19th century, G.B. Duchenne and W. Gowers described patients of both genders whose clinical symptoms and course were overlapping those of the Duchenne (DMD) type. In 1876-1880, a clinical form of atrophic pelvic muscle disease different from DMD was recognized by E. Leyden and P. Möbius (later referred as *atrophic, pelvic girdle or pelvi-femoral type of Leyden-Möbius*).

In 1954 J.N. Walton (Fig. 1) and F.J. Nattrass introduced the expression *limb girdle muscular dystrophy (LGMD)* to identify patients of both genders who presented onset of muscle weakness within the third decade of life, with weakness and atrophy of proximal muscles in the limb girdles, with sparing of facial muscles, and with moderately rapid course. They identified LGMD as a separate clinical entity from the more common, X-linked recessive DMD, and pointed out that LGMD most likely comprises a heterogeneous group of disorders [1].

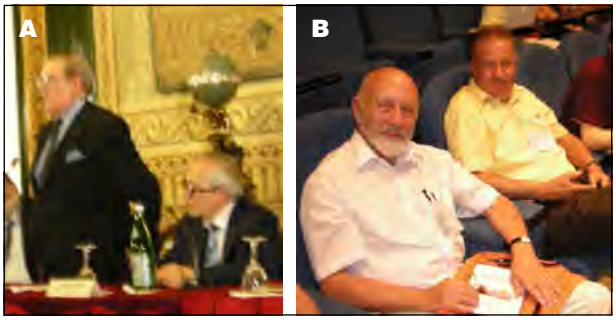

Figure 1. Lord John Walton of Detchant (the founder of Muscular Dystrophy Association in UK) and Giovanni Nigro (President and organizer) (A), and Alan E.H. Emery and Corrado Angelini (B) at the open ceremony of the 12th ICNMD congress held in Naples in 2010, taking place in the suggestive location of the San Carlo Royal Theatre.

The autosomal recessive LGMD of childhood, which was reported to be common in Brazil and North Africa, was initially referred as *Severe Childhood-onset Recessive Muscular Dystrophy (SCARMD)*, with clinical features indistinguishable from DMD [2]. The definition of LGMD soon emphasized its heterogeneity at both clinical and genetic level, and also the difficulty in discriminating between the different entities.

Identification of genetic loci of LGMDs

In the pre-molecular era, the diagnosis of LGMD was based only on clinical phenotype and by exclusion of other diagnoses, such as X-linked recessive DMD or its milder BMD variant (also using dystrophin gene and protein testing, available from 1987). In the following years, a molecular diagnosis of LGMD became possible using linkage analysis in large inbred families with many affected individuals.

In 1991 the first locus to be mapped in a recessive LGMD was to chromosome 15q15 (LGMD2A/R1) [3], probably due to the easy availability of large families, an observation that indirectly implies a high population frequency of the disease.

In the following years, many additional loci in inbred SCARMD or recessive LGMD families have been mapped to chromosome region 13q12 (LGMD2C/R5) [4], to 17q21 (LGMD2D/R3)[5], to 4q12 (LGMD2E/R4) [6], to 5q33 (LGMD2F/R6 [7,8], to 2p (LGMD2B/R2) [9].

Although possible in some instances, the genetic diagnosis of LGMD using linkage analysis was available only on research basis and unfeasible in the majority of patients who are isolated cases.

Such huge limitations in defining the genetic and molecular basis the LGMD patients, had for many years, important consequences on genetic counselling on family relatives because the mode of inheritance remained unclear in isolated cases.

In 2020, the large majority of LGMD patients achieve a genetic diagnosis, due to the recent development of advanced genome sequencing techniques, and the research is open to the future development of specific therapies. Patients' management is so far limited to physical rehabilitation, clinical follow up of cardiologic and respiratory complications, but several therapies have been tested (including corticosteroids [10] and myostatin inhibitors) with variable success [11].

Classification of LGMDs

Due to the genetic characterisation of a number of LGMDs in the late 20th century, in 1995 the first consortium meeting, under the auspices of the European Neuromuscular Center (ENMC), reached a consensus on a classification of LGMD subtypes based on molecular and genetic criteria [12,13] the autosomal dominant loci were designated as LGMD type 1, and the autosomal recessive loci were designated as LGMD type 2. LGMD nomenclature adopted a progressive alphabetical letter indicating the order of gene mapping identification (Tab. I), also avoiding imprecise and often lengthy nomenclature in use (e.g. SCARMD). During the following 10 years, the number of LGMD loci/genes identified increased rapidly, and occupied all the alphabetic letters (from LGMD2A to LGMD2Z); furthermore, the classification system applied by the Online Mendelian Inheritance in Man (OMIM) catalogue was no longer used by the clinical community, because it included also inherited myopathies with normal CK levels which should be more properly classified between categories other than LGMD (e.g. congenital myopathies, scapulo-peroneal myopathies, metabolic myopathies, etc.). Because of this dilemma, it was time to update and modify the LGMD nomenclature (Tab. I).

The 2nd ENMC workshop, which was held in March 2017 in Naarden, the Netherlands, on the classification and nomenclature of the LGMDs, was aimed to reach consensus of an updated definition of LGMD, and to review and evaluate suggestions of potential new classifications of LGMD subtypes [14]. At this meeting, the classification was revised naming the autosomal dominant LGMDs as D and numbering them from 1 to 5, and the recessive forms as R with numbers from 1 to 23. The classification of LGMD included dystrophies with proximal or disto-proximal presentation with evidence at biopsy of fiber degeneration and splitting, high CK, muscle MRI imaging consistent with degenerative change and fibro-fatty infiltration.

As of today, more than 30 different genetic subtypes of LGMD have been identified. Like for other inherited

Table I. Genetic classification of LGMD.

LGMD type	Disease OMIM#	Cytogenetic locus	Gene OMIM#	Gene symbol	Protein
1A	159000	5q31.2	604103	*MYOT*	Myotilin
1B	159001	1q22	150330	*LMNA*	Lamin A/C
1C	607801	3p25.3	601253	*CAV3*	Caveolin-3
1D/D1	603511	7q36.3	611332	*DNAJB6*	DNAJ/HSP40 homolog
1F/D2	608423	7q32.1	610032	*TPNO3*	Transportin-3
D3	609115	4p21.22	-	-	Heterogeneous molecular ribonucleic D-like protein
D4	613530	15q15.1	-	*CAPN3*	Calpain-3
2A/R1	253600	15q15.1	114240	*CAPN3*	Calpain-3
2B/R2	253601	2p13.2	603009	*DYSF*	Dysferlin
2C/R5	253700	13q12.12	608896	*SGCG*	γ-sarcoglycan
2D/R3	608099	17q21.33	600119	*SGCA*	α-sarcoglycan
2E/R4	604286	4q12	600900	*SGCB*	β-sarcoglycan
2F/R6	601287	5q33.3	601411	*SGCD*	δ-sarcoglycan
2G/R7	601954	17q12	604488	*TCAP*	Telethonin
2H/R8	254110	9q33.1	602290	*TRIM32*	Tripartite motif containing protein-32
2I/R9	607155	19q13.32	606596	*FKRP*	Fukutin-related-protein
2J/R10	608807	2q31.2	188840	*TTN*	Titin
2K/R11	609308	9q34.13	607423	*POMT1*	Protein O-mannosyl transferase-1
2L/R12	611307	11p14.3	608662	*ANO5*	Anoctamin-5
2M/R13	611588	9q31.2	607440	*FKTN*	Fukutin
2N/R14	613158	14q24.3	607439	*POMT2*	Protein O-mannosyl transferase-2
2O/R15	613157	1p34.1	606822	*POMGnT1*	Protein O-mannose N-acetyl-glucosaminyl Transferase-1
2P/R16	613818	3p21	128239	*DAG1*	Dystroglycan
2Q/R17	613723	8q24.3	601282	*PLEC1*	Plectin
2R/R18	615325	2q35	125660	*DES*	Desmin
2S/R19	615356	4q35.1	614138	*TRAPPC11*	Transport-protein-particle-complex-11
2T/R20	615352	3p21.31	615320	*GMPPB*	GDP-mannose-pyrophosphorylase B

conditions that display huge genetic heterogeneity, nomenclature has become a significant problem with the increased speed in which new disease genes are discovered. Because of new diagnostic techniques [15], such as exome and genome sequencing, it is likely that many more subtypes of LGMD will be identified in the future.

With the exception of autosomal inheritance, the different LGMD subtypes listed in the current classification system have little in common. The broad, original definition has led to potential inaccuracies over what is considered a form of LGMD. Over the past sixty years, the original clinical definition of LGMD has been useful, but our increased molecular and pathogenetic understanding of LGMD subtypes is beginning to call into question this definition and subsequent classification only by phenotype. Phenotypically LGMD subtypes are highly variable in their age of onset, speed of disease progression and overall severity; however, they do not share a common pathological mechanism that would distinguish them from other forms of muscular dystrophy and progressive limb girdle weakness is not always the leading clinical feature. Advances in genetic medicine and the identification of new genetic loci made the nomenclature increasingly difficult for LGMD, as mutations in a number of genes that have been assigned to subtypes of LGMD can also cause allelic conditions presenting with a different phenotype and be more commonly known under a different name. Mutations in the *TTN* or *DYSF* gene have e.g. also been associated with distal myopathic phenotypes. Several of the LGMDs are dystroglycanopathies that are also associated with a group of congenital muscular dystrophy syndromes, including Fukuyama congenital muscular dystrophy, Muscle-Eye-Brain disease, and Walker-Warburg-Syndrome. All dystroglycanopathies were grouped together due to the recognition that mutations in at least 18 different genes all interfere with the glycosylation of a-dystroglycan, and thus dystroglycan's function as a matrix receptor. Understanding the role of dystroglycan and its carbohydrate moieties as a basement membrane receptor will therefore be relevant for therapy

development for a large number of diseases, beyond the boundaries of traditional classification systems. Because industry is starting to show increased interest in LGMD, it will be important to have clarity about the classification of diseases caused by mutations in the same gene, as this will affect feasibility studies, inclusion criteria for clinical trials and recruitment strategies.

Nomenclature of disease is an important educational topic for both clinicians and patients and serves as a critical nosological reference point. Widely accepted nomenclature should therefore only be changed with caution and by consulting key clinical opinion leaders and patient advocacy groups. Any change of the classification also needs to take more general reforms of nosology into account (e.g. International Classification of Diseases (ICD)). Patients and their families will often have an emotional link with their diagnosis, identifying with the name of their disease. Changes to nomenclature therefore can have distressing effects for patients.

Description of LGMD

The term LGMD defines a progressive weakness with onset in the proximal limb girdle muscles, with age at onset of symptoms varying from early childhood (not congenital) to late adulthood. The progression of muscle weakness is usually symmetrical and variable among individuals and genetic type. The term LGMD used to molecularly classify the disease, is however sometime inappropriate for some patients when it is utilized to describe the clinical severity. Indeed, these disorders present a wide spectrum of muscle involvement and wasting, spanning from very severe forms, such as those with childhood onset and rapid progression, to relatively benign forms with late onset.

The clinical phenotypes due to mutation in the LGMD genes include severe childhood-onset forms, distal and proximal myopathies, pseudo-metabolic myopathies, eosinophilic myositis, and hyperCKemia. Because there is a spectrum of phenotypes under the same genetic entity, and a wide genetic heterogeneity under the same phenotype [16], it is crucial to identify suitable selection criteria to be used when screening patients for the proteins and genes responsible for LGMD.

LGMD is a genetically inherited condition that primarily affects skeletal muscle leading to progressive, predominantly proximal muscle weakness at presentation caused by a loss of muscle fibres. To be considered a form of LGMD the condition must be described in patients achieving independent walking, must have an elevated serum creatine kinase (CK) activity, must demonstrate degenerative changes on muscle imaging over the course of the disease, and have dystrophic changes on muscle histology ultimately leading to end-stage pathology for the most affected muscles.

Proximal muscle weakness and genetic inheritance were kept from the original definition as important factors of LGMD. To distinguish LGMD from congenital muscular dystrophies, patients must achieve independent walking, this criterium was felt to be superior to setting a defined age limit (i.e. two years). An elevated CK activity is seen in almost all LGMD patients in early stages of the disease process and is related to muscle fibre breakdown, which is considered a hallmark of muscular dystrophy. Degenerative changes on muscle MRI are defined as the replacement of skeletal muscle with adipose tissue as detected on standard T1 weighted axial images. Dystrophic changes on muscle histology include fibre necrosis and regeneration together with an increase in fibrosis and adipose tissue. The term 'end-stage pathology' refers to progressive replacement with fibro-adipose tissue seen on histological examination.

Calpainopathy identification (LGMD2A/R1)

Following the identification of the disease locus to chromosome 15 in 1991 [3], the first mutations in the calpain-3 gene have been identified in 1995 by Isabelle Richard[17] and the pioneer work of Michel Fardeau in 1996 (Fig. 2) analysed clinically a group of LGMD2A patients in a small community in the Reunion Island [18]. The discovery of this isolate was followed by the identification of other genetic clusters of LGMD2A/R1 throughout the

Figure 2. George Karpati and Michel Fardeau at the Myology Institute amphitheater, Hôpital Pitié-Salpetrière in Paris.

world, also in other specific isolates, e.g. in the venetian lagoon [19] or in the Alps [20].

LGMD2A is due to mutations in the gene encoding calpain-3 (*CAPN3*), a neutral protease [17]. This is the first muscular dystrophy to be recognized to be due to an enzyme defect rather than a structural protein defect [21]. The disease is typically characterized by a selective and progressive involvement of proximal limb-girdle muscles [22]. Two phenotypes have been identified based on the distribution of muscle weakness at onset: the pelvi-femoral form of Leiden-Möbius, which is the most frequently observed, in which muscle weakness is first evident in the pelvic girdle and later evident in the shoulder girdle, and the scapulo-humeral form of Erb, which is usually a milder phenotype with infrequent early onset, in which muscle weakness is first evident in the shoulder girdle and later evident in the pelvic girdle [23]. Another early and transient feature in LGMD2A/R1 is eosinophilic myositis, which has been reported in patients presenting with increased CK levels. CK levels are always elevated since infancy (5-80 times the normal). The age at onset of muscle weakness ranges between 2 and 40 years (in average 15 years). The first clinical symptoms are usually difficulty in running, the tendency to walk on tiptoes, and scapular winging caused by weakness of scapular girdle muscles. Weakness and wasting of the hip adductors/extensors and the posterior thigh muscles (gluteus maximus, semimembranosus, biceps femoris) are evident on both clinical examination and on muscle CT and MRI imaging scan. Joint contractures are typically seen and may be early present. As the disease progresses, waddling gait, difficulty in climbing stairs, lifting weights, and getting up from the floor or a chair are evident. Muscle involvement is mainly symmetrical and atrophic. Facial and neck muscles are usually spared. LGMD2A/R1 is the most prevalent form of LGMD in most European countries, where it would account for about 40-50% of total LGMD cases [24]. Its high prevalence, which was estimated to be about 1:15,000-1:150,000, is due to a high heterozygote frequency in the general population (about 1:100-120) [19]. Calpainopathy phenotype with Erb presentation shares some similarities with facio-scapulo-humeral muscular dystrophy (FSHD) and may be confused with this latter disorder especially in isolated cases: muscle weakness at onset in the shoulder girdle, scapular winging, elevated CK levels, and nonspecific myopathic changes on muscle biopsy; however, facial weakness and asymmetrical muscle involvement, which are frequent in FSHD, are uncommon in LGMD2A/R1. The diagnosis of BMD should be also excluded in isolated male patients who have weakness in the lower girdle muscles in adolescence or adulthood and high CK levels. Some patients with LGMD2A/R1 have been reported with pseudo-metabolic myopathy, presenting asthenia, myalgia, exercise intolerance, proximal muscle weakness, and excessive lactate production.

The lack of therapy is still distressing in a disease that have been described from 20 years. Attempts to replace the missing protein in an animal model has led to a cardiac phenotype.

A variety of clinical presentation and disease course may be due to primary calpainopathy including cases with respiratory insufficiency. Symptoms caused by respiratory failure may be specific, such as breathlessness, or more general, such as fatigue, lethargy, poor appetite, weight loss and impaired concentration. Patients may describe breathlessness at rest or on exertion, depending on the severity of the muscle weakness. With the association of the diaphragm, symptoms of orthopnoea or breathlessness may be apparent when bending over. Breathlessness experienced when a person is immersed in water above the waist is a rare but classical symptom of diaphragm weakness.

When the upper airway musculature is affected, speech and swallowing difficulties start to develop. Snoring, apnoeic episodes and daytime somnolence point to the possibility of obstructive sleep apnoea. If patients under-ventilate at night, the resultant hypercapnia may cause early-morning headaches, reduced concentration, or clouded consciousness. Blurring of vision from papillo-edema has been described but is rare and only seen in severe hypercapnia. A history of recurrent chest infections may indicate an ineffective cough. Coughing requires activation of the inspiratory muscles, closure of the glottis and then contraction of the expiratory muscles, particularly those of the abdominal wall; finally, the expulsive phase is initiated by opening the glottis. A poor cough can result from weakness or in-coordinated contraction of the inspiratory, glottic or expiratory muscles.

A patient that suffered from calpainopathy with respiratory insufficiency was Federico Milcovich, the founder of Italian Muscular Dystrophy Association (UILDM).

Sarcoglycanopathies identification (LGMD2C/R5, 2D/R3, 2E/R4, 2F/R6)

LGMD named SCARMD: Adhal (γ-sarcoglycanopathy, LGMD2C/R5) and Hetairosin (β-sarcoglycanopathy, LGMD2E/R4) proteins and genes discovery

Severe childhood-onset autosomal recessive muscular dystrophy (SCARMD) was first described by Ben Hamida and Fardeau in a symposium in Venice in 1980. In 1983 they reported 93 patients belonging to Tunisian inbred families with a severe form of muscular dystrophy, with childhood onset and early loss of ambulation (20-30 years of age) [2]; the CK was markedly raised in the early stages of disease, muscle wasting affected mainly

limb-girdle and trunk muscles, and calf muscle hypertrophy was usually present.

In 1992 the gene segregating in Tunisian, Moroccan, Algerian, Egyptian SCARMD families was mapped to chromosome region 13q12 [4]. In the same year, the deficiency of the 50k dystrophin-associated glycoprotein (DAG) (now called α-sarcoglycan, or α-SG) was identified in the muscle from 13q12-linked SCARMD patients, initially suggesting that the defect of this protein was the primary cause of the disease [25]. In 1994, Romero et al. [26] proposed to refer to the 50k DAG with the term "adhalin" from the arabic word for muscle, and consequently the disorder originating from this protein deficiency was originally called "adhalinopathy". This disorder was considered typical of Northern Africa, Arabian peninsula, but similar cases originated from Japan, Europe, and United States.

It was speculated that the gene encoding 50k DAG protein might be localized in 13q12, and that mutations in this gene may be responsible for SCARMD. However, SCARMD has been shown to be genetically heterogeneous, as families of Brazilian and European descent did not demonstrate linkage to 13q12 [27,28].

In 1995 Noguchi [29] discovered that the causative gene in Maghrebian SCARMD patients was that encoding for γ-SG, reported the first mutations in γ-sarcoglycanopathy (LGMD2C/R5), and introduced the term "sarcoglycanopathies" to refer to this group of disorders.

LGMD2C/R5 is often the most severe of autosomal muscular dystrophies [30] and since its first genetic characterization in 1995, a number of patients have been identified worldwide, with a high frequency in gypsy population or other genetic clusters [31-33]. Using linkage analysis in SCARMD patients in the inbred Amish population of North America, a second locus was mapped to chromosome 4q21[6]. Since the secondary deficiency of 50k DAG was common to all disorders due to a component of the sarcoglycan protein complex, the term "adhalinopathy" was initially used also for this second sarcoglycan gene mutated.

The gene was that encoding α-sarcoglycan protein, which was initially termed "hetairosin" (which means "accompanying"), an equivocal meaning according to Michel Fardeau, and therefore both terms adhalinopathy and hetairosin have now only an historical role. The first mutations in β-sarcoglycanopathy (LGMD2E/R4) were described contemporarily in patients from the Amish community [6] and in an Italian girl with SCARMD[34]. The clinical phenotype in this disorder was variable, including relatively mild LGMD cases (Amish patients) and patients with severe SCARMD phenotype.

α-sarcoglycanopathy (LGMD2D/R3) and δ-sarcoglycanopathy (LGMD2F/R6) identification

Linkage analysis in SCARMD patients with 50k DAG protein deficiency have excluded the 13q12 locus [26-28]. In 1993 another locus was mapped to 17q21 [5] and the first mutations in the gene encoding 50k DAG protein (or adalin or α-sarcoglycan) were identified in French patients [35]. The phenotype of LGMD2D/R3 is the most variable among all sarcoglycanopathies, including severe SCARMD patients and very mild myopathic patients [10,30].

In 1996 a locus for LGMD in brazilian families was mapped to 5q33-34 [7], and in the same year it was identified the gene and the encoded protein δ-sarcoglycan was discovered by Vincenzo Nigro in a brasilian family [8,36]. The phenotype of δ-sarcoglycanopathy (LGMD2F/R6) is that of a severe SCARMD phenotype associated with dilated cardiomyopathy.

Frequency of sarcoglycanopathies

Among total LGMD patients, sarcoglycanopathies constitute about 10-25% of cases in most countries, with higher frequency among inbred populations [24].

LGMD2D/R3 is the most frequent form of sarcoglycanopathies in most countries, followed by LGMD2C/R5 (which, however, is the most frequent form in Maghreb, India and Europe) and by LGMD2E/R4 and LGMD2F/R6. Severe childhood-onset LGMD patients have a higher probability of obtaining a molecular diagnosis than adult-onset LGMD patients. Among patients with the severe SCARMD phenotype, the frequency of sarcoglycanopathies ranges in various populations from between 22 to 69% of cases, whereas among adult-onset LGMD it is only 4-8% [24].

A few genetic epidemiological studies have estimated the prevalence of total sarcoglycanopathies to be about 1:178,000 and 1:370,000 inhabitants [37].

Clinical features of sarcoglycanopathies

The phenotypes of sarcoglycanopathies are rather, similar to those of dystrophinopathies (DMD, BMD), except for the absence of cognitive dysfunction [30] and more frequent occurrence of scapular winging. The most common presentation is a DMD-like phenotype with onset of weakness in childhood (especially in LGMD2C/R5, LGMD2E/R4, LGMD2F/R6), and the disease is more severe and rapid than in other LGMDs. Most patients have a severe and rapid course, leading to loss of independent walking ability before age 30-40 years. On average, the earlier the onset, the more rapid the progression, but in some cases the progression is not linear. Tiptoe walking in early childhood is often present before muscle weakness is detected. Adult-onset patients may be seen especially in LGMD2D/R3 and LGMD2C/R5. The ability to rise from the floor (presence of Gowers' sign), and to run, jump, and hop, as well as sporting ability may be affected in childhood or may be normal even until middle age. Muscle hypertrophy, especially of the calves and the tongue, is common [30,38].

Clinical variability in the phenotype and in severity has been observed between unrelated patients sharing the same mutation and even between patients belonging to the same family [10], suggesting that different factors of both genetic (intragenic polymorphisms, modulating genes) and non-genetic origin (nutrition, sport activity, body mass index, drugs, infections, inflammatory process) might have a role in determining the clinical phenotype and disease progression.

Cardiac, respiratory involvement and management in sarcoglycanopathies

Dilated cardiomyopathy may occur in all forms, but it is frequent and severe (sometimes fatal) in LGMD2E/R4 and LGMD2F/R6, while in LGMD2C/R5 and LGMD2D/R3 it is mild or occasional [39-42]. The subtypes mostly associated with cardiac involvement (manifest as conduction disorders and/or myocardial disease) are those associated with a defect in the genes encoding for the β-SG, or δ-SG proteins, which are expressed in heart and skeletal muscle,

Preclinical cardiomyopathy (44% of cases), and initial cardiomyopathy (19% of cases) are frequent, as well as arrhythmias and dilated cardiomyopathy [39,40]. Signs of hypoxic myocardial damage may occur in LGMD2E/R4, LGMD2F/R6, LGMD2C/R5. Abnormal coronary smooth muscle function has been suggested to be involved in the development of cardiomyopathy in LGMD2E and LGMD2F, since β-SG and δ-SG are also expressed in the coronary arteries [43].

Impaired vasoregulation occurs via marked reduction in membrane-associated neuronal nitric oxide synthase (nNOS) in both cardiac and skeletal muscle. Without dystrophin, nNOS mislocalizes to the cytosol; this greater distance between nNOS and the sarcolemma may impair NO diffusion through the myocyte membrane to the microvasculature. As a consequence, insufficient NO release follows muscle contraction resulting in muscle ischemia. Unopposed vasoconstriction may explain the necrosis observed in skeletal and cardiac muscle of dystrophinopathy patients. Microvasculature abnormalities have also been shown to result primarily from absence of dystrophin or sarcoglycan components in cardiomyocytes. The sarcolemmal nNOS expression correlated with the clinical severity [44] and muscle fatigue: absence or severe reduction of sarcolemmal nNOS expression was associated with a severe and childhood-onset form of muscular dystrophy and in most cases also with dilated cardiomyocytes.

Mice lacking either γ-SG or δ-SG display progressive focal cardiomyocyte degeneration that leads to reduced cardiac function and death. This model of cardiomyopathy closely parallels what is seen in humans with SG and dystrophin gene mutations. Null mice for β-SG and δ-SG (but not for αγ-SG) presented a disruption of the vascular smooth muscle SG complex. The perturbed vascular function induces ischemic injury in cardiac and skeletal muscle, suggesting that this mechanism could contribute to the development of cardiomyopathy and exacerbate skeletal myopathy.

It is well known that vascular spasm is an important contributor to cardiac pathology. Elevated levels of intracellular calcium, disturbances of the NOS pathway, and increased activity of protein kinase C, have been implicated in increased contractility and/or spasm of the microvasculature.

Therefore, the observation that sarcolemmal nNOS can be absent or mislocalized in sarcoglycanopathy muscle [45] provides a possible link between this pathogenetic mechanism and the development of cardiomyopathy in sarcoglycanopathies, offering further insights for therapeutic interventions.

NO stimulates soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), and in the absence of dystrophin the NO-sGC-cGMP pathway is disrupted. The nucleotide phosphodiesterases (PDEs) hydrolize the cGMP and regulate their downstream signaling. PDE5 expression in cardiomyocytes is low at baseline and increases in response to ischemia or pressure overload from heart failure. Impaired blood flow in muscle and heart in mdx dystrophin-deficient and NOS deficient mice was rescued by inhibition of PDE5. Unfortunately, in DMD and BMD patients a clinical trial with PDE5 inhibitor (Sidenafil) did not improve cardiomyopathy, since 30% of patients progressed to ventricular dilatation.

Long term dietary supplementation of L-arginine (a NOS substrate) was not a viable therapy for dystrophinopathy, but the use of antioxidants that attenuate the superoxide attack and restore the bioactive NO level, might be useful approaches for the treatment of these disorders.

Most patients present respiratory involvement of variable severity, which is especially relevant in the advanced stage of the disease [40], and sometimes results in respiratory failure while patients are still ambulant. Reduced respiratory function can be determined by measurements of forced vital capacity in both sitting and supine position. Respiratory insufficiency can be treated by non-invasive intermittent positive-pressure ventilation with nasal masks. Symptoms that suggest nocturnal hypoventilation are sleep disturbances, early morning headache, and daytime drowsiness. The demonstration of night-time hypoventilation by overnight pulse oximetry should lead to non-invasive nocturnal ventilation. Early detection of cardiomyopathy is important, since institution of cardio-protective medical therapies may slow adverse cardiac remodeling and attenuate heart failure symptoms in these patients. Although electrocardiogra-

phy and echocardiography are typically advocated for screening, cardiovascular magnetic resonance has shown promise in revealing early cardiac involvement when standard cardiac evaluation is unremarkable.

Joint range of movement necessitates physiotherapy and sometimes orthopaedic intervention. Scoliosis may be a problem in more severely affected patients, and they might need spinal surgery.

Dysferlinopathy identification (LGMD2B/R2)

A number of families of Palestinian and Italian origin suffered from a proximo-distal myopathy mapped to chromosome $2p^9$ (Fig. 3). Limb-girdle muscular dystrophy type 2B and the distal muscular dystrophy of Miyoshi (MM) are caused by mutations in the *DYSF* gene encoding the protein dysferlin [46,47]. Although the clinical features of LGMD2B/R2 and MM are different, both phenotypes can be detected among patients belonging to the same family [48]. The clinical heterogeneity might be attributed to additional epigenetic factors. Dysferlin immunolocalizes to the sarcolemma and has a central role in membrane fusion and repair of the plasmalemma lesions generated by eccentric muscle contraction, as demonstrated by the presence of many crowded vesicles just beneath the sarcolemma [49]. Several studies have reported a prominent inflammatory response in dysferlinopathy muscle [50] and increased ubiquitin-proteasomal and autophagic degradation secondary due to high levels of regeneration and inflammation [51]. The detection of dysferlin deficiency in muscle offers an important diagnostic tool [46,52].

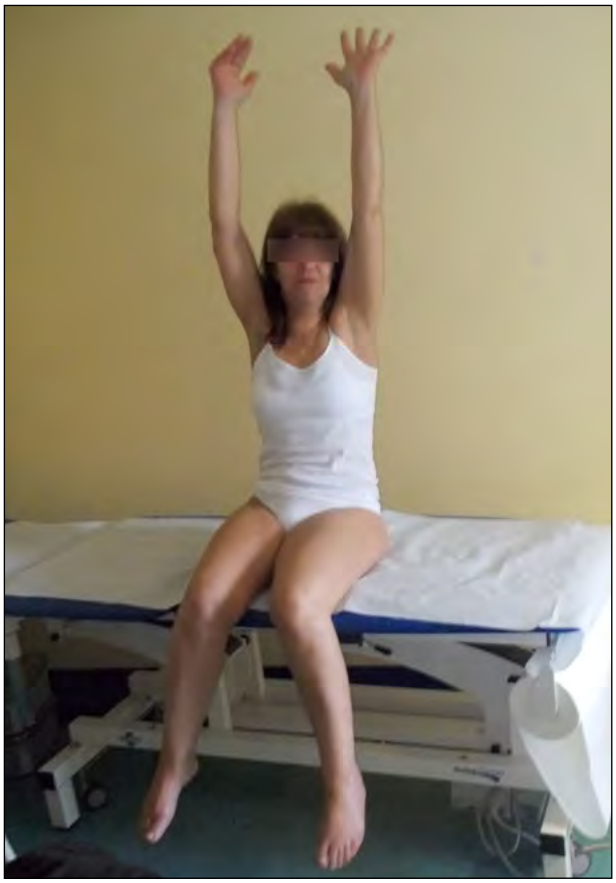

Figure 3. Dysferlinopathy-LGMD phenotype in a patient belonging to a family with affected individuals presenting various clinical phenotypes.

Transportinopathy identification (LGMD1F/D2)

The locus for one form of LGMD with autosomal dominant inheritance has been identified on chromosome 7 in 2003 thorough clinical and genetic analysis of a large Spanish family [53,54].

The identification of the gene and its encoded protein (transportin-3) was obtained in 2013 [55,56].

A large three generation family with several branches in Spain and Italy was previously examined and described in detail [54]. The clinical history in 29 patients was collected. There were early onset patients who became wheelchair bound around 30 years of age, while in milder cases, walking ability was preserved up to 65 years of age. Some patients had an early onset weakness, but others had the adult onset of the disease, with onset as late as 58 years. The severity appearance of phenotype does not always increase in successive generations [57], at difference to what originally reported by Gamez et al. [54]. In fact, anticipation phenomenon is generally seen in triplet expansion disorders, such as myotonic dystrophy or Huntington Chorea but not in this disorder. In this LGMD, the observed main features were dysphagia, dysarthria with bulbar, distal and axial weakness, variably occurring in the family members. The most involved muscles were at onset the lower limb muscles and then weakness spread to upper girdle muscles especially involving the triceps. Abnormal long fingers (arachnodactyly) characterize also the clinical phenotype [57]. There was a prominent neck axial weakness (flexor more than extensor). A main clinical sign was observed when patients were lying in bed in fact they were able to raise arm horizontally, but in standing position they were not able to fully lift arms over the head, because of scapular winging.

Transportin-3 protein is involved in HIV virus and other proteins transport in nucleus. The genetic mutations that cause transportinopathy make patients immune to AIDS, holding promise for research in this field and reassuring the patients regarding a possible HIV infection risk. The mutation could block the activity of the HIV-1 intasome and makes it unable to interact with cargo protein causing a block of nuclear import of proteins involved in lentiviral replication: CD3,CD28 peripheral blood cells

from transportinopathy patients of the italo-spanish family show in fact lower production of viral proteins in patients than control [58].

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Alpha-sarcoglycanopathy presenting as myalgia and hyperCKemia in two adults with a long-term follow-up. Case reports

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Two patients with a paucisymptomatic hyperckemia underwent a skeletal muscle biopsy and massive gene panel to investigate mutations associated with inherited muscle disorders. In the *SGCA* gene, sequence analyses revealed a homozygous c.850C > T/p.Arg284Cys in patient 1 and two heterozygous variants (c.739G > A/p. Val247Met and c.850C > T/p.Arg284Cys) in patient 2. Combination of histology and immunofluorence studies showed minimal changes for muscular proteins including the α-sarcoglycan. These two cases highlight the advantages of next-generation sequencing in the differential diagnosis of mild myopathic conditions before considering the more invasive muscle biopsy in sarcoglycanopathies.

Key words: hyperCKemia, Next Generation Sequencing, muscle biopsy, *SGCA*

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Introduction

Elevated blood creatine kinase (CK) is among the signs most likely to prompt referral of children and adults to neuromuscular specialists in Europe (ean.org/Guideline-Reference-Center.2699.0.html). Current international guidelines define persistent hyperCKemia as the presence of serum values more than 1.5 times the upper limit of normal in at least two independent measurements [1], but this definition does not consider the presence or absence of associated clinical manifestations. Indeed, asymptomatic hyperCKemia can run in families long before its chance detection. On the other hand, recent advances clarifying the genetic etiologies of muscle disorders have been crucial in reducing the number of undefined hyperCKemia cases and expanding the spectrum of manifestations associated with the condition. For example, using a multiple gene panel and next-generation sequencing (NGS) in a small cohort of undiagnosed patients with hyperCKemia, we recently detected a subset of paucisymptomatic individuals harboring biallelic variants in known muscular dystrophy genes [2].

We herein discuss two patients who showed persistent high serum CK levels and occasional exercise intolerance and myalgia in the absence of other neurological signs over a long-term follow-up. Both cases harbored biallelic variants of predicted pathogenic significance in *SGCA*. Patient

1 is 36-year-old man with an unremarkable prenatal and perinatal history and normal psychomotor development. He was first evaluated, because of persistent high CK levels at rest (ranging from 1700 to 8000 UI/L), at the age of 10 years. At that time his neuromuscular examination was unremarkable. At the age of 20, having more recently developed exercise intolerance and sporadic myoglobinuria after competitive exercise, he experienced two episodes of atrial fibrillation. After remission of his symptoms, he recovered completely and is still able to cycle and run for long distances, albeit with occasional muscle pain. At his latest neurological examination, which was unremarkable, moderate calf hypertrophy was observed.

Patient 2 is a 31-year-old woman with a history, from early childhood, of hyperCKemia (ranging from 500 UI/L to 4500 UI/L), almost invariably accompanied by myalgia and exercise intolerance. These complaints persisted unchanged over time in a context of normal psychomotor development. Her family history was significant for an asymptomatic uncle who had persistent high serum CK levels. The patient has undergone annual specialist evaluations over the past 25 years. Her latest neurological, cardiac and respiratory examinations were normal, but we observed minimal hypertrophy of the calves. A muscle MRI study of lower girdle muscles was unremarkable but we observed fat infiltration in the right gluteus minimus (Fig. 1).

In the course of their lives, both patients have twice undergone muscle biopsy; all these examinations showed only minimal myopathic changes on histological assessment and normal immunofluorescence staining for muscle proteins including α-sarcoglycan (Fig. 2). Using a massive gene panel to investigate genes associated with inherited muscle disorders [2], we identified a homozygous c.850C >T/p.Arg284Cys mutation in patient 1 and two variants (c.739G > A/p.Val247Met and c.850C > T/p.Arg284Cys) in compound heterozygosity in patient 2in the *SGCA* gene. Both variants were predictably deleterious, with high CADD scores (https://cadd.gs.washington.edu/); both segregated with the disease status in the respective families (Fig. 3), and met the American College Medical Genetics and Genomics recommended criteria for possible pathogenic variants [3].

The mutations detected in this study have already been reported in association with myoglobinuria, high CK levels and limb girdle muscle weakness [4,5]. Our report adds to the evidence that hyperCKemia can be the presenting feature of a sarcoglycanopathy associated with myoglobinuria, exercise-induced myalgia and/or rhabdomyolysis. Contrary to previous reports, our long follow-up of these patients, characterized by unremarkable neurological examinations and only minimal, pseudometabolic complaints, suggests that a sarcoglycanopathy can go undetected in patients and families. This suggests that caution should be exercised before dismissing cases of asymptomatic hyperCKemia as idiopathic, and that such cases should be monitored closely for potential severe myoglobinuria and rhabdomyolysis following minimal exercise. Our report is also significant as it might draw attention of neuromuscular experts to the value of the NGS approach, which can play a fundamental diagnostic role and may obviate the need to perform muscle biopsies in patients with asymptomatic hyperCKemia [6-8]. In our patients, the combination of muscle histology and immunofluorescence alone would not have identified the cause of the symptoms. This suggests that a first-tier NGS screening in blood should be favoured, especially in children, before considering the more invasive muscle biopsy option, which could instead be kept as a second-level diagnostic approach to be used in the event of uncertain or negative results. Whether other factors besides the genetic mutation influence phenotypic manifestations in sarcoglycanopathies remains unclear and should be further investigated.

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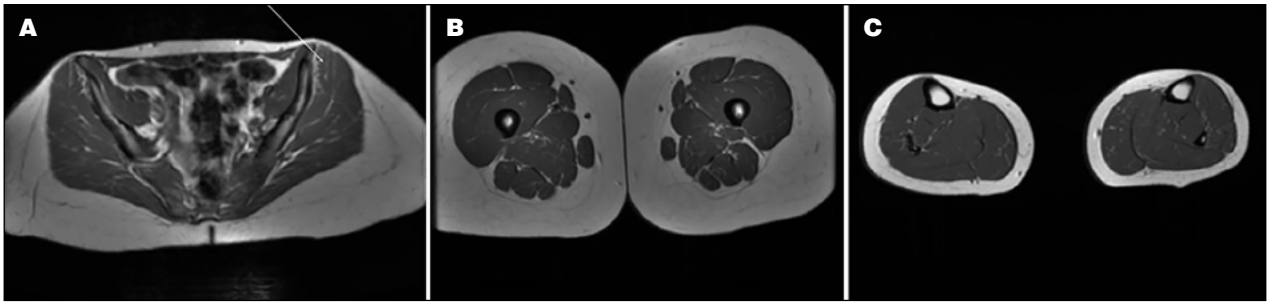

Figure 1. Muscle MRI findings in patient 2 at pelvi (A), thigh (B) and calf level were obtained using conventional T1-weighted spin-echo transverse images. No muscles fat infiltration were evident in all muscles except in the right gluteus minimus.

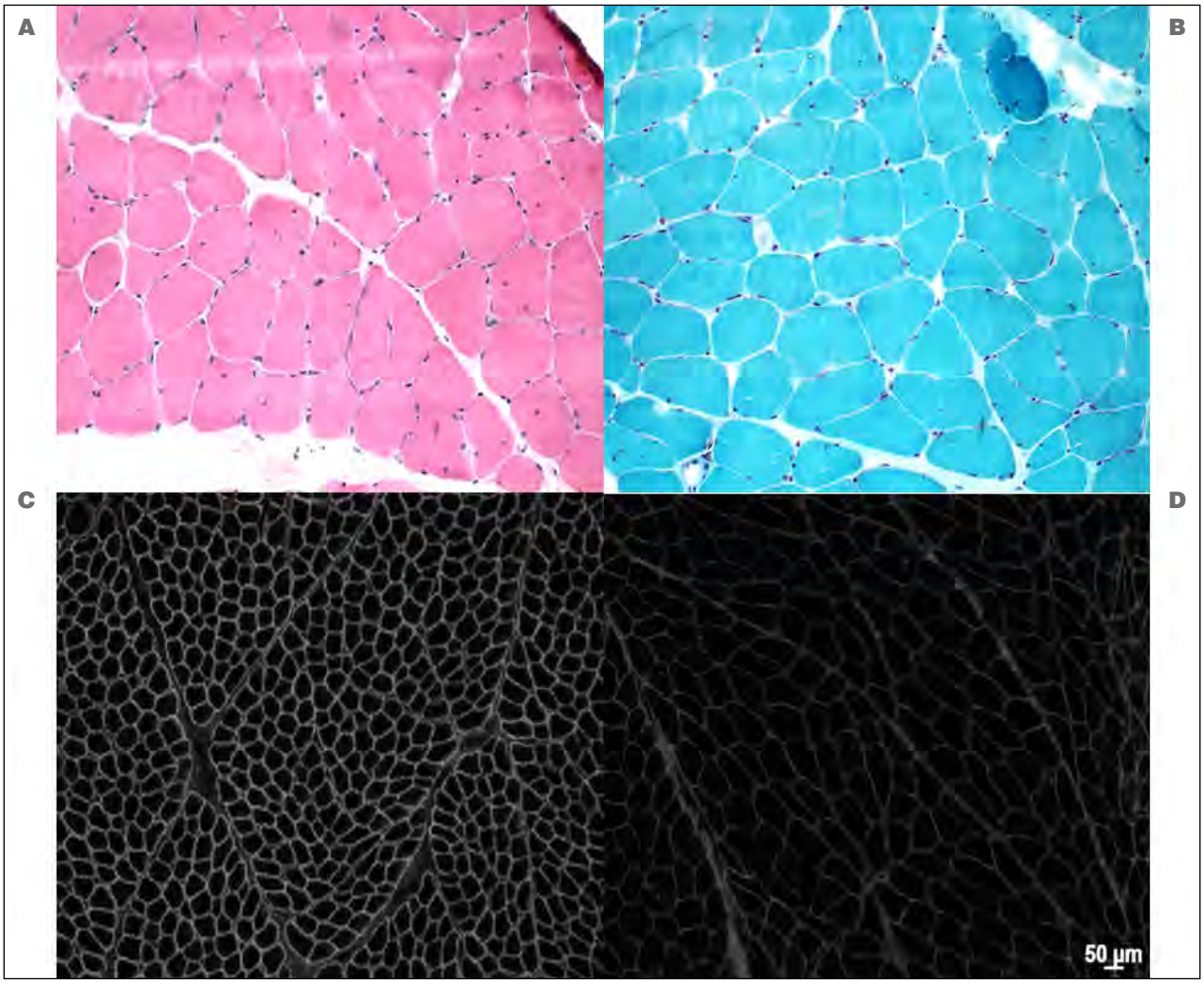

Figure 2. Myopathological changes in patient 2. Hematoxylin and eosin (A) and Gomori trichrome (B) staining demonstrating slight variation in fiber size and some central nuclei. Immunofluorescence labeling of α-sarcoglycan showing decreased expression in patient 2 (D) compared with an age-matched healthy control (C).

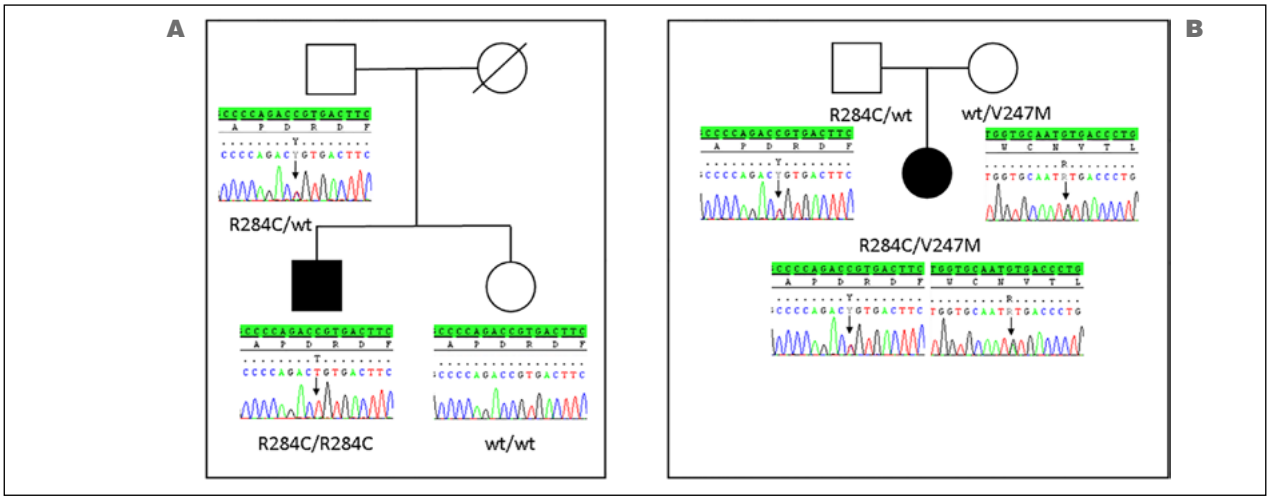

Figure 3(A-B) Genetic studies. Pedigree of the family and electropherograms showing the segregation of the variants in the family members.

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Myotonic dystrophy type 2: the 2020 update

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The myotonic dystrophies are the commonest cause of adult-onset muscular dystrophy. Phenotypes of DM1 and DM2 are similar, but there are some important differences, including the presence or absence of congenital form, muscles primarily affected (distal *vs* proximal), involved muscle fiber types (type 1 *vs* type 2 fibers), and some associated multisystemic phenotypes. There is currently no cure for the myotonic dystrophies but effective management significantly reduces the morbidity and mortality of patients. For the enormous understanding of the molecular pathogenesis of myotonic dystrophy type 1 and myotonic dystrophy type 2, these diseases are now called "spliceopathies" and are mediated by a primary disorder of RNA rather than proteins. Despite clinical and genetic similarities, myotonic dystrophy type 1 and type 2 are distinct disorders requiring different diagnostic and management strategies. Gene therapy for myotonic dystrophy type 1 and myotonic dystrophy type 2 appears to be very close and the near future is an exciting time for clinicians and patients.

Key words: myotonic dystrophy type 2, DM2, proximal myotonic myopathy, PROMM, *DMPK*, CNBP

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Introduction

The myotonic dystrophies are the more frequent muscle disorders in adulthood. So far 2 distinct entities have been described: myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2).

In this article I review the discovery of the gene, the clinical features, pathogenetic and management of more recently described DM2. All findings related mainly to clinical aspects, pathomolecular mechanisms, new guidelines of management have been updates to 2020.

Discovery of the genes

Myotonic dystrophies represent a group of dominantly inherited, multisystem (eye, heart, brain, endocrine, gastrointestinal tract, uterus, skin) diseases that share the core features of myotonia, muscle weakness, and early onset cataracts (before 50 years of age). The gene defect responsible for myotonic dystrophy described by Steinert on 1908, was discovered in 1992 and was found to be caused by expansion of a CTG repeat in the 3' untranslated region of myotonic dystrophy protein kinase gene (*DMPK*), a gene located on chromosome 19q13.3, encoding a protein kinase [1-3]. After the discovery of this gene defect, DNA testing revealed a group of patients with dominantly inherited myotonia, proximal more than distal weakness, and cataracts; these patients were previously diagnosed as having myotonic dystrophy of Steinert but lacked the gene defect responsible for this

disease. Subsequent clinical studies of kindreds with patients having these characteristics led to new diagnostic labels for these patients: myotonic dystrophy type 2 [4], proximal myotonic myopathy (PROMM) [5,6], or proximal myotonic dystrophy (PDM) [7]. Later studies demonstrated that many of the families identified as having myotonic dystrophy type 2, PROMM, or PDM had a single disorder that results from an unstable tetranucleotide CCTG repeat expansion in intron 1 of the nucleic acid-binding protein (CNBP) gene (previously known as zinc finger 9 gene, ZNF9) on chromosome 3q21 [8,9].

Myotonic dystrophy of Steinert, the classical form of myotonic dystrophy that results from an unstable trinucleotide repeat expansion on chromosome 19q13.3, was termed myotonic dystrophy type 1-DM1. Patients with the clinical picture of myotonic dystrophy type 2, PROMM, or PDM who have positive DNA testing for the unstable tetranucleotide repeat expansion on chromosome 3q21 were classified as having myotonic dystrophy type 2 (DM2). Reliability of DNA testing to establish or to exclude the diagnosis of myotonic dystrophy type 1 is close to 100% [10]. However, caution is necessary in the diagnosis of myotonic dystrophy type 2. At present, much more information is available on the natural history of DM1 than DM2, but knowledge of myotonic dystrophy type 2 will increase at a rapid pace over the next several years.

Biological basis: pathomolecular mechanisms

Myotonic dystrophy type 2 results from an unstable tetranucleotide repeat expansion, CCTG in intron 1 of the nucleic acid-binding protein (CNBP) gene (previously known as zinc finger 9 gene, ZNF9) on chromosome 3q21 [8,9,11,12]. The cause for the unstable expansion is unknown. In contrast to the (CTG)n repeat in myotonic dystrophy type 1, in myotonic dystrophy type 2/proximal myotonic myopathy the (CCTG)n repeat is a part of the complex repetitive motif (TG)n(TCTG)n(CCTG)n, and the (CCTG)n repeat tract is generally interrupted in healthy range alleles by 1 or more GCTG, TCTG, or ACTG motifs, whereas it is typically uninterrupted in the expanded alleles [9,11,13].

The size of the (CCTG)n repeat is below 30 repeats in normal individuals, whereas the range of expansion sizes in myotonic dystrophy type 2 patients is huge [13]. The smallest reported mutations vary between 55 and 75 CCTG [9,13] and the largest expansions have been measured to be about 11,000 repeats [9]. The expanded myotonic dystrophy type 2 alleles show marked somatic instability, with significant increase in length over time [9,14], thus the threshold size of the disease-causing mutation remains to be determined. The size of the CCTG repeat appears to increase over time in the same individual, and, like myotonic dystrophy type 1, this is a dynamic gene defect [14]. These 2 genetic findings complicate the correlation between genotype and phenotype (Tab. I). The gene mutation responsible for myotonic dystrophy type 2 appears to have arisen from a Northern European founder [11,12], but single-kindred Afghan [15] and Japanese [16] cases have been described. Both mutations are believed to have occurred after migration out of Africa, between 120,000 and 60,000 years ago. The age of the myotonic dystrophy type 2 founder mutation has been estimated at 4000 to 12,000 years (about 200 to 540 generations) [11]. The molecular pathomechanism leading to the manifestations of myotonic dystrophy type 2 is felt to be similar to that in myotonic dystrophy type 1 and relates to a toxic effect of the abnormally expanded RNA that accumulates in the muscle nuclei [17-21].

The fact that 2 repeat sequences located in entirely different genes can cause such similar disease features implies a common pathogenic mechanism. The clinical and molecular parallels between myotonic dystrophy type 1 and type 2 strongly suggest that the mutant RNAs containing the repeat expansions that accumulate in the cell nuclei as foci are responsible for the pathological features common to both disorders. It is now clear that the gain-of-function RNA mechanism is the predominant cause of myotonic dystrophy pathogenesis in which the CUG and CCUG repeats alter cellular function of several RNA-binding proteins. It has been demonstrated that mutant CUG and CCUG RNAs are very stable due to a deficiency of RNA helicase p68 [22]. The expanded CUG and CCUG RNA form hairpins, imperfect double-stranded structures that lead to dysregulation of 2 important RNA-binding proteins: muscleblind like 1 (MBNL1) and CUG-binding protein 1 (CUGBP1), which are antagonist regulators of alternative splicing of various genes [23,24]. Data demonstrate that MBNL1-containing foci in myotonic dystrophy type 2 cells also sequester snRNPs and hnRNPs, splicing factors involved in the early phases of transcript processing [25,26], thus strengthening the hypothesis that a general alteration of pre-mRNA posttranscriptional pathway could be at the basis of the multifactorial phenotype of myotonic dystrophy type 2 patients. In myotonic dystrophies, the downregulation of MBNL1, due to its sequestration in mutant RNA foci, and the upregulation of CUGBP1 result in abnormal expression of embryonic isoforms in adult tissues. The alteration of pre-mRNA processing strengthens the hypothesis of a spliceopathy that leads to an expression of isoforms inadequate for a particular tissue or developmental stage [27,28]. In both myotonic dystrophy type 1 and type 2, missplicing of insulin receptor gene (INSR) was associated with insulin resistance. However, Renna and colleagues re-

Table I. Etiology of DM1 and DM2.

	DM1	DM2
Chromosomal locus	19q 13.3	3q 21.3
Gene	DMPK	ZNF9/CNBP
Inheritance	Autosomal dominant	Autosomal dominant
Mechanism	CTG repeat expansion	CCTG repeat expansion
Normal repeat size	< 37	< 27
Pathologic repeat size	> 50	> 75?
Expanded repeat range	50-4000	75-5000 -> 11000
Anticipation	Yes	-----

ported that post-receptor insulin signal transduction via both IRS1-Akt/PKB and Ras-ERK pathway is impaired in myotonic dystrophy skeletal muscle, thus contributing to insulin resistance observable in myotonic dystrophy type 1 and type 2 patients [29]. Moreover, myotonic dystrophy skeletal muscle exhibits a lower expression of the insulin receptor in type 1 fibers, contributing to the defective activation of the insulin pathway [30]. It is now clear that the molecular pathomechanism of myotonic dystrophies is more complex than actually suggested [31].

miRNAs are small, noncoding RNA modulating gene expression at the posttranscriptional level, and their expression and intracellular distribution are deregulated in many human diseases, including muscular dystrophies [32-36]. Both in myotonic dystrophy type 1 and in myotonic dystrophy type 2 it has been demonstrated that the highly regulated pathways of miRNA are altered in skeletal muscle, potentially contributing to myotonic dystrophy pathogenetic mechanisms [34-36]. A deregulation of microRNA in skeletal muscle and plasma from myotonic dystrophy type 2 patients has been also reported [36,37]. The identification of minimally invasive analytical biomarkers for myotonic dystrophies and the established potential of circulating miRNAs as prognostic and diagnostic biomarkers are particularly important to monitor myotonic dystrophies progression and the effectiveness of new drug treatments.

A novel molecular mechanism that may contribute to the pathogenesis of myotonic dystrophies has been described by Zu and collaborators [38]. RNA transcripts containing expanded CAG or CUG repeats can be translated in the absence of a starting ATG, and this noncanonical translation, called repeat associated non-ATG translation (RAN-translation), occurs across expanded repeats in all reading frames to produce potentially toxic homopolymeric proteins [38,39]. It has been demonstrated that RAN-translation also occurs across transcripts containing the myotonic dystrophy type 2 CCUG or CAGG expansion mutation, producing tetra-repeat expansion proteins with a repeating Leu-Pro-Ala-Cys (LPAC) or Gln-Ala-Gly-Arg (QAGR) motif [40]. Both LPAC and QAGR RAN proteins accumulate in myotonic dystrophy type 2 human autopsy brains in distinct patterns. For LPAC, cytoplasmic aggregates are found in neurons, astrocytes, and glia in the gray matter regions of the brain. In contrast, QAGR RAN protein accumulation, which is nuclear, is found primarily in oligodendrocytes located in white matter regions of the brain. Moreover, it has been evidenced that RAN protein accumulation can be modulated by MBNL1 levels and that nuclear sequestration of CCUG, CUG, or CAG RNAs decrease steady-state levels of RAN proteins [40]. These data suggest that RAN-translation may be common to both myotonic dystrophy type 1 and type 2 and that RAN proteins may be responsible for some of the CNS features of myotonic dystrophies.

Another open question in the field of myotonic dystrophies is to clarify the pathomechanisms underlying the phenotypic differences between myotonic dystrophy type 1 and type 2. Clinical signs in myotonic dystrophy type 1 and type 2 are similar, but there are some distinguishing features: myotonic dystrophy type 2 is generally less severe and lacks a prevalent congenital form. This suggests that other cellular and molecular pathways are involved besides the shared toxic-RNA gain of function hypothesized. An important step forward in understanding the differences between myotonic dystrophy type 1 and type 2 has been made. Indeed, rbFOX1 has been reported as a novel RNA binding protein that specifically binds to expanded CCUG repeats, but not to expanded CUG repeats. rbFOX1 is enriched in skeletal muscle, heart, and brain and is involved in the regulation of various aspects of mRNA metabolism. In the study, it has been demonstrated that rbFOX1 co-localizes with CCUG RNA foci in muscle cells and skeletal muscle tissues of individuals with myotonic dystrophy type 2, but not with CUG RNA foci in myotonic dystrophy type 1 samples [41]. Interestingly, rbFOX1 competes with MBNL1 for binding to CCUG expanded repeats, and its overexpression partly releases MBNL1 from sequestration within CCUG RNA foci in muscle cells. Furthermore, expression of rbFOX1 corrects alternative splicing alterations and rescues muscle atrophy, climbing, and flying defects caused by

expression of expanded CCUG repeats in a Drosophila model of myotonic dystrophy type 2 [41].

Several studies have revealed a role for CNBP in myotonic dystrophy type 2. CNBP deletion in several animal models results in severe brain and muscle phenotypes and other abnormalities similar to those seen in myotonic dystrophy type 2 [42-45]. These defects can be rescued by reintroduction of wild-type levels of CNBP, suggesting that a loss of CNBP function likely contributes to myotonic dystrophy type 2. Two reports using cell models describe a reduction of the rate of protein translation in myotonic dystrophy type 2 muscle cells due to a decrease of CNBP protein levels in myotonic dystrophy type 2 myoblasts and adult muscle [46] and due to the interaction of CCUG repeats with cytoplasmic multiprotein complexes, which dysregulate translation and degradation of proteins in patients [47]. Sammons and colleagues report that CNBP activity is reduced in myotonic dystrophy type 2 human myoblasts leading to a decrease in CNBP activation of IRES-mediated translation of the human ODC and suggest that CNBP activity may contribute to myotonic dystrophy type 2 phenotype [48]. Moreover, the reduction of CNBP expression has been reported in myotonic dystrophy type 2 muscle biopsies but not in myotonic dystrophy type 1, thus explaining some of the phenotypic disparities between both types of myotonic dystrophies [49]. Taken together, these data suggest that myotonic dystrophy type 2 pathology may be due to a combination of an RNA gain of function and CNBP loss of function.

The role of CUGBP1 in myotonic dystrophy type 2 is particularly intriguing, with contradictory results being reported [47,49-51]. Cardani and colleagues demonstrated that this protein is overexpressed in muscle biopsies from patients affected by the adult classical form of myotonic dystrophy type 1 but not in muscle from myotonic dystrophy type 2 patients, suggesting that sequestration of MBNL1 evidently has a central role in splicing misregulation in both types of myotonic dystrophies, whereas CUGBP1 overexpression might be an additional pathogenic mechanism in myotonic dystrophy type 1 not shared by myotonic dystrophy type 2 [49]. However, it has been shown that MBNL1 overexpression in a mouse model of RNA toxicity (DM200) is not effective in reversing myotonic dystrophy type 1 phenotypes such as myotonia and cardiac conduction abnormalities. Also, the mice do not show improvement in function assays such as grip strength or treadmill running, and MBNL1 overexpression notably increases muscle histopathology and results in variable rescue of a number of splicing targets [52].

Vihola and collaborators investigated the molecular basis of muscle weakness and wasting and the differences in muscle phenotype between myotonic dystrophy type 1 and type 2. They identified differences in muscle gene expression and splicing between myotonic dystrophy type 1 and type 2 patients. In particular, the aberrant splicing isoform of TNNT3 is twice as frequent in myotonic dystrophy type 2 compared to myotonic dystrophy type 1. Moreover, in myotonic dystrophy type 1 and type 2, a different protein expression pattern has been found in the highly atrophic fibers [53]. Concerning myotonic dystrophy type 2, skeletal muscle phenotype has been studied in heterozygous Cnbp KO mice and in human muscle samples [54]. The study demonstrates that CNBP protein expression is reduced in cytoplasm of myotonic dystrophy type 2 muscle fibers, and it is predominantly localized at membrane level where its interaction with α-dystroglycan is increased compared to controls. These findings suggest that alterations of CNBP in myotonic dystrophy type 2 might cause muscle atrophy, not only via misregulation of mRNA but also via protein-protein interactions with membrane proteins affecting myofiber membrane function [54].

Epidemiology

Myotonic dystrophy type 2 appears to have a lower prevalence than myotonic dystrophy type 1 and primarily affects populations with a Northern European heritage [12]. For myotonic dystrophy type 2, there are currently no established prevalence estimates; myotonic dystrophy type 2 is generally thought to be rarer than myotonic dystrophy type 1, but large-scale population studies to confirm this have not been performed. In Germany, 267 mutation-verified molecular diagnoses were made between 2003 and 2005 compared with 277 myotonic dystrophy type 1 diagnoses within the same period. These data suggest that myotonic dystrophy type 2 appears to be more frequent than previously thought, with most myotonic dystrophy type 2 patients currently undiagnosed with symptoms frequently occurring in the elderly population [55]. However, many patients in older generations with myotonic dystrophy type 1 or type 2 with milder symptoms are clearly undiagnosed. It is noteworthy that recessive mutations in the chloride channel gene CLCN1, which have a high frequency in the general population, can act as modifiers in patients with myotonic dystrophy type 2 disease by amplification of their myotonia [56-58]. Meola's group has identified myotonic dystrophy type 2 patients presenting an atypical phenotype characterized by early and severe myotonia without mutation on the *CLCN1* gene but with mutations on *SCN4A* gene [59-61]. Thus, both *CLCN1* and *SCN4A* mutations may contribute to exaggerate the myotonia in myotonic dystrophy type 2 [60].

Clinical manifestation

There are no distinct clinical subgroups in DM2, and clinical presentation comprises a continuum ranging from

early adult-onset severe forms to very late-onset mild forms that are difficult to differentiate from normal aging. Only 2 cases of neonatal forms have been reported so far in the literature: 1 of these patients had reduced intrauterine movements and muscle hypotonia after birth [62], and the second had only congenital talipes equinovarus without any other clinical sign [63]. At present, there is no evidence of a congenital or childhood form of myotonic dystrophy type 2 [14]. The main difference in DM2 in comparison to DM1 is the absence of congenital form. Myotonic dystrophy type 2 typically presents in adulthood and has variable manifestations such as early onset cataracts (less than 50 years of age), various grip myotonias, thigh muscle stiffness, muscle pain, and weakness (in hip flexors, hip extensors, or long flexors of the fingers) [4-6,14,64-67]. These complaints often appear between 20 and 50 years of age. Posterior subscapular cataract before 50 years of age is a characteristic feature of myotonic dystrophy type 2, and early onset cataract can be a presenting feature of the disease, preceding all other symptoms [68]. Pain is a common as well as a highly relevant problem for many patients with myotonic dystrophy type 2, with an estimated lifetime prevalence of 76% and a negative effect on quality of life [69]. Patients and their care providers ascribe the symptoms to overuse of muscles, "pinched nerves", "sciatica", arthritis, or fibromyalgia. In comparison to other chronic muscle disorder patients, myotonic dystrophy type 2 patients more frequently describe a pain that is sometimes reported to be exercise-related, temperature-modulated, and palpation-induced (Tab. II) [70]. Younger patients may complain of stiffness or weakness when running up steps, whereas they infrequently complain of cramps. The muscle pain in myotonic dystrophy type 2 has no consistent relationship to exercise or to the severity of myotonia found on clinical examination. The pain, which tends to come and go without obvious cause, usually fluctuates in intensity and distribution over the limbs. It can last for days to weeks. This pain seems qualitatively different from the muscle and musculoskeletal pain that occurs in patients with myotonic dystrophy type 1. In a study on qualitative as well as quantitative aspects of pain in patients with myotonic dystrophy type 2, it has been observed that mechanical hyperalgesia is the main finding present in the rectus femoris, trapezius, and thenar, suggestive of at least a peripheral mechanism of pain [69]. Pain appears to be most often located symmetrically in the proximal limbs [69]. Myotonic dystrophy type 2 scored significantly lower than myotonic dystrophy type 1 on the bodily pain scale, indicating more body pain in myotonic dystrophy type 2. This finding has a high disease impact on physical as well as on mental health functioning [71], and on professional performance [72]. A transcriptomic analysis performed on 12 muscle biopsy specimens obtained from myotonic dystrophy type 2 patients has identified 14 muscle genes significantly up- or down-regulated in myalgic patients compared to nonmyalgic myotonic dys-

Table II. Multisystemic aspects of adult onset DM2.

Brain	• Similar visual-spatial executive function deficits to those present in DM1
Heart	• Significant disturbances in conduction much less common than in DM1
Respiratory	• Obstructive sleep apnea
Anesthesia	• Limited information is available to determine if there is a significant and increased risk of general anesthesia. Recommended careful monitoring in postoperative period until more information is published
Hypersomnia and fatigue	• Excessive daytime sleepness is not as prominent in DM1 • Obstructive sleep apnea • CNS and muscle related fatigue
Endocrine	• Gonadal insufficiency • Low testosterone • Erectile dysfunction • Insulin resistance • Hyperlipidemia • Hypothyroidism
Pregnancy	• Limited information is available to determine if there is significant risk of complication during pregnancy and delivery • Weakness and stiffness may worsen during pregnancy and improve following delivery
Muscle pain	• Often a major simptoms, expecially in the arms and upper lower back • Fluctuates in duration, location and intensity • Can worsen with exercise and cold temperature • Aches and stiffness

trophy type 2 patients. These data support the idea that molecular changes in the muscles of myotonic dystrophy type 2 patients are associated with muscle pain and suggest that muscle-specific molecular pathways might play a significant role in myalgia [73].

Early in the presentation of myotonic dystrophy type 2, there is only mild weakness of hip extension, thigh flexion, and finger flexion. Myotonia of grip and thigh muscle stiffness varies from minimal to moderate severity over days to weeks. Direct percussion of forearm extensor and thenar muscles is the most sensitive clinical test for myotonia in myotonic dystrophy type 2. Myotonia may appear only on electromyographic testing after examination of several muscles [14,64]. Facial weakness is mild in myotonic dystrophy type 2 as is muscle wasting in the face and limbs (Fig. 1). Weakness of neck flexors is frequent. Trouble arising from a squat is common, especially as the disease progresses (Fig. 2). Calf muscle hypertrophy occasionally is prominent (Fig. 3). Other manifestations, such as excessive sweating, hypogonadism, glucose intolerance, cardiac conductions disturbances, cognitive alterations, and neuropsychological alterations, may also occur and worsen over time [6,14,65,74]. Sleep complaints and breathing disorders are also frequent in myotonic dystrophy type 2 [75].

A study on frequency and progression of cardiac and muscle involvement in a large cohort of patients with myotonic dystrophy type 2 demonstrated that the frequency and severity of cardiac involvement and muscle weakness are reduced in myotonic dystrophy type 2 compared to myotonic dystrophy type 1 and that progression is slower and less severe [76]. Nevertheless, careful cardiac evaluation is recommended to identify patients at risk for potential cardiac major arrhythmia. A retrospective study comprised of 62 adult patients with myotonic dystrophy type 2 showed that cardiac conduction and rhythm defects are relatively rare in myotonic dystrophy type 2, although diastolic dysfunction is common, suggesting that regular ECG and echocardiography screening is needed in these patients [77]. Cardiovascular magnetic resonance

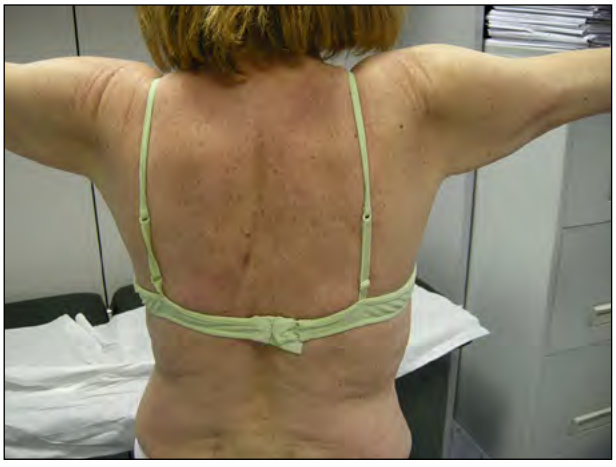

Figure 1. Mild atrophy, grade 4 MRC proximal muscle weakness in upper limbs in a patient affected by DM2.

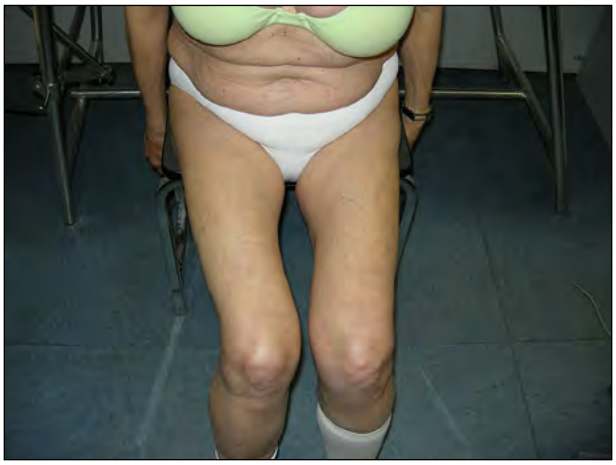

Figure 2. Moderate atrophy and weakness of proximal lower limbs (grade 3 MRC) with difficulty in arising from a chair in a patient affected by DM2.

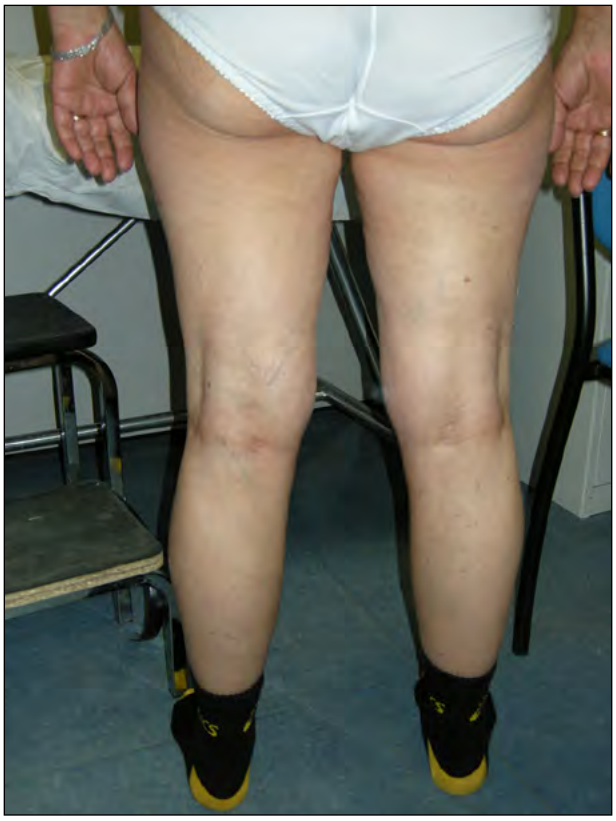

Figure 3. Calf hypertrophy in a patient affected by DM2.

demonstrates that in myotonic dystrophy type 2 patients subclinical myocardial injury was already detectable in preserved left ventricular ejection fraction. Moreover, extracellular volume was also increased in regions with no focal fibrosis and myocardial fibrosis was related to conduction abnormalities [78].

Patients with both myotonic dystrophy type 1 and type 2 have lower scores on frontal lobe functioning tests compared to controls and have an increased prevalence of avoidant personality disorders [6]. In a study aimed to analyze personality patterns in a cohort of myotonic dystrophy type 1 and type 2 patients, no significant personality impairments have been observed in patients with myotonic dystrophy type 2, and the most common clinical symptoms observed in these patients were anxiety and somatization [79]. In patients with type 2 disease, conventional brain MRI findings can be entirely normal. However, in advanced stages or more severe cases, diffuse white-matter changes can be present although be less pronounced than or different to that in myotonic dystrophy type 1 [80,81]. It has been reported that the main transcranial sonography finding in myotonic dystrophy type 2 patients is brainstem raphe hypoechogenicity, which is associated with fatigue and excessive daytime sleepiness. In addition, substantia nigra hyperechogenicity and increased diameter of the third ventricle has been observed [82]. The type of cognitive impairment that occurs in myotonic dystrophy type 2 is similar to but less severe than that of myotonic dystrophy type 1. A specific type of "avoidant" personality and a significant impairment in frontal lobe function (especially limited ability to perform executive functions) have been observed in myotonic dystrophy type 1 and type 2 patients, although these abnormalities were milder in myotonic dystrophy type 2 patients [82]. Similar observations have been reported in a study performed in a larger cohort of myotonic dystrophy type 2 patients [84]. In conclusion there are clinical, neuropsychological, and neuroimaging data that support the hypothesis of central nervous system involvement also in myotonic dystrophy type 2 [85].

Gastrointestinal manifestations are common in myotonic dystrophy type 2 patients, affecting their quality of life. A study on progression of gastrointestinal manifestations in these patients reports that during the 5 years of follow-up, the most common changes are the development of trouble swallowing and constipation and that female patients demonstrate a greater risk of a gastrointestinal manifestation [86]. A relatively high frequency of cholecystectomy on average before 45 years of age is also reported [86].

It has been reported that hearing impairment is a frequent symptom in myotonic dystrophy type 2 patients and that the sensorineural hearing impairment is located in the cochlea [87]. This suggests it is important to perform audiometry when hearing impairment is suspected in order to propose early hearing rehabilitation with hearing aids when indicated.

In a study conducted on a large cohort of 307 genetically-confirmed myotonic dystrophy type 2 patients, a profound gender and age influence on the phenotype has emerged, emphasizing that female gender and aging may be associated with a higher disease burden [88]. Indeed, it appears that with aging, there is a tendency towards the worsening of weakness, whereas myalgia and myotonia tend to decrease. Females seem to be more severely affected than men as they show more frequently muscle weakness, multisystem involvement, and need of using walking aids. This study suggests that these age- and gender-specific differences should be considered in diagnostics, management, and future clinical studies of myotonic dystrophy type 2.

It has been observed that metabolic syndrome is common in myotonic dystrophy type 2 patients but not more frequent than in healthy subjects. However, treatment of metabolic disturbances may reduce cardiovascular complications and improve quality of life in patients with myotonic dystrophy type 2 [89].

Body composition assessed by DEXA (dual-energy x-ray absorptiometry) reveals that patients with myotonic dystrophy type 1 and type 2 have similar total body mass, bone mineral content, fat mass, and lean tissue mass. Patients with myotonic dystrophy type 2 have less visceral fat deposition than those affected by myotonic dystrophy type 1. Also, right rib bone mineral density was lower in myotonic dystrophy type 2 patients [90].

Overall the prognosis for patients with myotonic dystrophy type 2 is more favorable than for individuals with myotonic dystrophy type 1. Patients usually have a slower, less severe, and less widespread progression of muscle weakness and less muscle wasting. Does not seem to be a more severe phenotype associated with the homozygotic form of this disease [15]. As in myotonic dystrophy type 1, patients with myotonic dystrophy type 2 who have an earlier onset of symptoms have an earlier onset of myotonia and weakness [91]. The natural history of myotonic dystrophy type 2 remains to be fully defined, but present information indicates that most patients have a normal lifespan. Respiratory failure, hypersomnia, and recurrent aspiration or pneumonia are not common in myotonic dystrophy type 2 [72]. Cardiac conduction disturbances occur [67], but they are less frequent compared to myotonic dystrophy type 1 [92,93]. An investigation using a variety of standard tests of autonomic function (response to Valsalva maneuver, deep breathing, change in posture, grip, analysis of heart rate variability) reveals no major abnormalities in patients with myotonic dystrophy type 2 [94].

Diagnosis

The gold standard for establishing the diagnosis of myotonic dystrophy type 2/proximal myotonic myopathy is to demonstrate the presence of abnormal CCTG repeats in the 3q21 zinc finger protein 9 (ZNF9/CNBP) gene involved with myotonic dystrophy type 2.

Leucocyte DNA testing is also available for myotonic dystrophy type 2, but previous DNA analysis for diagnosing myotonic dystrophy type 2 and proximal myotonic myopathy may have missed as many as 20% of affected individuals [14]. As for myotonic dystrophy type 1, a new ready to use genetic test has been validated to identify the myotonic dystrophy type 2 disease, with the advantage to reduce errors that can be introduced using homemade reagents [95]. However, the myotonic dystrophy type 2 diagnostic odyssey is complicated by the difficulties to develop an accurate, robust, and cost-effective method for a routine molecular assay [60].

A more practical tool for myotonic dystrophy type 2 diagnosis than the complex genotyping procedure is via in situ hybridization detection of nuclear accumulations of CCUG-containing RNA in myotonic dystrophy type 2 muscle biopsy using specific probes [21,96]. Moreover, because MBNL1 is sequestered by mutant RNA foci, it is possible to visualize the nuclear accumulation of MBNL1 by immunofluorescence on muscle sections. However, although MBNL1 represents a histopathological marker of myotonic dystrophies, it does not allow one to distinguish between myotonic dystrophy type 1 and myotonic dystrophy type 2 [96]. Another tool to investigate muscle weakness and wasting is muscle imaging with MRI. In type 2 disease, early muscular changes develop in the anterior vastus group of thigh muscles, with relative sparing of the rectus femoris [98]. The main aspects of multisystemic involvement are summarized in the Table II.

Management

In general, the management of myotonic dystrophy type 2 is similar to myotonic dystrophy type 1, but there is less need for supportive care like bracing, scooters, or wheelchairs. Cataracts require monitoring, and serial monitoring of ECG is necessary to check for covert arrhythmia. Disturbances in cardiac rhythm are less frequent in myotonic dystrophy type 2, but abnormalities do occur [14,67]. Hypogonadism and insulin resistance need monitoring as in myotonic dystrophy type 1. Myotonia tends to be less marked and less troublesome in myotonic dystrophy type 2, but in specific circumstances, especially if muscle stiffness is frequent and persistent, anti-myotonia therapy with mexiletine is helpful. Cognitive difficulties also occur in myotonic dystrophy type 2, as in myotonic dystrophy type 1, and appear to be associated with decreased cerebral blood flow to frontal and anterior temporal lobes [74] and decreased brain volume [94,99,100]. The changes are less severe than in myotonic dystrophy type 1. Their etiology is unknown but may relate to the toxic effect of intranuclear accumulations of abnormally expanded RNA. Management of these brain symptoms is similar to that for myotonic dystrophy type 1.

A frequent and difficult problem in myotonic dystrophy type 2 is the peculiar muscle pain described earlier. The exact mechanism underlying the pain is unknown, and there is no well-established effective treatment. Carbamazepine or mexiletine along with nonsteroidal anti-inflammatory medications ameliorate this pain in some patients. However, others with severe pain may require opiates on a regular basis to obtain relief. Fortunately, this peculiar muscle pain is not typical in myotonic dystrophy type 1. Guidelines on diagnosis and management have been published [98]. Care considerations and management issues on the wide spectrum of disease manifestations in DM2 have been published recently by a Consortium of international Experts [101].

For pregnancy and anesthesia there are some special considerations.

Pregnancy

Studies of prenatal diagnosis using sensitive DNA testing for myotonic dystrophy type 2 myopathy [14] are theoretically possible and more information is likely to become available in near future. If a mother has myotonic dystrophy type 2 with only minimal symptoms at the time of her pregnancy, she may have an increased risk of developing myotonia and weakness in the later stages of the pregnancy [14,102]. In 1 study of 96 pregnancies in 42 myotonic dystrophy type 2 women, it was found that 21% of the women had their first myotonic symptoms during their pregnancy. Additionally, 17% of their pregnancies ended in miscarriages, and 27% ended in preterm labor [103]. Two reports suggest that the symptoms that develop during pregnancy reverse after delivery [14,102], but more information is necessary to make such a prediction with certainty.

Anesthesia

One study of a large number of individuals with myotonic dystrophy type 2 has found no significant problems with the ability of patients to tolerate general anesthesia [14]. In a report of a large German patient cohort, the overall frequency of severe complications was 0.6% (2 of 340). The overall lower risk seems to be predominantly related to the minor respiratory involvement in myotonic dystrophy type 2 than in myotonic dystrophy type 1 [104].

Conclusions

Twenty-eight years have passed since the (CTG)n repeat expansion mutation was discovered in patients with myotonic dystrophy type 1, and 19 years ago the (CCTG) n mutation was identified in type 2 disease. Emerging data indicate that molecular pathomechanisms are much more complex than could have been envisioned when the respective mutations were just identified. RNA toxicity clearly has a major role, yet spliceopathy alone does not seem to fully account for all aspects of the multisystemic phenotype in myotonic dystrophies. Other pathomechanisms consistent with the toxic RNA model probably entail regulation of gene expression and translation and various cellular stress pathways and extend beyond the nucleus to the cytoplasm. Nevertheless, it is important to emphasize that despite clinical and genetic similarities, myotonic dystrophy type 1 and type 2 are distinct disorders requiring different diagnostic and management strategies.

Although treatment of myotonic dystrophy type 1 and myotonic dystrophy type 2 is currently limited to supportive therapies, new therapeutic approaches based on pathogenic mechanisms may become feasible in near future.

The future holds great promise for advances in translational research in DM2. The teamwork will expedite the development of targeted therapies and improve the lives of patients and their families [105].

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Late-onset myopathies: clinical features and diagnosis

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Late-onset myopathies are not well-defined since there is no clear definition of 'late onset'. For practical reasons we decided to use the age of 40 years as a cut-off. There are diseases which only manifest as late onset myopathy (inclusion body myositis, oculopharyngeal muscular dystrophy and axial myopathy). In addition, there are diseases with a wide range of onset including 'late onset' muscle weakness. Well-known and rather frequently occurring examples are Becker muscular dystrophy, limb girdle muscular dystrophy, facioscapulohumeral dystrophy, Pompe disease, myotonic dystrophy type 2, and anoctamin-5-related distal myopathy.

The above-mentioned diseases will be discussed in detail including clinical presentation – which can sometimes lead someone astray – and diagnostic tools based on real cases taken from the author's practice. Where appropriate a differential diagnosis is provided. Next generation sequencing (NGS) may speed up the diagnostic process in hereditary myopathies, but still there are diseases, e.g. with expansion repeats, deletions, etc, in which NGS is as yet not very helpful.

Key words: myopathies, late-onset, hereditary, acquired

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Introduction

Myopathies may be defined based on the clinical presentation (distal myopathy, limb girdle muscular dystrophy, facioscapulohumeral dystrophy, etc.) or on the onset (congenital, early-onset, childhood-onset, late onset myopathies), on histopathological findings (e.g., myofibrillar myopathies, central core disease), and in exceptional cases an eponym is used, like in Duchenne and Becker muscular dystrophy.

This paper will address myopathies with a late onset, but immediately we run into difficulties, because there is no cut-off for the age when the adjective 'late' is appropriate. The addition 'late onset' may sometimes give rise to confusion. It is common knowledge that oculopharyngeal muscular dystrophy (OPMD) is a late-onset disease, since the age at onset is always beyond 50 years, whereas late-onset Pompe disease starts at age 12 months since this subtype is distinguished from the infantile onset Pompe disease.

A pragmatic approach would be to distinguish two categories (see Table I):

1. Myopathies which only manifest with late onset muscle weakness;
2. Myopathies with a wide range of onset including late onset muscle weakness.

This overview will not be comprehensive, only the most frequent or well-known hereditary and acquired myopathies will be addressed. A case

Table I. Late onset myopathies.

Myopathies only manifesting with late onset muscle weakness		
Hereditary	***Gene***	***Salient clinical features***
Oculopharyngeal muscular dystrophy	*PAPBN1*	Asymmetric ptosis, dysphagia
Acquired		
Inclusion body myositis (IBM)	Not relevant	Asymmetric weakness of quadriceps muscles, deep finger flexors and oesophageal muscles
Isolated neck extensor weakness	Not relevant	Dropped head, no underlying disease
Slow late onset nemaline myopathy*[34]	Not relevant	Predominantly proximal and axial muscle weakness. Respiratory muscle involvement is common. Associated with a monoclonal protein (MP) or HIV infection. Treatable.
Myopathies with a wide range of onset including late onset of muscle weakness		
Hereditary		
Becker muscular dystrophy	*DMD*	Proximal muscle weakness, calf hypertrophy, exercise-related cramps, myalgia, rhabdomyolysis, dilated cardiomyopathy
Limb girdle muscular dystrophy, FKRP-related	*FKRP*	Proximal muscle weakness, calf hypertrophy, exercise-related cramps, myalgia, rhabdomyolysis, dilated cardiomyopathy
Facioscapulohumeral dystrophy	*DUX4*	Symmetric weakness of the facial and scapulohumeral muscles, descending to the axial and leg muscles. Rarely onset in anterior tibial or calf muscle
Late-onset Pompe disease	*GAA*	Proximal muscle weakness, diaphragm involvement at an early stage
Myotonic dystrophy type 2	*ZNF9*	Proximal muscle weakness, myalgia, cardiac involvement
Myofibrillar myopathy*[35]such as DES, PLEC 1, CRYAB, FLNC, MYOT, ZASP, BAG3, FHL1, and DNAJB6. Objective: To retrospectively analyze genetic mutations and demographic, clinical, and morphological aspects of PAM in a French population. Methods: Forty-eight PAM patients (29 men, 19 women	*DES, FLNC, MYOT, CRYAB, ZASP, BAG3, DNAJB6*	Distal muscle weakness (66%) most common, followed by simultaneous distal and proximal weakness. Respiratory and cardiac involvement occur frequently
Anoctamin-5 related distal myopathy	*ANO5*	Proximal or distal muscle weakness, cardiological involvement
Mitochondrial myopathy*[36]	Various in mitochondrial and nuclear genomes	Various phenotypes (chronic progressive external ophthalmoplegia), proximal myopathy (most common), exercise induced muscle pain, fatigue, often associated with involvement of other systems (cardiomyopathy, epilepsy, or stroke-like episodes)
Congenital myopathy*[37]	*RYR1 (most common), ACTA1, MYH7, DNM2, SELENON, MYH2, CACNA1S*	Proximal muscle weakness in all, axial muscle weakness (RYR1), rhabdomyolysis (RYR1, CACNA1S), malignant hyperthermia (RYR1), skeletal abnormalities
Acquired		
Idiopathic inflammatory myopathies (IIIM's) other than IBM* - immune mediated necrotizing myopathy, dermatomyositis (DM), non-specific myositis, antisynthetase syndrome (ASS)[38]	Not relevant	Subacute onset muscle weakness of the proximal muscles and dysphagia. Skin abnormalities in DM and in a proportion of ASS patients. DM and IMNM associated with cancer. Most IIM's, in particular non-specific myositis associated with connective tissue disorders. Treatable.

*These diseases will not be further discussed

vignette will be provided in selected cases, followed by general information on the disease, and where appropriate a differential diagnosis.

Hereditary myopathies only manifesting with late onset muscle weakness

Oculopharyngeal muscular dystrophy

Case 1 – A 76-year-old male was referred because of suspected ALS. He had developed gait difficulty due to weakness of the left leg after a fall. He admitted to having noticed a decrease in strength of the trunk and arm muscles. He had a hoarse voice and swallowing difficulty (later admitted to have progressive speech and swallowing difficulty since about 20 years). Previous history disclosed cataract surgery at age 74 and 75 years, respectively

Family history taking revealed that his mother may have had a 'muscle disease'.

On clinical examination he was found to have a facies myopathica and bilateral ptosis. He had a weak cough and generalized moderate weakness and atrophy of his arm and leg muscles. There was no myotonia and no fasciculation. Reflexes were normal except for a left plantar response which was extensor.

The following ancillary tests were done, electromyography (EMG) which was consistent with a myopathy. Serum creatine kinase (CK) activity was slightly elevated (205 IU/L (upper limit of normal (ULN) 190)). Videofluoroscopy showed silent aspiration. A muscle disclosed some neurogenic features, no rimmed vacuoles, DNA analysis showed a (GCN)12 repeat expansion exon 1 in the *PAPBN1* gene. Based on the family history a diagnosis of OPMD was considered very likely and DNA analysis confirmed a diagnosis of autosomal dominantly inherited oculopharyngeal muscular dystrophy.

General information

OPMD is caused by an abnormal (GCN) triplet expansion within the *PABPN1* gene

located on chromosome 14 (14q11.2-q13). While the wild-type *PABPN1* gene

contains 10 (GCN) repeats, the mutated form in OPMD is expanded to 11-18 repeats, adding

1-8 additional alanine residues at the N-terminus of the PABPN1 protein. A French study

showed that patients with OPMD with longer *PABPN1* expansion are on average diagnosed

at an earlier age than patients with a shorter expansion [1].

Inheritance is usually autosomal dominant, but autosomal recessive inheritance also occurs.

The age at onset in heterozygotes is in the fourth to sixth decade.

The most salient features include often asymmetric ptosis, incomplete external ophthalmoplegia and dysphagia. Slowly progressive limb muscle weakness, usually proximal, and legs more than arms occurs in the course of the disease.

As regards ancillary investigations CK is normal or slightly elevated, EMG shows myopathic changes and sometimes features compatible with 'denervation'. In a proportion of the muscle biopsies rimmed vacuoles can be found and at the ultrastructural level intranuclear filaments, albeit a muscle biopsy is no longer needed, since diagnosis is currently made by molecular testing.

Acquired myopathies only manifesting with late onset muscle weakness

Inclusion body myositis

Case 2 – A 57-year-old male was referred because of gradually progressive muscle weakness of the upper legs since about 5 to 6 years. He had no swallowing difficulty. Previous and family history were unremarkable.

On clinical examination he was found to have severe wasting and weakness of the quadriceps muscles. In addition, we noticed slight weakness of the facial muscles, a positive Gowers' phenomenon, slight weakness of the iliopsoas muscles and of the m. extensor hallucis longus weak (MRC grade 4). Vibration sense of the big toes was absent and there was areflexia.

Ancillary testing: CK was slightly elevated (2 x ULN). Anti-cN1A autoantibodies were positive. EMG showed spontaneous muscle activity in the quadriceps and gastrocnemius muscles. An MRI disclosed fatty replacement and atrophy of the quadriceps muscles. A muscle biopsy showed mononuclear cell infiltrates invading non-necrotic muscle fibres and rimmed vacuoles (Fig. 1).

He was diagnosed with inclusion body myositis based on the clinical findings and the ancillary tests. During follow-up proximal muscle weakness further deteriorated and he became wheelchair bound. He also developed dysphagia.

General information

Inclusion body myositis (IBM) is both an inflammatory and myodegenerative condition. The presence of inflammatory cells mostly cytotoxic CD8+Tcells sur-

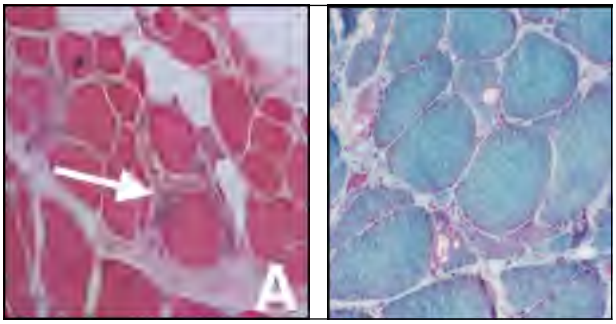


Figure 1. A) (pt 2): H&E stain showing a mononuclear cell infiltrate surrounding and invading a non-necrotic muscle fibre (arrow); B): Modified Gomori stain showing two muscle fibres with rimmed vacuoles (arrows).

rounding and focally invading non-necrotic muscle fibers indicate an immune attack [2]. The evidence of rimmed vacuoles with abnormal protein aggregation and deposition of congophilic inclusions within the muscle fibers, in association with mitochondrial dysfunction, supports the presence of a degenerative component [2].

IBM is the most common late onset acquired myopathy. The age at onset is later than 45 years and the duration of symptoms should be more than 12 months according to a consensus workshop [3]. In 35% of the patients falls and difficulty standing are the first manifestations. Decreased dexterity and swallowing difficulty may also be presenting symptoms. Muscle weakness is often asymmetrical and slowly progressive.

There is a characteristic distribution of muscle weakness including the thigh muscles (quadriceps), deep finger flexors, oesophageal muscles and facial muscles. Sometimes weakness in other distal muscles (foot extensors or the calf muscles) may also be early manifestations. Usually there is no muscle pain.

There has been a heated debate about the minimum requirements for a diagnosis. The 'Griggs criteria' were initially considered to be mandatory [4]. However, gradually we and others found that rimmed vacuoles were not always present in the muscle biopsies of patients who otherwise fulfilled the clinical criteria for IBM [5,6]. Based on an evaluation by Lloyd et al. on the specificity and sensitivity of published diagnostic criteria the following diagnostic criteria for IBM were established: weakness in the quadriceps muscles more than hip flexors, as well as in finger flexors more than shoulder abductors. In addition, patients are required to have at least one of the following pathological features: endomysial inflammation, rimmed vacuoles, increased MHC-I, 15 to 18 nm filaments, or accumulation of amyloid or other proteins [7].

Serum CK activity can be normal and moderately elevated to a maximum of 15 times the upper limit of normal [8]. EMG is consistent with a myopathy showing increased spontaneous activity and fibrillation potentials, associated with short duration, low-amplitude, motor unit potentials often mixed with long duration, high-amplitude motor unit potentials. These findings are also present in non-IBM myositis [9]. Elevated cN-1A antibodies are reported to be 33 to 76% sensitive and 92 to 96% specific for IBM. They were reported in various autoimmune disorders, including Sjögren's syndrome, systemic lupus erythematosus and dermatomyositis [2].

Isolated neck extensor weakness ('dropped head')

Case 3 – A 74-yr-old male experienced a painful neck (VAS score 42 out of a maximum of 100. Fourteen days later he developed a dropped head which hampered him in his activities (walking, biking, social activities).

On examination there was anteroposition of the head, which was not redressible. Hypertrophy and increased tone of the bilateral m. trapezius descendens were found. Range of motion was limited in all directions. No fasciculations, normal reflexes.

Ancillary tests: MRI showed fatty replacement of the multifidus muscles (Fig. 2).

Based on the history, clinical examination, and additional investigations amyotrophic lateral sclerosis (ALS), progressive muscular atrophy (PMA), myositis, and myasthenia gravis were ruled out and he was diagnosed with isolated neck extensor weakness.

He received a collar for a couple of months and physiotherapy was given. There was a follow-up for more than 4 years and the patient had less pain (VAS 10), did not

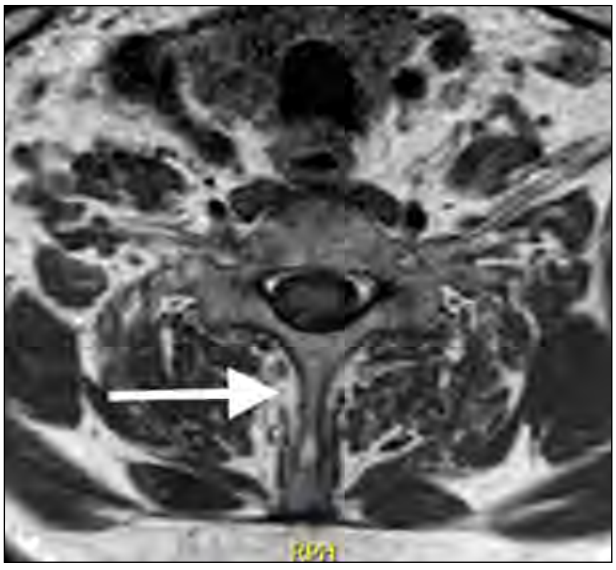


Figure 2 (Pt 3). MRI of the neck muscles showing fatty replacement of the multifidus muscles (arrow).

develop weakness in other muscles and had been able to restart his activities.

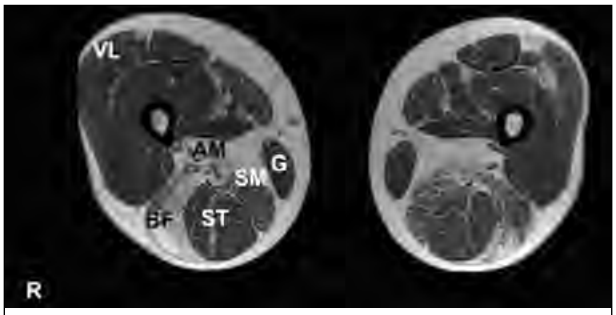
General information

Dropped head is defined by weakness of the cervical paraspinal muscles resulting in flexion of the head and cervical spine that is typically passively correctable. Patients typically present with neck pain. Dropped head as first or prominent manifestation of neuromuscular disease can be found in several non-myopathy diseases, including ALS/PMA, myasthenia gravis, in particular associated with anti-muscle-specific kinase antibody (MuSK) antibodies, and Lambert-Eaton syndrome. It has also been described as presenting symptom in IBM, other myositis subtypes, Pompe's disease, facioscapulohumeral dystrophy, myofibrillar myopathy, mitochondrial myopathy, sporadic late onset nemaline myopathy (SLONM), but usually on clinical examination other muscles appeared to be affected as well [10]. The term isolated neck extensor weakness was coined by Katz and should be reserved for patients with only severe extensor muscle weakness showing a benign course in whom the muscle biopsy findings were non-specific [11]. A systematic review showed that the mean age of patients was 63.6 (95% CI 60.9-66.2 y) and the majority were female [12]. In a large cohort of 92 patients diagnostic work up was conclusive in 57 patients (53%) [10]. Among these patients, inflammatory myopathy (often associated with a connective tissue disorder, in particular scleroderma) and SLONM were the most frequent ones. The authors recommend perform a comprehensive work up in patients presenting with dropped head [10].

Hereditary myopathies with a wide range of onset including late onset muscle weakness

Becker muscular dystrophy

Case 4 – A 44-year-old man was referred because of an elevated CK (1300-2200 IU/L (normal < 171)). On history he did not complain about muscle weakness. Previous history disclosed diabetes mellitus type II. On examination he was found to have hypertrophic calves and focal atrophy of the left thigh, but no muscle weakness. MRI showed replacement of the adductor magnus and the long head of the biceps femoris muscle by fat (Fig. 3), a muscle biopsy revealed reduction of dystrophin, and a subsequently performed DNA analysis of the dystrophin gene disclosed an in-frame deletion of exons 2-7 compatible with a diagnosis of Becker muscular dystrophy. Cardiological examination did not reveal any abnormalities but the patient was scheduled to undergo cardiological screening on a regular basis.



AM: m. adductor magnus; SM: m. semimembranosus; ST: m. semitendinosus; BF: m. biceps femoris, long head; VL: m. vastus lateralis; G: m. gracilis. Fatty replacement of bilateral AM and BF (partially on the left), left-sided SM and VL (partially)

Figure 3 (Pt 4): MRI of the upper legs.

General information

Becker muscular dystrophy (BMD) is an X-linked recessive disease caused by a mutation in the *DMD* (dystrophin) gene, which is the largest human gene, consisting of 79 exons. Approximately 60% of the patients have a deletion of one of more exons, and in 5-10% of the BMD/DMD patients, duplications are found. Approximately 10% of the patients have a point mutation. The mutations in BMD are usually in-frame, leading to an altered but still functioning dystrophin. Symptoms typically begin between ages 3 and 21, with a mean age of onset of 11 years. However, onset at a much later age and even in the seventh decade has also been reported [13].

In BMD muscle symptoms and signs are more heterogeneous than in DMD, ranging from very mild, such as exercise-induced myalgia or cramps, to severe progressive muscle weakness in thigh muscles. Weakness and wasting of the quadriceps femoris muscles can be the only symptom for a long time. Usually in due course the upper limbs become affected as well. Cardiac involvement is similar to that in DMD – degeneration of cardiac muscle fibres leading to rhythm disturbances and dilated cardiomyopathy - and ultimately present in all BMD patients. Severe dilated cardiomyopathy requiring heart transplantation may be the presenting symptom in BMD [14].

CK is usually more than five times elevated. BMD may present with hyperCKemia without clinical symptoms as in our patient. Electromyography usually does not contribute to the diagnosis, especially not if CK is more than 10 times elevated. Muscle imaging of the thigh muscles may be helpful showing fatty replacement of the adductor magnus muscle, semimembranosus muscle and long head of the biceps femoris muscle as the first manifestations which are specific findings [15,16]. Pathological changes include an abnormal variation in muscle fiber size due to atrophic and

hypertrophic fibers, focal necrosis, and regeneration, and extensive endomysial fat and connective tissue. For diagnostic confirmation immunohistochemistry with antibodies raised against different parts of dystrophin are used as a qualitative measure for dystrophin in muscle tissue. In BMD muscle immunohistochemistry may show that dystrophin is distributed normally but globally reduced or that the staining is discontinuous with either a normal or reduced intensity. However, often this test is not sufficient to confirm the diagnosis of BMD. Western blot analysis, which is a semi-quantitative measurement of dystrophin, can detect abnormal amounts of dystrophin and/or dystrophin with a different molecular weight. For accurate genetic classification there is a variety of sequencing and genetic diagnosis methodologies ranging from Sanger sequencing which is a low throughput, conventional strategy with lower cost than more advanced sequencing and allowing for sequencing the dystrophin gene to next generation sequencing [17].

Differential diagnosis may include limb girdle muscular dystrophy, spinal muscular atrophy type 3 and Pompe disease which all manifest with a limb girdle distribution of weakness and may be associated with a markedly elevated CK and calf hypetrophy.

Limb-girdle muscular dystrophy

Case 5 - A 41-year-old male was referred because of back ache. On history taking he admitted that had not been able to keep up with his peers at sport. He experienced exercise-related myalgia from early childhood onwards and had once had myoglobinuria. Since the neurologist from elsewhere had noticed firm calves and wasted thighs, he was referred.

On examination there was calf hypertrophy and atrophy of the thigh muscles. He was found to have weakness of the m. iliopsoas and adductors and a positive Gowers' phenomenon indicating muscle weakness of the gluteal and quadriceps muscles.

An immunostained muscle biopsy showed reduced alpha-dystroglycan and subsequently a homozygous missense mutation c.826C > A; p.Leu276Ile was found in the fukutin-related protein gene. Thereupon the patient was diagnosed with LGMDR9 FKRP-related, formerly known as LGMD2I (see below).

Since this type of LGMD is associated with cardiac involvement he was referred for cardiological screening albeit he was asymptomatic. A dilated cardiomyopathy was found and unfortunately, he died from heart failure, as a result of decompensation of the cardiomyopathy waiting for heart transplantation.

General information

Limb girdle muscular dystrophy (LGMD) is named after the distribution of muscle weakness. The term 'limb girdle muscular dystrophy' was coined by Walton and Nattrass in 1954 who identified LGMD as a separate clinical entity from X-linked recessive Duchenne muscular dystrophy, autosomal dominant facioscapulohumeral muscular dystrophy and autosomal dominant myotonic dystrophy [18]. After the first molecular genetic characterisation of a number of LGMDs, a European Neuromuscular Centre (ENMC) consortium reached a consensus on the classification of LGMD subtypes in 1995 [19]. An alphanumeric system was introduced, in which the number 'one' or 'two' was assigned to a dominant or recessive mode of inheritance, respectively. A letter was assigned based on the order of discovery of linkage assignment to a certain genetic locus or of a new disease gene. Since the letter of the alphabet had been reached as far as the autosomal recessive LGMDs are concerned, but even more importantly disease entities were included which did not predominantly manifest with limb-girdle distribution of muscle weakness (e.g., laminopathy or myofibrillar myopathy) or were already known as a separate disease entity (e.g., Pompe disease, also known as LGMD2V) another ENMC workshop was organized in 2017 [20]. The participants formulated a definition of LGMD: "[…] a genetically inherited condition that primarily affects skeletal muscle leading to progressive, predominantly proximal muscle weakness at presentation caused by a loss of muscle fibres. […] the condition […] must have an elevated serum creatine kinase activity, must demonstrate degenerative changes on muscle imaging over the course of the disease, and have dystrophic changes on muscle histology, […]".

Subsequently the definition was applied to the existing classification and ten conditions did no longer fulfill the criteria of LGMD. Four novel conditions which did were added. The formula for the new classification was as follows: "*LGMD, inheritance (R or D), order of discovery (number), affected protein*". In our case the diagnosis is LGMDR9 FKRP-related.

FKRP mutations cause a number of rare, autosomal recessive muscular dystrophies, the most common of which is LGMDR9, FKRP – related and muscular dystrophy – dystroglycanopathy type C, 5 (MDDGC5), and even rarer congenital conditions. Even within LGMDR9 there is a wide variation as regards age at onset and disease severity. Recently the data of a large cohort assembled in a registry (Global FKRP Registry) was analyzed [21]. The mean age of diagnosis for LGMDR9 was 30.1 ± 17.3 years. The presenting symptoms were muscle weakness of the lower limbs and hyperCKemia. Respiratory insufficiency requiring (non)invasive ventilation was found in a fair proportion of patients (18.2%) and 23.3% reported a heart condition, i.e. dilated cardiomyopathy.

Exercise-related rhabdomyolysis and myalgia can

occur and may be the sole manifestation for a number of years [22].

Diagnosis is most reliably made by molecular analysis. Since LGMDR9 has much in common with other LGMDs and Becker muscular dystrophy (limb girdle distribution of muscle weakness, calf hypertrophy, high CK, dystrophic pattern on the muscle biopsy) usually NGS with a targeted gene panel is done. Immunostaining of a muscle biopsy may reveal a reduced alpha-dystroglycan but can be normal in less severely affected patients.

Pulmonary and cardiological screening should be carried out after the diagnosis has been established.

Facioscapulohumeral dystrophy

Case 6 – A 50-year-old male patient was referred because of calf muscle weakness noticed since age 45 years. The patients was previously described [23]. He did not complain about back ache, sciatic pain, or paresthesia.

On examination there was atrophy of both calves, left more than right and he was not able to walk on tiptoes. Achilles tendon jerks were absent. He was also found to have axillar folding, no facial weakness, no scapulae alatae.

Based upon the presence of axillar folding a diagnosis of FSHD was considered and indeed a short (20) EcoRI fragment on chromosome 4q was found confirming the diagnosis.

General information

Facioscapulohumeral dystrophy (FSHD) type 1 is a slowly progressive, autosomal dominantly inherited myopathy defined by a contraction of the D4Z4 repeat array on chromosome 4q35, with a residual EcoR1 fragment of 10-38 kb.

FSHD generally starts during adolescence, but with a wide range of onset (4-60 years). The presenting symptoms are typically variable degrees of muscle weakness, involving the facial and shoulder girdle muscles, leading to the characteristic scapulae alatae, often in an asymmetrical manner. Rarely, an onset in the anterior tibial muscle or calf muscle was reported and sometimes without apparent facial involvement [23,24]. In due course other muscles are involved as well, i.e. the quadriceps muscles, hamstrings, abdominal muscles, upper arm muscles, pelvic girdle, and distal leg muscles. In a proportion of the patients swallowing difficulty and respiratory involvement occur. On the other end of the spectrum are patients who are asymptomatic and show some subtle symptoms on clinical examination. This variability may also occur within families.

CK may be elevated but is usually normal, EMG is not helpful and the same holds true for the muscle biopsy which often shows non-specific features, albeit inflammation may occur. MRI can be useful showing (asymmetrical) replacement of the hamstring muscles, adductor muscles, rectus femoris, and gastrocnemius medialis muscles, also in early stage disease and in patients without leg muscle weakness [25].

Diagnosis is established by genetic analysis. The smallest remaining fragments may be correlated with the most severe phenotype, i.e. infantile FSHD manifesting with paralysis of the facial muscles.

There is also another FSHD subtype (2) caused by mutation in the structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) gene with a similar phenotype to FSHD type 1.

Pompe's disease

Case 7 – A 51-yr-old female was referred because of difficulty getting up the stairs and mounting a horse since about 2 years. In retrospect, symptoms may have been present since age 40 years. She had always been good at sports as an adolescent and young adult.

Family history disclosed that there were 9 sibs of who the eldest sister may have the same problems.

On examination there was a positive Gowers' phenomenon caused by weakness of the pelvic girdle and thigh muscles. Otherwise no abnormalities.

Ancillary investigations showed that CK was elevated (5 x ULN). A muscle biopsy revealed accumulation of glycogen in vacuoles. There was deficiency of acid maltase in leukocytes: 21 (N 60-250) nmol/hr/mg.

All these ancillary investigations suggested the diagnosis of Pompe's disease and this was confirmed by DNA analysis showing that she was a compound heterozygote for c.IVS1-13T > G and C.379-380delTG (p.Cys127fs) mutations in the *GAA* gene.

After the diagnosis the lung function was assessed, and she was referred for enzyme replacement therapy.

General information

Glycogen storage disease type II or Pompe disease, also referred to as acid maltase deficiency, is a rare lysosomal storage disease characterized by the accumulation of glycogen primarily in muscle tissue. It is an autosomal recessive inherited disease in which there is a deficiency in the activity of the lysosomal enzyme acid α-glucosidase. The α-glucosidase gene is located on the long arm of chromosome 17 and myriad mutations have been identified.

The disease presents as a spectrum of phenotypes, ranging from a rapidly fatal phenotype in infants to slower progressive phenotypes in older children and adults. We will confine ourselves to the late onset Pompe disease (LOPD). The disease can start at any age after the age of 12 months. In addition to limb-girdle muscle weakness, the diaphragm is often involved irrespective of the

severity of the limb muscle weakness. Other features include ptosis, bulbar dysfunction, and scapular winging. Since the early symptoms of late-onset Pompe disease are non-specific, it often takes several years to reach the correct diagnosis. It is of utmost importance to diagnose the patients as early as possible since they can be treated with alglucosidase alfa enzyme replacement therapy.

CK is usually moderately elevated. EMG may show a myopathic pattern and signs of membrane irritability with myotonic discharges. A muscle biopsy may show vacuoles filled with glycogen as in our patient but is often non-specific. Measurement of α-glucosidase activity in dried blood spots (or in leukocytes) is essential for the diagnosis of late-onset Pompe disease. Given its high specificity and sensitivity, mutation analysis of the α-glucosidase gene may be performed as a confirmatory test. Currently, next generation sequencing with a targeted gene panel including the *GAA* gene is applied on patients with a clinical picture that may fit with Pompe disease. In a large cohort of unselected adult patients with hyperCKemia and/or limb girdle muscular weakness, a prevalence of late-onset Pompe disease of 2.4% was found by target gene panel screening [26].

Myotonic dystrophy type 2

Case 8 – A 60-year-old female was referred because of a 10-year history of progressive weakness. In particular, she had difficulty with climbing stairs and exercise-induced myalgia. She underwent cataract surgery at age 58 years. Family history disclosed that her brother had died but was known to have a pacemaker installed. Her father and two paternal uncles were known with 'heart conditions' and cataract surgery or walking difficulty.

On examination there was moderately severe limb-girdle muscle weakness. She had firm calves and myotonia was absent.

Ancillary tests showed a slightly elevated CK, myopathic changes on EMG but no myotonic discharges. In light of the family history a diagnosis of myotonic dystrophy type 2 was considered, and genetic analysis showed a repeat expansion in intron 1 of the ZNF9 gene.

A cardiac monitor was implanted because of two syncopes.

General information

Myotonic dystrophy type 2 (DM2), also known as proximal myotonic myopathy, is a rare, multi-systemic disease. DM2 has a later onset and a usually milder phenotype as compared to DM1. DM2 lacks the severe congenital form seen in DM1.The gene defect is an unstable CCTG repeat expansion in the cellular nucleic acid-binding protein gene, formerly known as the zink finger protein (ZNF) 9 gene. Like DM1, DM2 has an autosomal dominant inheritance pattern and expansion lengths of 75 repeats or longer are considered pathogenic. There is no clear correlation between the length of the CCTG expansion and clinical severity.

Symptoms in DM2 patients usually begin in the third to fifth decades of life. Muscle weakness (proximal and axial, not facial, not distal) and/or myalgia are the most common symptoms at onset. As the duration of DM2 increases many organs and systems may be affected and therefore the patient's management should include amongst others screening for heart diseases (cardiomyopathy, arrhythmias), diabetes mellitus/insulin resistance, thyroid dysfunctions, cataract, and gastrointestinal disturbances [27]. Symptom severity widely varies among patients, even within members of the same family.

There are no specific ancillary tests. Clinical myotonia or myotonic discharges on EMG are only found in a proportion of the patients [28].

Anoctamin-5 related distal myopathy

Case 9 – A 55-year-old man was referred because of a 5-year history of difficulty getting up the stairs. Family history was negative. On examination atrophy of the thighs and calves was found. He was not able to walk on tiptoes.

CK was markedly elevated (30x times the ULN). We established a diagnosis of a Miyoshi-like distal myopathy. Muscle imaging showed fatty replacement of the medial head of the gastrocnemius and of the gluteus minimal muscles on both sides.

A muscle biopsy revealed a dystrophic pattern and a normal dysferlin stain. DNA analysis: showed compound heterozygous mutations in the *ANO5* gene: c.191dupA (exon 5) and c.1898+1G>A (intron 17).

We followed the patients for more than 20 years and muscle weakness remained restricted to the lower extremities.

General information

Mutations in the anoctamin5-gene which encodes a putative calcium-activated chloride channel causes LGMDR12 formerly known as LGMD 2L, Miyoshi-like distal myopathy (MMD3) and asymptomatic hyperCKemia. Historically LGMD and distal myopathy caused by ANO5 mutation (MMD3) - and this also holds for MMD1 caused by dysferlin mutation – were considered two separate disease entities. However, natural history studies including muscle strength assessment and MRI showed that there is a spectrum in which distal-onset phenotypes already show proximal involvement in early stages and vice versa as regards proximal-onset phenotypes [29]. There are subtle differences between MRI findings in dysferlin and ANO5-related LGMD/distal myopathy [30].

Figure 4. Meeting of the Executive Committee of the World Muscle Society in Naples, 2002.

The median of age at onset in ANO5-related myopathy is 38 years, ranging from 4 to 67 years with a male preponderance [31]. Exercise-related myalgia and rhabdomyolysis have been described in several papers [31,32]. Cardiac involvement does occur and therefore cardiological screening should be carried out after the diagnosis has been established [31,33].

Ancillary tests are non-specific showing a markedly elevated CK, myopathic EMG sometimes also associated with positive sharp waves and fibrillations, and a dystrophic pattern on muscle biopsy. Attempts to detect ANO5 on immunohistochemistry were not successful.

Conclusions

There is a variety of myopathies with may manifest with late onset muscle weakness, both hereditary and acquired conditions. Even a so-called congenital myopathy may present as late-onset muscle weakness.

I am indebted to Giovanni Nigro, with whom I shared the fascination of cardiological involvement in myopathies. The picture (Fig. 4) shows a meeting of the Executive Committee of the World Muscle Society in Naples in 2002.

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Panorama of the distal myopathies

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Distal myopathies are genetic primary muscle disorders with a prominent weakness at onset in hands and/or feet. The age of onset (from early childhood to adulthood), the distribution of muscle weakness (upper versus lower limbs) and the histological findings (ranging from nonspecific myopathic changes to myofibrillar disarrays and rimmed vacuoles) are extremely variable. However, despite being characterized by a wide clinical and genetic heterogeneity, the distal myopathies are a category of muscular dystrophies: genetic diseases with progressive loss of muscle fibers. Myopathic congenital arthrogryposis is also a form of distal myopathy usually caused by focal amyoplasia.

Massive parallel sequencing has further expanded the long list of genes associated with a distal myopathy, and contributed identifying as distal myopathy-causative rare variants in genes more often related with other skeletal or cardiac muscle diseases.

Currently, almost 20 genes (*ACTN2, CAV3, CRYAB, DNAJB6, DNM2, FLNC, HNRNPA1, HSPB8, KHLH9, LDB3, MATR3, MB, MYOT, PLIN4, TIA1, VCP, NOTCH2NLC, LRP12, GIPS1*) have been associated with an autosomal dominant form of distal myopathy. Pathogenic changes in four genes (*ADSSL, ANO5, DYSF, GNE*) cause an autosomal recessive form; and disease-causing variants in five genes (*DES, MYH7, NEB, RYR1* and *TTN*) result either in a dominant or in a recessive distal myopathy. Finally, a digenic mechanism, underlying a Welander-like form of distal myopathy, has been recently elucidated. Rare pathogenic mutations in *SQSTM1*, previously identified with a bone disease (Paget disease), unexpectedly cause a distal myopathy when combined with a common polymorphism in *TIA1*.

The present review aims at describing the genetic basis of distal myopathy and at summarizing the clinical features of the different forms described so far.

Key words: distal myopathy, rimmed vacuoles, myofibrillar myopathy

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Introduction

The term distal myopathy refers to a long list of genetic muscle diseases presenting at the onset with weakness of distal extremities, usually combined with progressive atrophy of the corresponding distal muscles. Other muscles, including proximal muscles and/or cardiac and respiratory muscles, can be affected at a later stage of the disease. The clinical phenotype is extremely variable, ranging from severe forms with earlier onset and loss of ambulation to very mild late adult onset forms. Other muscle

diseases (genetically determined or acquired) may present with a distal phenotype, making the diagnostic process more complex.

Although two patients with weakness in hands and in legs or feet were first described as distal myopathy by Gowers over 100 years ago [1], only in 1998 the first genetic defect underlying a distal myopathy was identified [2]. Ten years ago, in 2010, only fourteen causative genes were known. In the last years, massive parallel sequencing has contributed to identify disease-causing variants in novel genes and to elucidate the first example of a digenic mechanism causing a distal myopathy (Tab. I). At the same time, the number of causative variants, identified in large resequencing projects, has exponentially increased [3-7]. Interestingly, most currently known genes are also responsible for separate different clinical entities, confirming the extreme phenotypic divergence observed in the field of genetic myopathies [8].

More advanced histopathological techniques and refined cell and molecular biology studies have resulted in a better understanding of the pathophysiology of distal myopathies. Clinical, histopathological, and imaging features of each form have been partly clarified, addressing the diagnosis, and supporting a proper interpretation in case of novel variants identified in previously known genes.

Adult – late onset distal myopathies

Welander distal myopathy (WDM) – TIA1

WDM was first described in several Swedish families in 1951 as an autosomal dominant late adult-onset (usually over 50 years) disease with a prominent early involvement of fingers and wrist extensors [9]. As the disease progresses, weakness involves also finger flexors, toe and ankle extensors. The disease course is usually slowly progressive, and patients remain ambulant. Histopathology features include rimmed vacuoles.

A missense variant (p.E384K) in *TIA1* gene causing the disease was identified in 2013 [10]. *TIA1* encodes an RNA-binding protein involved in the alternative splicing of specific pre-mRNAs [11-14], and is a key molecule in stress granules, regulators of RNA-translation metabolism that show altered dynamics in WDM [10].

Digenic SQSTM1 and TIA1 mediated distal myopathy

Patients with a Welander distal myopathy phenotype but negative for causative rare mutations in *TIA1* were discovered to have instead a common polymorphism in the *TIA1*, which, with a population frequency of 1%, could not be the cause of the disease. Further gene panel sequencing in these patients showed the presence of *SQSTM1* mutations previously known to cause the Paget's disease of the bone, a dominant disease with reduced penetrance [15]. Functional studies showed that the *SQSTM1* gene product, p62, interferes with the same stress granule dynamics pathway as *TIA1* explaining the background for the digenic mechanism [15]. This genetic combination of rare *SQSTM1* causative variants and the common *TIA1* polymorphism did not result in a Paget disease of the bone but caused the canonical Welander phenotype. On the other hand, a cohort of 50 patients with Paget disease of the bone carrying the same *SQSTM1* mutations did not have the *TIA1* polymorphism [15].

Tibial muscular dystrophy (Udd myopathy) – the first human titinopathy

Tibial muscular dystrophy (TMD) or Udd myopathy was described in 1993 in Finnish patients [16]. Weakness in ankle dorsiflexion and atrophy of anterior lower leg muscles (often asymmetric) start after age of 35 or much later. Progression is slow and walking is usually preserved. Extensor digitorum brevis and hand muscles are normally spared. Serum CK is normal or mildly elevated and muscle imaging shows fatty degeneration in anterior tibial muscles and at later stage in all long toe extensors, hamstring and medial gastrocnemius muscles.

Muscle biopsy shows myopathic changes with acid phosphatase, ubiquitin, p62 and LC3 positive in the affected muscles, but in preserved muscles there is only a slight increase of internal nuclei.

In Finnish TMD patients, a common founder mutation (FINmaj) in the last exon of titin gene (*TTN*) was identified in 2002 [17]. FINmaj is a complex 11-bp insertion–deletion resulting in substitution of four amino acids without any frameshift and preserving the downstream amino acid sequence. Following the FINmaj identification, missense variants in the same exon (364) were also identified in non-Finnish patients [17-19].

TTN gene encodes titin, the third filament system of the sarcomere [20]. Titin interacts with several important proteins, including calpain-3 that binds the C-terminal portion of titin [21,22]. Through a large number of alternative splicing events, *TTN* encodes for a large number of different transcripts, developmental-stage or tissue specific [23,24]. Reflecting the size and complexity of titin, causative variants result in allelic diseases affecting skeletal muscle, heart or both of them, referred to as 'titinopathies' [25,26]. Dominant titinopathies include the aforementioned TMD, and the hereditary myopathy with early respiratory failure (HMERF) caused by missense variants in exon 344 [17,27-29]. Recessive titinopathies include a wide spectrum of diseases with a prenatal, congenital, childhood or later onset [30,31]. A recessive form of early/juvenile onset recessive distal titinopathy is further

Table I. List of distal myopathies and causative genes.

Clinical entity	Gene(s)	Trasmission	References
Adult – late onset distal myopathies			
Welander distal myopathy	*TIA1*	AD	Hackman et al., 2012
Digenic SQSTM1 and TIA1 mediated distal myopathy	*SQSTM1+TIA1*	DG	Lee et al., 2018
Tibial muscular dystrophy (Udd myopathy)	*TTN*	AD	Hackman et al., 2002
Vocal cord and pharyngeal distal myopathy	*MATR3*	AD	Senderek et al., 2009
Distal Actininopathy	*ACTN2*	AD	Savarese et al., 2019
Distal Myopathy with sarcoplasmic bodies	*MB*	AD	Olive et al., 2019
Oculopharyngeal distal myopathy	*NOTCH2NLC, LRP12 and GIPC1*	AD	Deng et al., 2020; Ishiura et al., 2019; Saito et al., 2020; Sone et al., 2019
PLIN4 mutated distal myopathy	*PLIN4*	AD	Ruggieri et al. 2020
VCP distal myopathy	*VCP*	AD	Palmio et al 2011
Myofibrillar distal myopathies			
Distal myopathy with myotilin defect	*MYOT*	AD	Penisson-Besnier et al., 2006
Late onset distal myopathy (Markesbery-Griggs, Zaspopathy)	*LDB3*	AD	Griggs et al., 2007
Desminopathy	*DES*	AD > AR	Sjoberg et al., 1999
Alpha-B crystallin-mutated distal myopathy	*CRYAB*	AD	Reichlich et al. 2010
Early adult onset distal myopathies			
Miyoshi myopathy	*DYSF*	AR	Liu et al., 1998
Recessive distal titinopathy	*TTN*	AR	Evila et al., 2017
Distal myopathy with rimmed vacuoles (Nonaka and GNE myopathy)	*GNE*	AR	Kayashima et al., 2002
Distal ABD-filaminopathy	*FLNC*	AD	Duff et al., 2011
DNAJB6 distal myopathy	*DNAJB6*	AD	Ruggieri et al., 2015 - Palmio et al., 2020
Rimmed vacuolar neuromyopathy	*HSPB8*	AD	Ghaoui et al., 2016
ANO5 distal muscular dystrophy	*ANO5*	AR	Bolduc et al., 2010
RYR1 mutated calf predominant distal myopathy	*RYR1*	AD/AR	Laughlin et al., 2017 - Jokela et al., 2019
Early-childhood onset distal myopathies			
Early onset distal myopathy (Laing)	*MYH7*	AD > AR	Meredith et al., 2004
Early onset distal myopathies with nebulin defect	*NEB*	AR > AD	Wallgren-Pettersson et al., 2007, Kiiski et al., 2019
Early onset ADSSL distal myopathy	*ADSSL*	AR	Park et al., 2016
Early onset distal myopathy with KLHL9 mutations	*KLHL9*	AD	Cirak et al., 2010
Other myopathies and dystrophies with distal weakness			
Distal myopathy with caveolin defect	*CAV3*	AD	Tateyama et al., 2002
DNM2 related distal myopathy	*DNM2*	AD	Bitoun et al., 2005

AD: autosomal dominant; AR: autosome recessive; DG: digenic

discussed below in this review. With the increasing number of reported patients, first insights on the genotype-phenotype correlation are achieved [30]. *TTN* variants are also associated with dilated and hypertrophic cardiomyopathy [32,33].

Vocal cord and pharyngeal distal myopathy – MATR3

First described in a large North American family [34] and later in a large Bulgarian pedigree [35], vocal cord and pharyngeal distal myopathy (VCPDM) is characterized by adult-onset (between 35 and 60 years) distal weakness and weakness of vocal cord and pharyngeal muscles. Limb weakness can be asymmetric and the phenotype is highly variable in terms of age of onset, progression and muscle weakness distribution [34,36,37]. Most patients develop respiratory failure [38]. CK levels are normal or mildly

elevated. EMG shows myopathic changes and rimmed vacuoles are present in the biopsy. Muscle MRI shows a predominant involvement of the lower legs both anterior and posterior compartment and hamstrings in thighs [39].

The underlying re-occurring p.S85C mutation was identified in *MATR3* gene [35]. *MATR3* encodes matrin-3, a protein located in the nuclear matrix where it regulates several processes related to gene expression, RNA splicing and export of RNA and nuclear proteins [40,41]. Variants in *MATR3* have also been identified in patients with amyotrophic lateral sclerosis (ALS) [42].

Distal Actininopathy – ACTN2

Distal actininopathy is an autosomal dominant, adult onset distal myopathy starting usually with foot drop. The disease later progresses to proximal lower limb muscles while upper limbs remain relatively spared [43]. Serum CK levels are mildly elevated and muscle biopsy shows rimmed vacuoles with some myofibrillar disarrays and undulation of the Z-disk on electron microscopy [43].

The underlying genetic defects in the four families reported so far are heterozygous missense variants in the *ACTN2* gene. *ACTN2* encodes alpha-actinin2, a structural molecule of the Z-disks that interacts with titin and acts a scaffold of many other Z-disk located proteins such as myotilin [44-47].

Variants in *ACTN2* also cause congenital myopathy with structured cores and Z-line abnormalities [48]. Moreover, dilated cardiomyopathy and hypertrophic cardiomyopathy have been associated with missense variants in *ACTN2* [49-53].

Distal Myopathy with sarcoplasmic bodies – MB

In 1980 Edström et al. published a Swedish family with this title [54]. Only recently, the genetic cause of the disease was identified with one unique causative variant in Myoglobin (*MB*), reoccurring in several unrelated families [55]. In these later studied families, the characteristic muscle pathology was evident but the clinical phenotype was more proximo-distal and not particularly distal [55].

Oculopharyngeal distal myopathy OPDM – CGG and GGC expansions

The peculiar combination of severe adult onset distal atrophies in limb muscles and facial weakness, ptosis and dysphagia can occur both in dominant and recessive families and in sporadic patients [56,57]. In the studied patients, the muscle pathology is a rimmed vacuolar myopathy. In the two last years the cause of many Asian dominant families have been clarified as caused by triplet repeat expansions, both CGG and GGC, in three different genes *NOTCH2NLC*, *LRP12* and *GIPC1* [58-61]. The repeats are translated into aggregating protein products and the host gene functions are not supposed to contribute to the disease mechanism.

PLIN4 mutated distal myopathy – PLIN4

A large Italian family with an autosomal dominant adult-onset distal myopathy and histopathological features of rimmed vacuoles was first described in 2004 [62]. Linkage analysis suggested that the causative gene could have been localized in the 19p13.3 locus [62].

Recently, Ruggieri et al. identified the underlying genetic defect in the *PLIN4* gene, encoding for perilipin-4 [63]. Thirty-one repeats of 99 nucleotides in exon 4 of *PLIN4* encode the 31x33 amino acid amphipathic domain of perilipin-4. An expansion of the normal repeat to 40 × 99 bases, resulting in 297 (9 × 33) extra amino acids, has been identified in the affected members of the family.

Perilipin-4 is a member of the perilipin family, a group of proteins that coat the surface of lipid droplets [64]. Perilipin-4 is highly expressed in skeletal muscle with a possible role in lipid metabolism. The identified repeat expansion in patients with *PLIN4*-related distal myopathy seems to cause a misfolding and leads to protein accumulation in vacuoles disrupting the myofibrillar organization [63].

VCP distal myopathy – VCP

Initially described by Palmio and colleagues in a large dominant Finnish family, *VCP*-related distal myopathy has an onset in mid-adulthood mainly affecting anterior leg muscles [65]. After 25 years of disease, the patients became affected by a progressive frontotemporal dementia. None of the patients had signs of Paget disease of the bone. Serum CK levels are normal or slightly elevated. Myopathic changes with rimmed vacuoles are observed in the muscle biopsy. MRI shows degenerative changes of anterior lower leg muscles.

Although a clinical variability has been observed [66-73], the most common phenotype of pathogenic *VCP* variants is proximal myopathy with scapular winging, Paget disease and frontotemporal dementia (IBMPFD) [74-76].

Myofibrillar distal myopathies

Distal myopathy with myotilin defect – MYOT

A late-onset distal myopathy has been associated with heterozygous variants in *MYOT* gene [77-79]. The first symptoms, weakness of ankle dorsiflexion and/or calf muscles, occur after age 50 years but, despite late onset, the further progression can be rapid. Respiratory and cardiac muscles are spared.

Histopathological features are consistent with myofibrillar myopathy and include rimmed and non-rimmed vacuoles, and myofibrillar disorganization with myotilin accumulations [79-81]. Muscle imaging shows that soleus is typically the first muscle affected followed by tibialis anterior and gastrocnemius medialis muscles [82,83].

The most common causative variants in *MYOT* are missense changes affecting serine and threonine amino acids in the serine rich domain. *MYOT* gene encodes myotilin, a key component of the Z-disc, directly binding F-actin [84]. Some patients have been described as affected by a dominant limb-girdle muscular dystrophy (previously LGMD1A) [85,86], but distal myopathy is the main phenotype. A proximal muscle involvement is only observed in later stages or in homozygosity for known dominant variants [87,88]. The term 'spheroid body myopathy' was also used since the protein aggregates in some cases have the corresponding shape [89,90].

Late onset distal myopathy (Markesbery-Griggs, Zaspopathy) – LDB3

The dominant *LDB3*-related distal myopathy usually starts with ankle weakness after the age of 40 years with later involvement of proximal muscles [91-94]. Cardiomyopathy can occur very late; facial and respiratory muscles are preserved. Muscle biopsy reveals myofibrillar myopathy with rimmed and non-rimmed vacuoles [95]. Myofibrillar protein accumulations are similar with myotilinopathy and desminopathy [96].

LDB3 encodes the lim domain-binding 3 protein, also called Z-band alternatively spliced PDZ motif-containing protein (ZASP) that interacts with other Z-disk proteins [97,98]. Hypertrophic and dilated cardiomyopathies (with or without left ventricular noncompaction) are allelic disorders [99,100].

Desminopathy – DES

Desmin-related distal myopathy is a myofibrillar myopathy with cytoplasmic accumulation of desmin in cardiac and skeletal muscles. The first family was described in 1943 long before the gene was known [101,102]. Cardiomyopathy and cardiac conduction defects are frequent, and the weakness/atrophy involves both hands and lower legs with later spread to proximal muscles. MRI shows the early involvement of peroneal muscles followed by tibialis anterior, gastrocnemius and soleus muscles [80,82]. CK is usually slightly elevated.

The fist causative variants in DES were identified in 1998 [103]. DES encodes desmin, a protein of the intermediate filament connecting Z-band with the plasmalemma and the nucleus[104]. As suggested by a recent study, desmin forms seeding-competent amyloid that is toxic to myofibers and disease-causing mutations enhance the amyloid formation [105]. Most patients have a dominant disease with onset in early adulthood but a later onset is possible [106]. Rare cases with a recessive, more severe, form have been reported [107]. Dominant cardiomyopathy without skeletal muscle disease, scapuloperoneal and other phenotypes, due to the increasing number of causative variants identified, are also reported [108-112].

Alpha-B crystallin-mutated distal myopathy – CRYAB

In 1998, Vicart and colleagues identified the first causative variant in the *CRYAB* gene causing a myopathy with accumulation of aggregates of desmin [113]. In 2003, Selcen et al described patients with a generalized proximal and distal myopathy affecting also the cardiac and respiratory function and carrying mutations in *CRYAB* [114]D. In 2010 and in 2012, two studies identified patients with *CRYAB* mutations and a distal adult-onset myofibrillar myopathy [115,116]. *CRYAB*-related distal myopathy mainly involves the anterior part of the distal leg at the early stage and progresses with a milder proximal weakness. Cataracts are the hallmark and dysphagia, dysphonia, respiratory failure, and cardiomyopathy may be associated. Muscle MRI shows fatty degenerative changes in tibialis anterior, gastrocnemius medialis muscles and vastus muscles [82,115,117,118].

CRYAB encodes alpha-B-crystallin, also called HSPB5, a member of the small heat-shock protein family, a molecular chaperone that interacts with desmin in the assembly of intermediate filaments [119-122].

Causative *CRYAB* variants also cause a dominant dilated cardiomyopathy, congenital cataract (dominant and recessive) and a more severe, usually recessive myopathy (fatal infantile hypertonic myofibrillar myopathy) [123-127].

Early adult onset distal myopathies

Miyoshi myopathy – DYSF

Miyoshi and colleagues first described patients in the sixties with early adult-onset weakness, myalgia and atrophy in calf muscles [128]. Serum creatine kinase (CK) is highly elevated already in the early stages of the disease or even in presymptomatic patients. Muscle imaging shows marked involvement of posterior lower legs. Muscle biopsy shows myopathic changes with necrotic fibers in the calf muscles and inflammation is a common finding.

Dysferlin (*DYSF*) as causative gene with biallelic recessive mutations was identified in 1998 [2]. Dysferlin is a ubiquitous transmembrane protein with a high skeletal muscle expression. The protein most probably acts

in calcium-mediated sarcolemmal fusion events and resealing [129-131]. Dysferlin expression by immunostaining or western blot (even from blood leucocytes) is useful in the diagnostic process, although the protein can be also secondarily reduced [132,133].

Myoshi myopathy and LGMDR2 Dysferlin-related (previously LGMD2B), one of the most common LGMD form in several countries [4,134,135], are allelic diseases with overlapping symptoms and signs [136,137]. LGMD patients have a more proximal involvement at the onset but, after 20 years of disease progression, the two phenotypes usually merge as dysferlinopathies [138-140].

Recessive distal titinopathy – TTN

Some nonsense, small indels causing a frameshift or splice site variants in the last and second last exons of *TTN,* initially also thought to cause dominant TMD because of dominant-looking pedigrees, later proved to be recessive [141-145]. The presence of second causative variants *in trans* explains the novel entity of early/juvenile onset recessive distal titinopathy, a more severe condition than the late onset TMD [141-143]. In some families, multiple second causative variants segregating with the disease would mimic the presence of a dominant inheritance, making the diagnosis even more complex [141,142,146].

The complexity of *TTN* gene may result in elusive variants not identified on DNA by the traditional pipelines [147]. Second tier tests, such as copy number variant (CNV) analysis and RNA sequencing, contribute to identify unrecognized pathogenic variants [148-152].

Distal myopathy with rimmed vacuoles (Nonaka or GNE myopathy) – GNE

Independently described by Nonaka et al. and by Argov and Yarom, the *GNE* distal myopathy is a rimmed vacuolar recessive myopathy with an early adult onset [153,154]. It first affects the anterior compartment of lower legs and thigh hamstring muscles with sparing of the quadriceps, but the progression is rather severe, and half of the patients loose ambulation within 10 years. Serum creatine kinase is mildly elevated and muscle histopathology is characterized by rimmed vacuoles.

The causative gene (*GNE*) was identified in 2001 [155] and, since then, patients have been reported worldwide. *GNE* encodes an epimerase-kinase enzyme involved in the sialic acid biosynthesis. Glycoproteins and glycolipids located in the membrane often undergo a sialic acid modification that seems to be crucial for their function [156]. Nevertheless, in a recent study, no consistent major change in sialylation has been observed comparing patients and matched control samples, suggesting that the pathophysiology of the disease is still unclear [157].

More than 180 variants are currently known and founder mutations first reported from Middle East and Japan have been described in many populations [158-163]. *GNE* is susceptible to Alu-mediated recombination, and copy number variants (CNV) have been reported suggesting the utility of second-tier tests in case of an uninformative sequencing analysis aiming at the identification of single nucleotide variants [164-167]. Moreover, a vast clinical heterogeneity, only partly explained by the *GNE* genotype, is observed in families with *GNE* mutations [160,168-170].

Sialuria is an allelic dominant metabolic disease characterized by the accumulation of N-acetylneuraminic acid (NeuAc) due to missense variants in GNE [171].

Distal ABD-filaminopathy – *FLNC*

A large Australian family with a dominant, adult-onset, slowly progressive distal myopathy was described in 2005 by Williams and colleagues [172]. In a second Italian family with otherwise similar phenotype reported by Duff et al. cardiac involvement was also present [173]. Weakness of handgrip is the usual presentation followed by calf muscle plantar flexion weakness. The progression is slow, and patients remain ambulant. CK is normal or mildly elevated, and muscle MRI shows fatty replacement in posterior compartment of lower legs. Histopathology is unspecific myopathic without vacuoles or myofibrillar abnormalities.

Combining linkage data and resequencing of candidate genes in these two families, two different missense changes in the N-terminal actin-binding domain (ADB) of *FLNC* were identified [173]. The *FLNC* gene encodes filamin, an actin ligand that plays an important role in mechanical stabilization, mechanosensation and intracellular signalling through a large network of interactors [174,175]. Mutations in other parts of the gene may cause late onset myofibrillar myopathy with generalized weakness and cardiomyopathy [176-178]. After the gene identification in 2011, novel *FLNC* causative variants have been identified, expanding the spectrum of *FLNC*-related myopathies [179-181].

Recent findings suggest a more complex genotype-phenotype correlation. A missense variant, p.M222V, in the N-terminal actin-binding domain, causing a distal myofibrillar myopathy, has been reported [182]. Another missense change, p.C203Y, has been recently found to cause an upper limb distal myopathy with nemaline bodies [183].

DNAJB6 distal myopathy – DNAJB6

The disease was originally reported by Servidei and colleagues in a large Italian family with onset of ankle weakness between the second and sixth decades of life [184], and usually progressing to proximal muscles and upper

limbs. Muscle biopsy showed dystrophic changes and rimmed vacuoles. In the Italian family, the causative variant was found in *DNAJB6*, in a different locus from the one initially reported [185]. *DNAJB6* encodes a ubiquitously expressed member of the DNAJ/HSP40 family of co-chaperones [119,186]. Mutations in *DNAJB6*, specifically in the G/F domain, cause more often a proximal myopathy (LGMD1D) [187-193]. Recently, a form of DNAJB6-related distal calf-predominant myopathy has been reported in patients with particular mutations in the N-terminal J-domain [194].

Rimmed vacuolar neuromyopathy – HSPB8

Ghaoui and colleagues reported two families with a dominant *HSPB8*-related disease showing early adult neurogenic leg weakness and progressing towards a distal and proximal myofibrillar and rimmed vacuolar myopathy in the later stage of the disease [195]. EMG revealed denervation in the distal lower limbs and myopathic proximal changes. MRI of the lower limb muscles showed first diffuse neurogenic changes in gastrocnemius, deep toe flexors, and peroneus with later fatty replacement in proximal thigh and lower legs muscles.

The *HSPB8* gene encodes the small heat-shock protein-beta 8, acting as stress protein with a chaperone-like activity and part of the chaperone-assisted selective autophagy (CASA) complex [119,196]. *HSPB8* missense variants had been previously associated with distal hereditary motor neuropathy 2A (dHMN2A) and Charcot-Marie-Tooth disease (CMT2L) [197-201]. The myofibrillar myopathy with aggregates and rimmed vacuoles mimics the histopathological changes seen in myopathies caused by defects in *BAG3* and *DNAJB6* [202,203].

Later other families with a combined neuromuscular disorder, encompassing dHMN and MFM have been described[204]. In one family decrease of *TARDBP* mRNA levels causing a consistent alteration of TDP-43-dependent splicing was reported [204]. Recently, a novel *HSPB8* variant has been found in a patient with limb-girdle myopathy without associated neuropathy [205].

ANO5 distal muscular dystrophy – ANO5

Distal anoctaminopathy has an age of onset in early/mid adulthood (18-40 years) [206,207]. Early manifestations include difficulties in sport activity and in walking on tiptoes but often the clinical presentation is mild, or the disease does not even result in overt clinical signs. The early stage hypertrophy of calf muscles progresses into muscle atrophy [208]. At a later stage, proximal muscle weakness and wasting is observed. Typically, the cardiac muscle is spared. CK levels are usually highly elevated (over 10 times the upper limits). Non-specific myopathic changes with scattered necrotic fibers are observed in the muscle biopsy.

The disease is due to bi-allelic causative variants in the *ANO5* gene, encoding for anoctamin-5, a putative cytoplasmic calcium-activated chloride channel, with a possible role in membrane fusion and repair [209,210]. The more common phenotype of bi-allelic variants in *ANO5* is late onset proximal (LGMDR12) [206,207,211-214]. Variants causing *ANO5*-related recessive anoctaminopathies mostly result in a reduced protein expression and missense changes likely destabilize the protein, causing its degradation [215,216]. We still lack a clear genotype-phenotype correlation explaining the high intrafamilial and interfamilial clinical variability observed, also considering that female patients often have a milder disease than males [206,212,217-221].

A dominant form of gnathodiaphyseal dysplasia is (GDD) an allelic disorder caused by *ANO5* missense variants in heterozygosity [222,223]. The pathomechanism of the *ANO5*-related GDD is still unclear. However, the protein seems to have an important role in the embryonic development and most probably in the osteoblast differentiation [223].

RYR1 mutated calf predominant distal myopathy – RYR1

A very mild dominant distal myopathy with preferential fatty degeneration of medial gastrocnemius, clearly shown by muscle MRI, has been recently reported in one Italian and two Finnish families [224]. Some patients exhibit toe walking in the childhood with spontaneous remission. In adulthood, patients complain of exercise myalgia in the calves, and show 5-10 fold elevated CK. No limitation of walking was present even in elderly patients. Muscle biopsy reveals core pathology. Three different *RYR1* mutations were identified in different parts of the gene, which encodes ryanodine receptor 1, a calcium release channel of the sarcoplasmic reticulum that, together with sarcolemmal voltage-gated calcium channels (DHPR), is responsible for the excitation-contraction coupling.

Dominant and recessive mutations in the *RYR1* gene present with a multitude of phenotypes including malignant hyperthermia (MH) susceptibility and congenital central core disease (CCD), centronuclear myopathy, multiminicore myopathy, congenital fibre type disproportion, axial myopathy, King-Denborough syndrome, atypical periodic paralysis and exertional rhabdomyolysis/myalgia [225-235]. A childhood-onset distal myopathy presenting with hand stiffness and facial weakness has been associated to bi-allelic *RYR1* variants [236].

Early-childhood onset distal myopathies

Early onset distal myopathy (Laing myopathy) – MYH7

Laing myopathy was the first distal myopathy with established genetic linkage [237]. The onset is in early child-

hood with the ankle dorsiflexor and toe extensor (hanging big toe) weakness and the disease has a slow progression. Severe forms develop scoliosis and involves proximal, neck and facial muscles. CK levels are normal or mildly elevated. The most consistent histopathology is hypotrophy of type 1 slow fibers, often combined with core/minicore lesions [238]. Muscle MRI shows the involvement of anterior compartment lower leg muscles and, eventually, of the sartorius, with relative sparing of the lateral gastrocnemius muscle and rectus femoris [239-242].

In 2004 the causative variant was identified in the *MYH7* gene encoding the beta heavy chain of myosin [243]. Since then, a large number of causative variants in the tail of the protein were reported, with a large proportion (30%) of re-occurring de novo mutations masking the dominant effect of the variants [244-248]. Causative variants in the head and neck domains at the N-terminal of the protein have been mainly associated with hypertrophic cardiomyopathy (without skeletal muscle involvement) [249-251]. Variants in the ultimate C-terminal region most often result in other skeletal myopathies (hyaline body myopathy) with or without cardiac involvement [252,253]. Rare recessive forms of *MYH7*-related myopathy have been reported [254-256].

Early onset distal myopathies with nebulin defect – NEB

Bi-allelic, mainly missense, variants in *NEB* gene may result in an early-onset distal myopathy with a predominant weakness of extensor muscles of feet and later hands [257,258]. The progression is very slow and adult patients do not have major disability.

Muscle imaging shows a selective fatty degeneration in the anterior tibial muscles, EMG is myopathic and CK is normal or mildly elevated. Scattered and grouped atrophic fibers (that can be misinterpreted as neurogenic changes) are detectable in the biopsy of affected muscle without rods on light microscopy [258,259]. Small rods associated with Z-disks may be present on electron microscopy [259].

A large in-frame deletion, dominantly inherited in a three-generation family with a distal nemaline rod/cap myopathy, was recently described [260]. The in-frame deletion results in a protein of reduced size with a dominant-negative effect [260]. Patients present with foot drop in childhood and the disease progresses with the involvement of distal upper limbs. CK can be slightly elevated, EMG is myopathic and muscle MRI shows fatty degeneration of the anterior compartment lower leg muscles. A large heterozygous de novo deletion in young patient with asymmetric distal and facial weakness has just been identified confirming the dominant effect of an abnormal protein (submitted).

The 183-exon *NEB* gene encodes nebulin, a protein of 600-900 kDa that regulates the length of actin filaments [261,262]. Causative variants in *NEB* (mainly nonsense, out-of-frame indels or copy number variants, and splicing variants) are the most common cause of congenital nemaline myopathy [263,264]. Additional allelic diseases are core rod myopathy, and fetal akinesia/lethal multiple pterygium syndrome [259,265,266].

Copy number variant (CNV) analysis and RNA sequencing are essential to identify possible elusive pathogenic *NEB* variants [148-150,267,268].

Early onset ADSSL distal myopathy – ADSSL

In 2016, Park and colleagues reported two unrelated Korean families with an autosomal recessive adolescent-onset distal myopathy with facial muscle weakness, mild CK elevation and rimmed vacuoles in the muscular biopsy [269,270]. Two different variants in the *ADSSL* gene were identified by exome sequencing. The *ADSSL* gene encodes the muscle isozyme of adenylosuccinate synthase, the enzyme catalysing the initial reaction in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) [271,272]. Two following studies identified novel Korean and non-Korean (Turkish and Indian) patients, confirming the gene-disease association [273,274]. Most patients presented with a distal myopathy with onset in childhood or adolescence progressing to involve weakness of proximal muscles in early adulthood. However, one patient, homozygous for a missense variant, shows a proximal myopathy with contractures and muscle atrophy, expanding the *ADSSL*-related spectrum of phenotypes [274].

Early onset distal myopathy with KLHL9 mutations – KLHL9

Cirak and colleagues described a German family with an autosomal distal weakness caused by a heterozygous variant in the *KLHL9* gene [275]. Despite extensive later studies, this gene has not been confirmed in any other myopathy families.

Other myopathies and dystrophies with distal weakness

Distal myopathy with caveolin defect – CAV3

In 2002, Tateyama and colleagues described a form of sporadic distal myopathy caused by an heterozygous missense variant in *CAV3* gene [276]. Since then, additional patients and further causative variants have been reported [277,278]. Onset is in early adulthood, muscle atrophy and weakness are often limited to the small muscles of the hands and feet. Other features included calf hypertrophy, pes cavus, myalgias, slightly increased serum CK. EMG studies show a myopathic pattern and histological find-

ings include nonspecific myopathic changes [278,279]. *CAV3* expression can be reduced.

Variants in *CAV3* can also cause an isolated hyperckemia or a rippling muscle disease [280-285] and even cardiac phenotypes (hypertrophic cardiomyopathy and long QT syndrome) [286,287]. Some patients with *CAV3* related myopathy have been previously described as LGMD1C patients [288].

DNM2 related distal myopathy – DNM2

DNM2-related myopathy is an autosomal dominant slowly progressive centronuclear myopathy characterized by the presence of centrally located nuclei in a several muscle fibres [289]. The clinical onset is usually in childhood or early adulthood [290]. The distal muscle weakness is marked although facial weakness is the clinical lead to diagnosis [291-300]. More severe forms, clinically resembling myotubular myopathy, have been described [301]. MRI studies shows fatty infiltration of the calf muscles [302]. Electromyography may show a mix pattern with myopathic and neuropathic changes.

DNM2 encodes dynamin 2, a ubiquitously expressed GTPase that is involved in endocytosis and intracellular trafficking [303-306]. Dynamin 2 interacts with actin and has an active role in regulating microtubule networks and in centrosome function [307]. *DNM2* mutations can also cause an intermediate or axonal CMT disease with and without cataracts [308]. A lethal congenital syndrome associating akinesia, joint contractures, hypotonia, skeletal abnormalities, and brain and retinal haemorrhages has been observed in three consanguineous families with a missense variant (p.Phe379Val) in homozygosity [309].

Differential diagnostics

A long range of other myopathies needs to be considered in the differential diagnostics since they may show prominent distal weakness and/or atrophy:

- Facioscapulohumeral muscular dystrophy;
- Myotonic dystrophy type1;
- HMERF titinopathy (i.e. p.C31712R mutation);
- Scapuloperoneal syndromes (FHL-1, *TRIM32*,…);
- Other nemaline and rod-core myopathies (*TPM2*, *ACTA1*,…);
- Inclusion body myositis;
- Telethoninopathy;
- Glycogenoses: brancher and debrancher enzyme defects;
- Lipidosis in *PNPLA2* mutated disease;
- Mitochondrial distal myopathy (*POLG1*);
- Nephropathic cystinosis;
- Amyloid myopathy (myeloma induced).

Conclusions

Despite the huge developments in the last 20 years to uncover the genetic cause of distal myopathy, some families and patients still remain without a final diagnosis. The introduction of long read sequencing and RNA sequencing in clinical care and the constitution of large international consortia will most probably further increase the diagnostic rate [310-316].

The reason for some genetic defects to have preference for the distal limb muscles in causing loss of muscle tissue is unclear and understanding the molecular background for this preference may also harbour insight for therapeutic opportunities.

Considering the crucial advancements in the last decade, we can look forward optimistically to the upcoming decade. We will most probably identify an increasing number of digenic diseases and of genetic and non-genetic modifiers influencing the phenotype. The next challenge is to translate the genetic and molecular advancements in clinics, thereby contributing to the development of a personalized medicine aiming at providing a tailored approach to each patient with a distal myopathy [317,318].

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Core myopathies – a short review

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Congenital myopathies represent a clinically and genetically heterogeneous group of early-onset neuromuscular diseases with characteristic, but not always specific, histopathological features, often presenting with stable and/or slowly progressive truncal and proximal weakness. It is often not possible to have a diagnosis on clinical ground alone. Additional extraocular, respiratory, distal involvement, scoliosis, and distal laxity may provide clues. The "core myopathies" collectively represent the most common form of congenital myopathies, and the name pathologically corresponds to histochemical appearance of focally reduced oxidative enzyme activity and myofibrillar changes on ultrastructural studies. Because of the clinical, pathological, and molecular overlaps, central core disease and multiminicore disease will be discussed together.

Key words: core myopathies, central core, multiminicore, malignant hyperthermia

Introduction

The scope of this short review is pure core myopathies: central core disease and multiminicore disease often associated with mutations in the skeletal muscle ryanodine receptor 1 (*RYR1*) and selenoprotein N1 (*SEPN1*; also known as *SELENON* according to new nomenclature).

Mutations in the skeletal muscle ryanodine receptor 1 (*RYR1*) gene are associated with dominantly inherited central core disease and subgroups of recessively inherited multiminicore disease, centronuclear myopathy (CNM), and congenital fiber type disproportion. Malignant hyperthermia susceptibility trait is a dominantly inherited allelic trait and is described as a pharmacogenetic predisposition to severe and potentially life-threatening reaction in response to halogenated anesthetic agents and depolarizing muscle relaxants.

RYR1-related malignant hyperthermia susceptibility is allelic to central core disease and has also been described as a common cause of induced and episodic phenotypes such as exertional rhabdomyolysis or periodic paralysis, which present throughout life. Late-onset presentations in the adulthood period highlight relevance of the congenital myopathies for adult neuromuscular practice.

A number of distinct phenotypes are seen in multiminicore disease, which is most commonly caused by recessive mutations in the *RYR1* and *SEPN1* genes. It has been linked to dominant mutations in the gene for beta-myosin heavy chain protein (*MYH7*) and autosomal recessive mutations of titin (*TTN*). Recessive mutations of satellite cell gene (*MEGF10*) are defined in patients with early-onset myopathy, areflexia, respiratory distress, and dysphagia (EMARRD) [1].

Central core disease is probably the most common form of the congenital myopathies[2]. Recessive *SEPN1*-related minicore myopathy is the

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second most common core myopathy[3]. The exact prevalence of the condition is unknown. In prevalence study of congenital myopathies in a representative pediatric United States population, overall point prevalence of congenital myopathies was 1 out of 26,000, with mutations in *RYR1* being the most common cause of congenital myopathies at 1 out of 90,000 [2].

History

In 1956, Magee and Shy described "a new congenital nonprogressive myopathy" characterized by a distinctive microscopic appearance on skeletal muscle biopsy[4]. It was the first recognition of the congenital myopathies, the diagnosis of which was based on the distinct structural or histochemical changes in biopsied skeletal muscle, as a distinct group of diseases.

This disorder was then named central core disease [5], and an absence of oxidative enzyme and phosphorylase reactivity in the cores were demonstrated on muscle biopsy in these patients [6].

In 1971, Engel and colleagues described a patient with multiple small cores within muscle fibers [7], which were later renamed as "multicores," "minicores," "focal loss of cross-striation," "target-like lesions," and "miniature cores;" multiminicore disease is now the preferred terminology [8].

Clinical manifestations

There is a wide clinical spectrum of core myopathies, with severity ranging from mild to severe. Hypotonia, joint laxity, developmental delay in motor milestones, hip girdle or axial weakness, and congenital hip dysplasia may be among early clinical presentations. Arthrogryposis represents the severe end of the spectrum. There may be a variability even within the same family; some of the individuals being asymptomatic and others presenting with hyperCKemia, exertional myalgia, rhabdomyolysis or periodic muscle stiffness, and paralysis (Fig. 1).

Typical central core disease patients present with mild and symmetrical weakness, hypotonia, and delayed motor milestones, and although late, patients achieve independent ambulation. The course is usually nonprogressive or slowly progressive. Patients presenting with severe neonatal weakness, arthrogryposis, and respiratory failure require early respiratory support and corrective scoliosis surgery. More significant respiratory muscle weakness is seen in infants with recessive core disease [9]. Extraocular muscles are spared in the dominant forms. Because of musculoskeletal deformities including congenital hip dislocation, kyphoscoliosis, pes cavus, pes planus, and thoracic deformities, patients are frequently referred from orthopedic clinics. Heart disease is not considered as a part of the typical "core myopathy" spectrum. Some cardiac abnormalities described include mitral valve prolapse, arrhythmias, and asymptomatic right bundle branch block[10]. Intelligence is generally normal in central core disease.

Genetic resolution of core myopathies in recent years further lead to "mutation-specific" clinical presentations and phenotype-genotype correlations. Availability of next-generation sequencing (NGS) techniques lead to improved detection rate for mutations and expanded the clinical spectrum [3,11].

Central core disease is associated mainly with dominant *RYR1* mutations, and multiminicore disease is genetically a more heterogenous condition [12].

Dominantly inherited *RYR1*-related central core disease is characterized by mild to moderate muscle weakness presenting from infancy to childhood. Congenital hip dislocation, scoliosis, and generalized joint laxity are common. In contrast to the recessive forms with a more severe clinical phenotype, there is no extraocular muscle involvement. Bulbar, respiratory, and cardiac involvement is uncommon. Myalgia may be prominent. Central core disease tends to be stable over long periods with a possible progression in adulthood, and due to intrafamilial variability, there may be a delay in diagnosis [13].

Malignant hyperthermia is a disorder of calcium metabolism. Central core disease is associated with an increased risk of malignant hyperthermia. Most patients with malignant hyperthermia have normal muscle biopsy features, and less than 30% of the patients with central core disease have malignant hyperthermia susceptibility [14]. Two well-known malignant hyperthermia-related syndromes are King-Denborough syndrome and Native American myopathy (NAM).

The association between malignant hyperthermia and central core disease patients was first described in 1973 and has since been confirmed in many other reports [15,16]. King-Denborough syndrome is characterized by facial and skeletal dysmorphism, malignant hyperthermia sus-

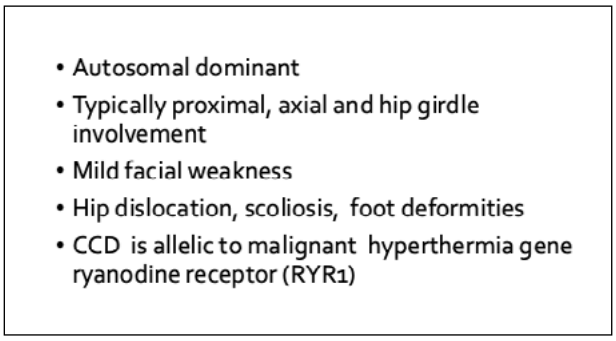

Figure 1. Central core disease (CCD).

ceptibility, and myopathy. All genetically solved patients to date are due to *RYR1* mutations.

Native American myopathy is characterized by mild facial dysmorphism, skeletal abnormalities, and mild extremity weakness. All individuals to date are from Lumbee Native Americans in North Carolina, with recessive mutations in *STAC3*, coding a protein regulating excitation-contraction coupling [1,17].

In a retrospective cohort study including 277 pediatric and adult patients referred for malignant hyperthermia and inherited myopathies, *RYR1* mutations were detected in 77 unrelated patients with a detection rate of 28%, and exertional rhabdomyolysis phenotype was prominent in this Dutch series [18].

RYR1-related malignant hyperthermia susceptibility is allelic to central core disease, and some patients with central core disease may also have malignant hyperthermia susceptibility. Exertional rhabdomyolysis and periodic paralysis may present throughout life [3]. Late-onset presentations in the adulthood period highlight relevance of the congenital myopathies for adult neuromuscular practice (Fig. 2).

Multiminicore disease is a clinically heterogeneous disorder. Four major clinical subgroups are recognized [4,8]. Core phenotype due to *SEPN1*-related myopathies can be defined as predominant axial weakness, early spinal rigidity, scoliosis, and respiratory involvement. There is a disproportion between axial and skeletal muscle weakness. This is the most common (approximately 75% of all cases) "classic" form, which presents in the neonatal period or first year of life. Affected infants are hypotonic and weak and have delayed motor development. Some children have associated congenital abnormalities such as cleft palate, dislocated hips, or arthrogryposis [19]. Physical examination reveals generalized hypotonia, joint hyperlaxity, and asthenic phenotype with decreased muscle bulk. Short stature and failure to thrive are common in children with significant weakness. Intelligence is normal. Weakness in the classic form is predominantly axial. The neck flexors are usually the most affected muscles, with poor or absent head control in infancy being characteristic of this disorder. There is mild to moderate weakness of the proximal limb muscles. Facial weakness is common but of variable severity, whereas the extraocular muscles are spared. The deep tendon reflexes are absent or diminished. Extremity weakness may be static or slowly progressive or may even appear to improve slightly with increasing age. Paraspinal rigidity and kyphoscoliosis frequently lead to early-restrictive respiratory dysfunction with progressive respiratory insufficiency, which may be rapid in onset. Weakness and spinal deformity are usually static after adolescence. Because limb weakness is often relatively mild, most patients are ambulant well into adulthood, even in the presence of significant respiratory compromise. Up to two thirds of patients with the classic form of minicore disease develop respiratory insufficiency in late adolescence or early adulthood [8]. Cardiac involvement is usually in the form of cor pulmonale secondary to respiratory insufficiency, rather than primary myocardial involvement. About 50% of cases of "classic" multiminicore disease are caused by recessive mutations in the *SEPN1* gene [20].

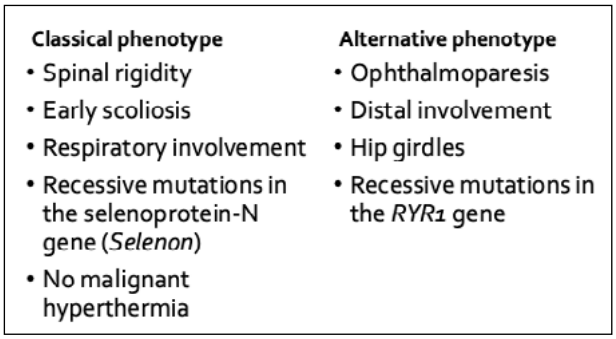

Figure 2. Multi-minicore disease.

The moderate form of multiminicore disease with hand involvement is relatively rare (fewer than 10% of all cases) and is characterized by relatively mild distal weakness of the upper limbs with hand amyotrophy and marked joint hyperlaxity. The lower extremities are mildly affected with proximal pelvic girdle weakness. Scoliosis and respiratory involvement are minimal or absent [8,18].

Fewer than 10% of cases of multiminicore disease are of the ophthalmoplegic form, in which there is external ophthalmoplegia in addition to proximal limb weakness [21]. This form of minicore disease may be associated with recessive mutations in *RYR1* [22].

There is a huge phenotypic variability in multiminicore disease. Spanish kindreds with a dominantly inherited distal myopathy with weakness of the great toe and ankle dorsiflexors and often associated neck flexor, finger extensor, and mild facial weakness were found to have minicore myopathy caused by the common mutation in the *MYH7* (beta-myosin heavy chain protein) gene, which

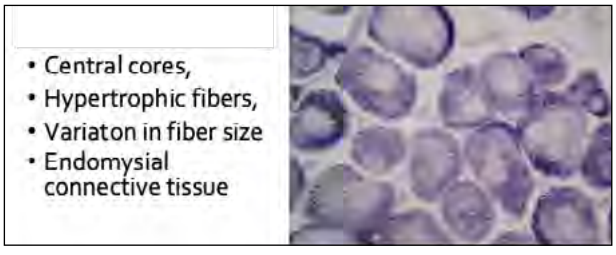

Figure 3. Central core disease muscle biopsy findings (courtesy of Dr. Beril Talim), NADH staining.

is more commonly known to cause Laing distal myopathy [23]. A report described 2 kindreds with minicore disease caused by dominant mutations in *MYH7*, presenting in childhood with proximally predominant weakness with progression in adulthood to distal weakness and dilated cardiomyopathy [24].

Recessive mutations of a satellite cell gene (*MEGF10*) are also implicated in multicore disease [25]. Clinical phenotype is characterized by early-onset myopathy, areflexia, respiratory distress, and dysphagia (EAMRDD).

In 2015, a single case with a severe congenital myopathy, ophthalmoplegia, and recessive variants in the gene encoding the alpha-1 subunit of the dihydropyridine receptor (*CACNA1S*) is described [26].

At the beginning of 2020, *TRIP4* mutations leading to loss of the coactivator protein ASC1, which direct binds to transcription factors, have been shown to cause multiminicore disease with a contractural phenotype [27].

Prognosis and complications

The course is static or slowly progressive in most central core disease patients. Involvement of the respiratory muscles may be of insidious onset and may remain subclinical until unmasked by intercurrent illness or anesthesia. Lung function should be monitored with serial pulmonary function tests, and where indicated, polysomnography. Cardiac complications are uncommon, but baseline electrocardiography and echocardiography are appropriate in most cases of suspected myopathy. Children should be monitored for the development of scoliosis and other skeletal deformities [28]. Many patients with central core disease are at risk of developing malignant hyperthermia during a general anesthetic. As the first exposure to trigger substances elicits an event in only 50% of malignant hyperthermia susceptibility patients, a previous history of tolerance of halogenated anesthetic agents or depolarizing muscle relaxants does not guarantee that these agents can be used safely in future anesthetics. Appropriate anesthetic precautions should be taken in all instances [28].

Minicore myopathy follows a variable course. In many patients, the condition remains benign with static or slowly progressive weakness of the extremities and retention of independent mobility. In some, spinal rigidity becomes a clinically predominant feature in late childhood or adolescence. Severe progressive scoliosis is apparent in a minority. Surgical fixation of the spine is required in most such cases. Minor contractures of the elbows, knees, and hips may develop after infancy and are generally amenable to physiotherapy.

Progressive scoliosis or respiratory insufficiency is seen in up to two thirds of patients with the "classic" form of multiminicore disease, although the ability to walk

Figure 4. Multiminicore disease biopsy findings (courtesy of Dr. Beril Talim), NADH staining.

independently is often preserved even in adults with respiratory failure. There is a marked discrepancy between profound respiratory impairment and preserved ambulation [4]. Mortality from multiminicore disease is usually related to complications of respiratory disease. Mortality related to cardiac involvement is uncommon but has occasionally been reported [16,24].

Pathogenesis

Central core disease is caused by mutations in the *RYR1* gene on chromosome 19q13.1. The *RYR1* gene encodes a protein product, the calcium release channel ryanodine receptor (RyR1), which is highly expressed in skeletal muscle sarcoplasmic reticulum, B lymphocytes, and lymphoblastoid cells [29].

The *RYR1* gene is large (containing 106 exons), rendering genotype-phenotype correlation difficult. Mutations in the ryanodine gene can be identified in more than 90% of patients with central core disease when all parts of the *RYR1* are carefully sequenced [30]. Mutations causing central core disease and malignant hyperthermia cluster to 3 regions of the *RYR1* gene. Approximately 50% of patients with clinical evidence of a myopathy and central cores on muscle biopsy have a mutation in region 3 in exons 93 to 104 of *RYR1* [31]. Recessive *RYR1* mutations may cause a marked decrease in sarcoplasmic reticulum calcium release during excitation-contraction coupling [32].

Central core disease has traditionally been regarded as an autosomal dominant disease with variable penetrance. De novo mutations are relatively common [31]. The new mutation rate is estimated to be about 10%.

Autosomal recessive inheritance of central core disease has been recognized in a number of families [33,34]. Recessive central core disease links to *RYR1* but demonstrates more clinical and pathologic heterogeneity than that is seen with dominant inheritance. Protein expression studies variably suggest a correlation between specific mutations, protein levels, and phenotype. Recessive core disease may be more common than has previously been recognized.

The pathological hallmark of central core disease is the presence of well-demarcated cores (round or oval

shaped regions within a muscle fiber that lack oxidative enzyme activity on histochemical stains) within type 1 muscle fibers (Fig. 3). There is usually type 1 fiber predominance, which may be most marked in those with *RYR1* mutations at the C-terminal [30]. Cores usually extend along most or all of the length of the fiber. There may be 1 or more cores within a fiber. Although the biopsy may show other myopathic features such as fiber size variability, increased internal nuclei, and fiber splitting, the presence of cores as the predominant pathological feature in the biopsy establishes the diagnosis of central core disease. Necrosis and significant fiber regeneration are uncommon, but extensive fibrosis and fatty infiltration are seen occasionally [35].

Cores may be centrally or eccentrically placed and, in a minority of cases, may resemble minicores, raising the possibility of the separate but related disorder minicore myopathy, which is generally autosomal recessive in inheritance. In such cases, microscopic evaluation of cross sections may not distinguish between central core disease and multiminicore disease, whereas longitudinal sections reveal the cores of central core disease to run the whole length of the fiber (Fig. 4). Those of multiminicore disease are usually shorter and are seen in both type 1 and type 2 fibers. Diagnosis is also difficult in the small number of patients whose muscle biopsy demonstrates uniformity of type 1 fibers without cores. This entity, now known as congenital neuromuscular disease with uniform type 1 fiber and *RYR1* mutation (CNMDU1), is caused by mutations in *RYR1* in 40% of cases [36].

Desmin (an intermediate filament protein found in muscle fibers) reactivity, detected by an indirect immunofluorescence assay, is abnormal in cores [37]. Accumulations of desmin are seen in many other myopathies. Myotilin, a Z-disc protein that binds alpha-actinin, gamma-filamin, and F-actin, is also present in central cores [38]. Immunocytochemistry is helpful in demonstrating cores but shows no other specific abnormalities in central core disease [35].

Electron microscopic examination of cores shows an absence of mitochondria, the anatomical correlate to the loss of oxidative enzyme activity in histochemical reactions [39].

Muscle MRI may help to define distinct pattern of involvement and can be used as a potential biomarker for disease severity in neuromuscular conditions [40].

Selenoprotein N, a glycoprotein, localizes to the endoplasmic reticulum and is found at low levels in virtually all body tissues. Selenoprotein N mutations have also been implicated in myogenesis, with work suggesting a possible role in muscle sarcomeric organization and myofiber attachment [41]. *SEPN1* mutations cause 40 to 50% of cases of classic minicore myopathy [20].

Mutations in 2 genes are responsible for approximately 50% of cases of minicore disease. In a study from Italy, mutations of *SEPN1* represented 6% of congenital muscular dystrophy patients [42].

Recessive mutations in the gene for *SEPN1* account for 30% of all multiminicore disease and represent 40 to 50% of all "classic" form (20). Homozygous mutations of the same gene were originally described in congenital muscular dystrophy with rigid spine (rigid spine muscular dystrophy) [43], an entity that is now felt to represent severe "classic" minicore myopathy presenting in early childhood [33].

Minicore myopathy is described in subjects with mutations in *MYH7*, a gene for beta-myosin heavy chain protein usually associated with Laing distal myopathy [23,24,44]. Weakness in such cases may be of childhood or adult onset, may be proximally or distally predominant, and may be associated with an adult-onset cardiomyopathy.

Diagnostic work-up

The traditional approach to the diagnosis of a congenital myopathy is combining detailed clinical and family history with physical examination findings in order to recognize clues to clarify a phenotype. The International Standard of Care Committee for Congenital Myopathies provides a diagnostic approach and highlights the fact that other than muscle biopsy investigations are rarely specific for congenital myopathies but are widely used to exclude other possible diagnoses. Evaluation of muscle biopsy is important; however, one should also keep in mind the overlap and morphological abnormalities seen in these conditions, marked variability in their clinical progression, and severity [45].

Serum creatine kinase is usually normal or mildly elevated.

Electromyography (EMG) and nerve conduction studies (NCS) are useful to exclude denervation disorders. EMG is typically normal or shows myopathic features, with short-duration, small-amplitude, polyphasic motor unit potentials.

Muscle imaging (ultrasonography and MRI) may be useful in diagnosis demonstrating a characteristic pattern of selective muscle involvement, which may be used in conjunction with clinical features to guide genetic testing. Muscle biopsy and analysis of muscle histology, histochemistry, immunohistochemistry, and ultrastructure by light and electron microscopy (EM) have been the mainstay of reaching the diagnosis of a specific form of congenital myopathies [46].

From a few laboratories worldwide offering diagnostic mutational screening, there is a shift in paradigm, from invasive procedures to the new era of NGS strategies [47]. Cost-effectiveness and increasing availability of these techniques in different centers give the opportunity to directly perform molecular analysis.

Diagnostic work-up finally includes molecular genetic testing. In a patient with a phenotype consistent with core myopathy first *RYR1* mutations, minicore myopathy first *SEPN1* mutations and second *RYR1* mutations should be screened. In case of associated cardiomyopathy, *MYH7* and *TTN* analysis is recommended [46].

Whole-exome sequencing and whole-genome sequencing approaches, wherever available, may help diagnosis in a cost-effective way [46].

Management

Core elements of treatment include physical therapy, orthopedic interventions, management of respiratory complications, and feeding problems.

Involvement of the respiratory muscles may be of insidious onset and may remain subclinical until unmasked by intercurrent illness or anesthesia. Respiratory function should be monitored with serial pulmonary function tests, and where indicated, sleep studies.

Cardiac complications are uncommon in central core disease, but baseline electrocardiography and echocardiography are appropriate in most cases [16].

Children should be monitored for the development of scoliosis and other skeletal deformities. Scoliosis may develop quickly and may be of a severity disproportionate to that of limb weakness. Congenital dislocation and dysplasia of the hip require orthopedic treatment. Gamble and colleagues reported a higher number of treatment failures and hip contractures after surgery in children with central core disease than in other myopathies [48].

Because malignant hyperthermia susceptibility is common in central core disease, all patients subjected to general anesthesia require preoperative anesthetic consultation and planning to ensure that a nontriggering anesthetic technique is used. Individuals with multiminicore disease are usually able to care for themselves in all activities of daily life. Schooling is unaffected. A regime of physical therapy wherein muscle strength is maintained, and range of motion preserved is generally appropriate. Minor contractures of the elbows, knees, and hips may develop after infancy and may respond to physical therapy or splinting. Surgical release is rarely necessary.

One study examined the effect of salbutamol, a beta-2 agonist, on effect on muscle strength in central core and minicore disease. Five children with minicore myopathy (average age 13.6 years) received 2 mg salbutamol orally 4 times a day for 6 months. One stopped the medication because of tremors and palpitations. The other 4 completed 6 months of therapy, at the end of which, they reported improved stamina and had a small but significant improvement in strength measured by myometry and MRC scores, and in their vital capacity, relative to baseline [49].

The 2 major complications of multiminicore disease are the development of progressive kyphoscoliosis and progressive deterioration of respiratory function. Monitoring of spinal growth is important, especially during growth spurts. Given the possibility of "malignant" kyphoscoliosis, it is advisable to obtain a surgical opinion as soon as kyphoscoliosis becomes evident, and early spinal operation should be considered in those with progressive respiratory involvement [50]. The possible predisposition to malignant hyperthermia should be kept in mind in all patients with multiminicore disease [51].

Respiratory function should be monitored in all affected children, but especially those with significant scoliosis and spinal rigidity. The degree of respiratory insufficiency seen in minicore disease is often disproportionate both to the extent of peripheral weakness and to the severity of scoliosis, and in those with significantly diminished reserve, there is potential for life-threatening decompensation of respiratory function with intercurrent illness. Polysomnographic evaluation should also be undertaken in order to evaluate for subclinical nocturnal hypoventilation.

Evaluation of cardiac function, in the form of a clinical assessment, ECG, and echocardiogram, should be undertaken at baseline and in patients with progressive skeletal weakness or respiratory insufficiency. Cardiac involvement in multiminicore disease is most commonly in the form of cor pulmonale as a sequela of progressive respiratory failure, but occasional cases have been associated with primary cardiomyopathy [52,53].

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Dominant or recessive mutations in the *RYR1* gene causing central core myopathy in Brazilian patients

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Central Core Disease (CCD) is an inherited neuromuscular disorder characterized by the presence of cores in muscle biopsy. CCD is caused by mutations in the *RYR1* gene. This gene encodes the ryanodine receptor 1, which is an intracellular calcium release channel from the sarcoplasmic reticulum to the cytosol in response to depolarization of the plasma membrane. Mutations in this gene are also associated with susceptibility to Malignant Hyperthermia (MHS).

In this study, we evaluated 20 families with clinical and histological characteristics of CCD to identify primary mutations in patients, for diagnosis and genetic counseling of the families.

We identified variants in the *RYR1* gene in 19/20 families. The molecular pathogenicity was confirmed in 16 of them. Most of these variants (22/23) are missense and unique in the families. Two variants were recurrent in two different families. We identified six families with biallelic mutations, five compound heterozygotes with no consanguinity, and one homozygous, with consanguineous parents, resulting in 30% of cases with possible autosomal recessive inheritance. We identified seven novel variants, four of them classified as pathogenic. In one family, we identified two mutations in exon 102, segregating in cis, suggesting an additive effect of two mutations in the same allele.

This work highlights the importance of using Next-Generation Sequencing technology for the molecular diagnosis of genetic diseases when a very large gene is involved, associated to a broad distribution of the mutations along it. These data also influence the prevention through adequate genetic counseling for the families and cautions against malignant hyperthermia susceptibility.

Key words: central core disease, *RYR1*, Next Generation Sequencing

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Introduction

Central core disease (CCD) is one of the most common genetic congenital myopathies, characterized by muscle weakness, atrophy, hypotonia, hyporeflexia, and delayed motor development, starting commonly in the perinatal period. Muscle weakness is usually proximal and symmetrical, stable or slightly progressive [1].

The probable incidence of congenital myopathies has been estimated in ~1:25,000, and has been reported to account for 14% of all cases of neonatal hypotonia, or one out of ten of all cases of neuromuscular disorders [1].

The classification of congenital myopathies is under constant review, as more genes and forms are identified and associated with various phenotypic and muscle histological alterations. In structural forms, the classification is based on the characteristics observed on muscle biopsy. The histopathological hallmark of CCD is the presence of cores, areas with reduced oxidative activity, observed in muscle fibers under the reaction for oxidative enzymes (NADH or SDH). The genetic variants causing core myopathies primarily affect proteins involved in skeletal-muscle excitation-contraction coupling (ECC) by altering calcium ion (Ca2+) transits between the sarcoplasmic reticulum (SR) and sarcoplasm. Ineffective ECC causes muscle weakness and is also associated with the formation of mitochondria-depleted core lesions. However, the processes governing core formation are far from completely understood [2].

The *RYR1* gene encodes the major sarcoplasmic reticulum calcium release channel of the skeletal muscle [3], and mutations in this gene cause CCD and also lead to several other types of myopathy subtypes, such as Multiminicore Disease (MmD), Centronuclear myopathy (CNM) and Malignant Hyperthermia susceptibility (MHS, MIM# 145600) [3]. Malignant hyperthermia is a pharmacogenetic disorder of skeletal muscle, triggered by exposure to volatile anesthetic gases like halothane and depolarizing muscle relaxants such as succinylcholine [4]. Patients with CCD usually also present Malignant hyperthermia susceptibility.

RYR1 is located at 19q13.2 and contains 106 exons. The protein product of *RYR1* is composed by 5037 amino-acids and 535 kDa. The combination of four of these subunits, together with a number of accessory proteins, forms the major calcium channel in the skeletal muscle. RYR1 combined molecules are embedded in the membranes of sarco/endoplasmic reticula (SR/ER) and regulate the rapid intracellular release of Ca2+ following transverse tubule depolarization. RyR isoforms also contribute to maintaining cellular Ca2+ homeostasis under resting conditions [5]. Over 450 variants were identified in the *RYR1* gene causing CCD and MH, and these mutations were mainly located in three hotspots of the gene. The hotspots, also referred to as regions 1-3 (D1, D2, and D3), include N-terminal residues 1-614 (sarcoplasm), central region residues 2163-2458 (sarcoplasm), and C-terminal residues 4136-4973 (Pore-forming, SR lumen, and membrane). MH causing disease are predominantly located in D1 and D2, and mutations causing CCD are predominant in the C-terminal D3 region [3].

For many years, due to the large size of the *RYR1* gene and the broad distribution of the mutations along the gene, screening for mutations in candidate patients was done predominantly in the hotspot regions, restricting the effectiveness of the molecular diagnosis of the patients. In our days, a significant improvement has started with the introduction of sequencing using next-generation sequencing methodologies, which became a more economical and efficient way to study a large number of genes and regions simultaneously. Custom panels can be designed to include several hundred genes of interest, or ready to use panels, such as the Illumina Trusight panels, that are available with more than 6,700 genes for Mendelian diseases [6].

Here, we studied 20 families with CCD, aiming the molecular characterization of the patients, and evaluation of the frequency of mono versus biallelic mutations in the *RYR1* gene. The results have important implication for the study of physiopathological mechanisms involved in the disease, and for the prevention through genetic counseling in the Brazilian families.

Patients and methods

Patients

The ethics committee of the Biosciences Institute of the University of Sao Paulo approved this work, and the DNA samples are stored in the biobank repository of the Human Genome and Stem Cells Research Center of IB-USP. All patients agreed in participating in this study and signed an appropriated informed consent.

The patients included in this study have been followed in the last 20 years in the Myopathies Laboratory of clinic for neuromuscular diseases at the Human Genome and Stem Cells Research Center, Institute of Biosciences, University of Sao Paulo, Brazil. Patients were also referred from other hospitals in Sao Paulo and medical centers from Belo Horizonte, Brazil, where a complete clinical and neurological evaluation was also performed. The inclusion criteria was patients of any age and sex with clinical diagnosis of congenital myopathy, and a muscle biopsy with histopathological findings including cores in oxidative enzymes reaction in muscle fibers.

Molecular analysis

The DNA of the selected patients was extracted from peripheral blood lymphocytes using routine methodology. Parents were also studied, when available, for segregation analysis.

The genetic investigation was carried out by Next-Generation Sequencing, using first a customized panel including *RYR1* and additional 95 genes associated with neuromuscular diseases (NMD) genes. After, in order to expand our investigation, we begin to use the Illumina TruSight One Expanded panel, which targets more than 6700 genes and exonic regions that were associated to a described clinical phenotype

The SureSelect QXT library preparation kits and the SureSelect Human all exons and V6 capture kit (Agilent, United States) were used. The Hiseq2500 equipment (Illumina, United States) performed the sequencing. The data were aligned according to the reference version GRCh37/hg19 of the human genome.

Variants were filtered and compared to control populations of 1000 Genomes, NIH, gnomAD, 6500 Exome Sequencing Project (Washington University), and the recently created Online Archive of Brazilian Mutations – AbraOM (http://www.abraom.ib.usp.br). Rare variants were checked in the *RYR1* gene (OMIM#180901), and analyzed using bioinformatic tools. Pathogenic variants already described were checked in Gene Mutations Databases HGMD, LOVD, and Clinvar. The pathogenicity of de novo variants was analyzed in prediction sites including: Mutation taster, Predict SNP1, CADD, DANN, FATHMM, FunSeq2, GWAVA, VEP, SIFT, Polyphen2 and Human splicing finder3.0.

Sanger sequencing of specific exons was done to confirm the mutation and screen other affected patients in the family, or to study the segregation of the mutation within the family.

The classification of the variants was carried out according to the American College of Medical Genetics and Genomics (ACMG) pathogenicity classification guidelines [7]. For this, we relied on the help of the Intervar software (http://wintervar.wglab.org)

Results

Patients characterization

Twenty-five patients, belonging to 20 unrelated families with at least one CCD affected patient were studied. Four families presented more than one patient, three with autosomal dominant (P9, P16 and P18 - Index patient from each family is identified as P#) and one with autosomal recessive inheritance (P8- two affected sibs), and 16 patients were isolated cases. Consanguinity among parents was present in only one family (P11).

Molecular analysis

Molecular screening for variants included the application of several filters of frequency and genes selection. 23 different variants in the *RYR1* gene were identified: 22 of them were missense, and one, a frameshift mutation. In only one patient (P20), no mutation in the *RYR1* gene was identified. Therefore, it was possible to molecularly characterize 19 of the 20 families. The majority of the variants, 21 of them, were unique, each family present a different variant. However, two variants were present in two families: p.Arg4861His was found in patients P12 and P13; variant p.Arg4861Cys, in patients P7 and P14. In addition, different variants were found in the same codon, such as p.Arg4861His (P13), p.Arg4861Cys (P14), p.Arg4914Met (P18), p.Arg4914Thr (P19).

Among the 23 variants, 16 were previously described in patients with CCD, HM or congenital myopathies, while seven variants are being described for the first time in this manuscript (P2, P3, P4, P7, P9, P10 and P18). Among them, four were classified as pathogenic or likely pathogenic (P2, P9, P10, P18), two were classified as variant of uncertain significance (VUS) (P4, P7), and one was considered as likely benign (P3). Therefore, molecular diagnosis with pathogenic mutations in the *RYR1* gene could confirm the diagnosis in 16 of the families. It is important to note that two among the three VUS were accompanied by a pathogenic mutation in the other allele.

The distribution of the variants along the coding sequence of the *RYR1* gene showed a predominance of CCD patients with mutations in the C-terminal domain in exons 94-102: P2, P4, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18 and P19. One variant was localized in the N-terminal region in exon 2 (P1), and three in the central region of the gene, in exons 35 (P3), exon 66 (P5) and exon 73 (P6).

Monoallelic heterozygous variants were found in 12 patients, while patients with mutations in both alleles were identified in seven cases: one (P11) was homozygous for the same mutation (and the parents are consanguineous), five patients (P1, P2, P4, P7, and P8) were compound heterozygous. In one family (P18) with autosomal dominant pattern of inheritance, the two mutations in exon 102 segregate together in cis in the same allele. Therefore, biallelic cases constitute 6/20 of our cohort, or 30% of the cases.

Segregation analysis confirmed parental segregation of the mutation/s in families 1, 2, 8, 11, 16 and 18.

Histological analysis were possible in nine of the patients (Tab. I), and all presented visible cores with a predominant frequency of affected fibers - 80% in 7/9 cases (Fig. 1). In addition, four patients presented a big, unique and structured core (P4, P7, P10 and P12), while five patients showed non-structured cores, both small, multiple

and unique large cores inside muscle fibers (P1, P3, P5, P11 and P17) (Tab. II).

In one patient (P7), a severe clinical phenotype was associated to biallelic mutation in the *RYR1* gene, and a histopathological pattern on muscle biopsy showing very severe muscle degeneration and connective tissue replacement. However, in the remaining muscle fibers, big unique or multiple cores could be observed (Fig. 2).

Discussion

Central Core Myopathy (CCD) is caused predominantly by mutations in the *RYR1* gene, which is a huge gene composed by 106 exons. More than 450 different mutation causing disease were identified along the coding sequence of the gene, which makes the molecular screening through Sanger Sequencing methodology difficult, expensive and time consuming. For this reason, for many years, the screening was done focusing on three enriched hotspot regions: N-terminal region 1, amino acids 35-614; central region 2, amino acids 2163-2458; and C-terminal region 3, amino acids 4550-4940. Region 1 and 2 variants are predominantly associated with the MH susceptibility phenotype and region 3 variants with the classic CCD phenotype [5].

RYR1-related congenital myopathies present a significant genetic heterogeneity and the increasing utility of next generation sequencing (NGS) approaches to variant identification, coupled with reduction in sequencing cost, has enlarged the access to this methodology worldwide. Therefore, to screen patients for pathogenic variants using NGS sequencing of the entire *RYR1* gene rather than only the three hotspots is now considered the best practice. In fact, using this new approach, we were able to identify mutations in the *RYR1* gene in 19 of the 20 tested families, confirming the utility of this powerful molecular tool.

Allelic heterogeneity

The large number of variants identified in the *RYR1* gene in patients with CCD has shown the occurrence of significant molecular variability, constituting the vast majority of particular mutations for each family, with only 10% of the variants in *RYR1* being functionally characterized [5]. This fact was also confirmed in our patients by the number of different variants found: a total of 23 different variants in 19 families. 20 of these variants were particular mutations. In addition, seven novel variants were identified in our patients, suggesting that the number of variants with possible clinical significance in this gene may increase with more studies using new molecular technologies.

Some mutations have been frequently described in different populations, such as p.Arg4861Cys (Davis et al., 2003 - LOVD: 12 reports), p.Arg4861His (Monnier et al., 2001 - LOVD: 16 reports), and p.Arg614Cys (Gillard et al. [8] - LOVD: 43 reports). In Brazilian patients, we also identified these variants, which were even present in more than one unrelated patient. We identified three recurrent mutations, each one in two unrelated families: p.Arg4861Cys in families P7 and P14, p.Arg4861His in families P12 and P13, and p.Arg4914Met or Thr in families P18 and P19. These mutations were located in hotspot of exon 101 or 102 of the *RYR1* gene, and also already described in other families [9-11].

The distribution of variants in the *RYR1* gene showed that fifteen variants localized in the C-terminal region (exons 94-104); two were located in the N-terminal re-

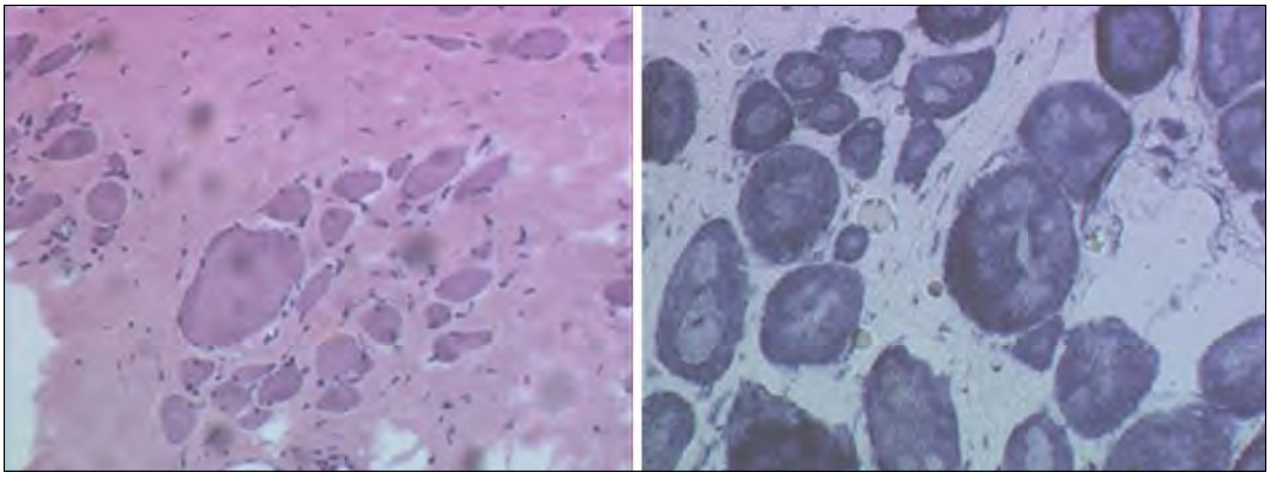

Figure 1. Examples of type of cores observed in the patients: big and structured cores in almost all fibers in P4, and in less fibers in P10, few small and less structures cores in P11 and P3.

Table I. Genetic data of the 20 studied families.

Fam	I / F	Consang	Exon	Mutation (segregation)	CADD/ Phred	Classification	References
1	I	N	2	c.122 T > C:p.(Phe41Ser)	24.7	Likely pathogenic	Klein et al., 2011 [18]
			25	c.3362 C > G:p.(TyrY1121Cys) (maternal)	25.5	Likely pathogenic	Wilmshurst et al., 2010 [19]
2	I	N	**17**	c.C1840T:p.(Arg614Cys)	28.8	Pathogenic	Gillard et al., 1991 [8]
			104	c.14938_14939del:p.(Thr4980Ala*fs) (maternal)	-	Pathogenic	this MS
3	I	N	**35**	c.5723 A > G:p.(Lys1908Arg)	22.3	Likely benign	this MS
4	I	N	**46**	c.7433 C > A:p.(Thr2478Asn)	18.66	Uncertain significance	this MS
			94	c.13703 T > C:p.(Leu4568Pro)	29.6	Pathogenic	Wu et al., 2006 [12]
5	I	N	**66**	c.9758 T > C:p.(Ile3253Thr)	23.8	Uncertain significance	Böhm et al 2013 [22]
6	I	N	**73**	c.10747 G > C:p.(GluE3583Gln)	18.39	Uncertain significance	Robinson et al., 2006 [23]
7	I	N	**79**	c.11321 C > T:p.(Ala3774Val)	25.2	Uncertain significance	this MS
			101	c.14581 C > T:p.(ArgR4861Cys)	32	Pathogenic	Davis et al., 2003 [9]
8	F - AR	N	94	c.13673 G > A:p.(Arg4558Gln) (maternal)	32	Pathogenic	Kossugue et al., 2007 [4]
			101	c.14537 C > T:p.(Ala4846Val) (paternal)	25.8	Likely pathogenic	Gambelli et al., 2007 [21]
9	F- AD	N	**95**	c.13952 A > G:p.(His4651Arg)	25	Likely pathogenic	this MS
10	I	N	**100**	c.14411 A > C:p.(His4804Pro)	26.9	Likely pathogeinc	this MS
11	I	Y	**101**	c.14545 G > A:p.(Val4849Ile)	24.5	Pathogenic	Jungbluth et al., 2002 [16]
			101	c.14545 G > A:p.(Val4849Ile)	24.5	Pathogenic	Jungbluth et al., 2002[16]
12	I	N	**101**	c.14582 G > A: p.(Arg4861His)	32	Pathogenic	Monnier et al., 2001 [10]
13	I	N	**101**	c.14582 G > A: p.(Arg4861His)	32	Pathogenic	Monnier et al., 2001 [10]
14	I	N	101	c.14581 C > T:p.(ArgR4861Cys)	32	Pathogenic	Davis et al., 2003 [9]
15	I	N	**101**	c.14677 C > T:p.(Arg4893Trp)	29.9	Pathogenic	Monnier et al., 2001 [10]
16	F-AD	N	**102**	c.14690 G > T:p.(Gly4897Val) (affected father)	29.2	Pathogenic	Kossugue et al., 2007 [4]
17	I	N	**102**	c.14693 T > C:p.(Ile4898Thr)	27.9	Pathogenic	Lynch et al., 1999 [24]
18	F-AD	N	**102**	c.14763 C > G:p.Fen4921Lys (affected mother)	25.4	Pathogenic	Todd et al., 2018 [11]
			102	c.14741 G > T:p.(Arg4914Met) (affected mother)	31	Pathogenic	this MS
19	I	N	102	c.14741 G > C:p.(Arg4914Thr)	28.7	Pathogenic	Davis et al., 2003 [9]
20	I	N		No mutations in RYR1			

Including inheritance (I- isolated case, F – familial case, AD – autosomal dominant inheritance, AR – autosomal recessive inheritance, Consanguinity in the parents- Y-yes, N-no, Exon with the mutation, description of the mutation using the NM_000540 transcript, the CADD (Combined Annotation Dependent Depletion) score for variation, the classification of the mutation according to the ACMG guidelines, and the references for the mutations previously described

Table II. Data on muscle biopsies: type of cores, proportion, and distribution inside the muscle fiber.

Patient	Exon mutation	% Fibers with cores	Number of cores	Type of core	Position of cores
P1	2/25	96	Few small	Less structured	Central
P3	35	86	Few small	Less structured	Peripheral
P4	46/94	99	Big unique	Structured	Central
P5	66	52	Big unique	Less structured	Central
P7	19/101	99	Big unique	Structured	Central
P10	100	84	Big unique	Structured	Peripheral
P11	101/101	41	Few small	Less structured	Peripheral
P12	101	100	Big unique	Structured	Central
P17	102	100	Few small	Less structured	Peripheral

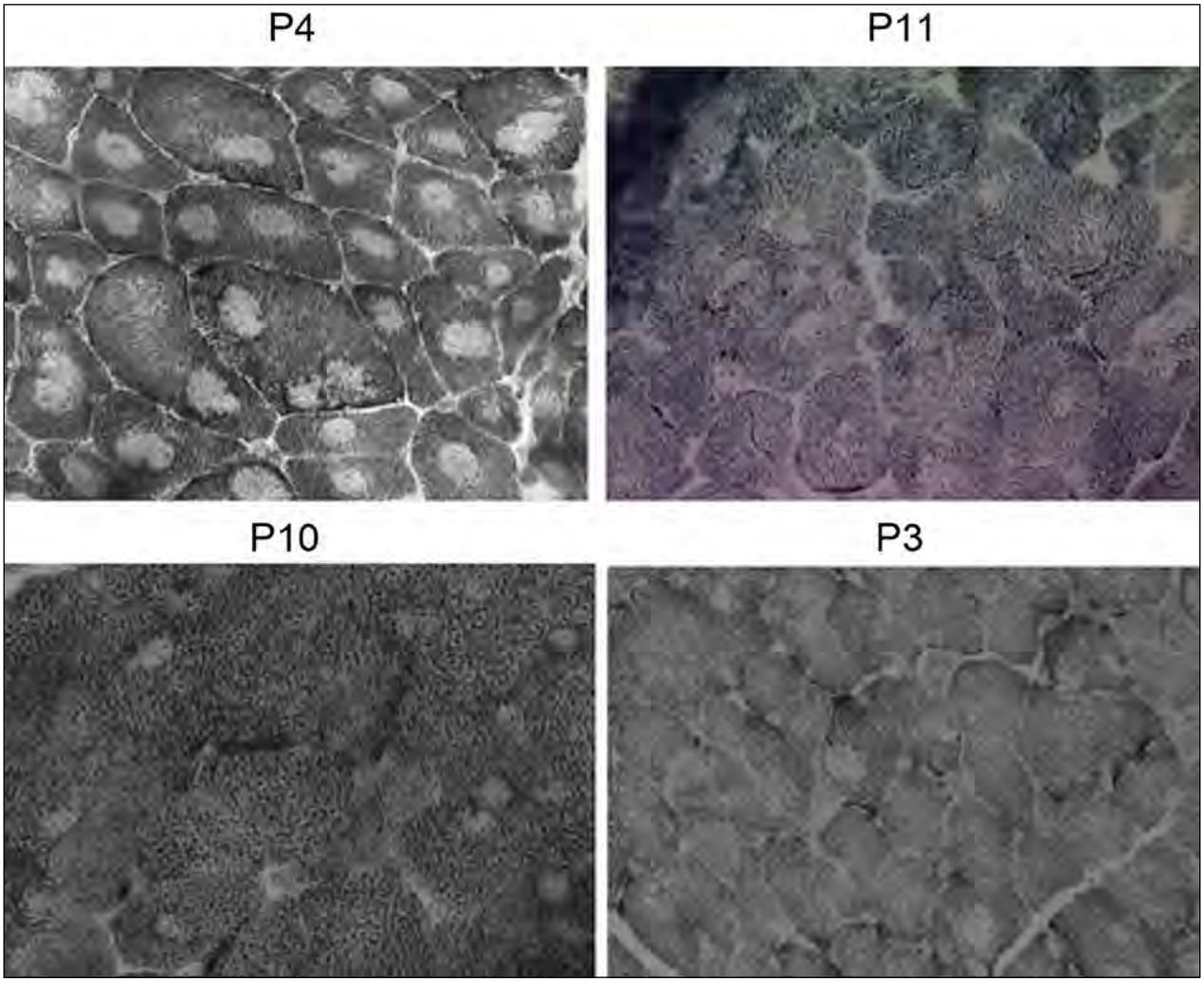

Figure 2. Histological characterization of patients P7: A) HE staining illustration massive muscle degeneration with a few variable muscle fibers in a massive connective tissue replacement; B) NADH staining showing the presence of unique or multiple large and structured-like cores in the remaining muscle fibers. Amplification: X 400.

gion (exons 2 and 17); one was found in the central region of the protein (exon 46); and five variants were distributed between the N-terminal and central region of the *RYR1* sequence which is not in the typically studied regions (Tab. I). This illustrates how the extension of the screening is improving the identification of more muta-

tions in this gene. But, the predominance of mutations in the C-terminal region continues higher [9,10,12].

As summarized by Fusto [2], two main pathological mechanisms are triggered by *RYR1* defects according to which protein domains are affected. Mutations in hotspots one and two lead to channel hyperactivity and an early release of Ca2+ from the sarcoplasmic reticulum [13]. Another consequence is the reduction of the threshold required for channel activation. All these effects together result in lack of a fine control of the channel, as it can be activated easier and longer than it is supposed to be. Patients harboring mutations in these hotspots are under susceptibility of Malignat hyperthermia syndrome [5]. The second pathological mechanism is associated with mutations in hotspot three that causes a defective coupling between membrane depolarization and calcium release from the SR [13]. Further evidence and the identification of novel *RYR1* mutations along the gene are showing that the pathological mechanism is not dependent only on mutation positioning. A study by Dirksen and Avila [14] reported the effect of mutations in hotspot two of the gene causing a channel that is both hypersensitive to agonist and voltage activation and has its basal activity increased.

Type of mutation

Of the approximately 460 already identified mutations in *RYR1* (HGMD) 399 or ~86% were point mutations (missense/nonsense); 23 were mutations in splicing sites; 26 were small deletions; 9 were small inserts; 4 were small indels; 3 were large deletions; and 2 were major insertions and duplications. Accordingly, we also identified in our patients a predominance of missensse variants, constituting 22 of the 23 variants found in the Brazilian patients with CCD. One of our patients presented a frameshift mutation, but it was associated to a second previously described p.Arg614Cys mutation [8], in the other alelle (P2). In a study by Wu et al., (2006) [12], missensse mutations were found in 25 of the 27 studied patients with CCD. In fact, loss of function mutations, which lead to the absence of *RYR1* expression in the muscle, are incompatible with life, as demonstrated by Takeshima et al. [15], in a mouse knockout model for the *RYR1* gene. In the absence of the protein, the mouse presents perinatal death with gross muscle abnormalities. This study also showed that *RYR1* is essential both for muscle maturation and for E-C coupling to occur since homologous proteins to RYR1 like RYR2, (which is also expressed in muscle) do not have the ability to replace the effect caused by the absence of protein, leading to defects in the release of intracellular Ca2+ ions. The function of the RYR1 protein would therefore be essential in skeletal muscle during E-C coupling and could not be replaced by other receptor subtypes. This fact could explain the predominance of *RYR1* missense mutations causing disease.

Biallelic mutations

The classic form of CCD has been classified as autosomal dominant inherited disease. However, there are several reports in the literature of patients with autosomal recessive inheritance pattern [4,12,16,17]. In the present study, patients with biallelic mutations constitute 30% of the cases, compatible with the finding of 12/47 or 25% of the cases described by Todd et al. [11], illustrating how the recent introduction of studies using NSG approaches for the *RYR1* gene sequencing can help to identify more cases with biallelic mutations. Therefore, the autosomal recessive form of CCD would be more common than expected, as already proposed in a previous study by our group [4].

It is also possible that other mutations, previously found in individuals and considered non-pathogenic, are responsible for muscle weakness if present in compound heterozygosis or in homozygosis, as observed in P11, a 9-year-old affected girl with delayed neuropsychomotor development and difficulties to run and climb stairs. Molecular analysis identified the homozygous mutation p.Val4849Ile in exon 101 of the *RYR1* gene. The consanguineous non-affected parents were both heterozygous for this mutation. Muscle biopsy of P11 showed non-structured cores, both small and large inside about 40% of muscle fibers. In fact, this mutation was already described [16] in homozygous in a patient with MmD and a similar pattern of cores in the muscle biopsy.

Regarding the other five patients with biallelic mutataions, four presented at least one of the variants localized in the C-terminal hotspot domain of *RYR1* gene.

Interestingly, only two among the six cases showed a severe phenotype of CCD. In addition to P11, already discussed, the other patients are described bellow:

- **Patient P1,** a 4-year-old girl, an isolated case in the family, was diagnosed in early childhood, with hypotonia, and late deambulation, at 3 years of age. On muscle biopsy, she presented small multiple unstructured cores. Molecular analysis identified two variants in the *RYR1* gene, previously described as pathogenic, p.Phe41Ser and p.Try1121Cys, in exons 2 and 25 (this one, inherited from the mother). Interestingly, these two variants have been previously reported [18,19] causing the CCD phenotype also in compound heterozygosis: p.Phe41Ser was combined with p.Thr3933Cys variant [18] and p.Tyr1121Cys variant was in combination with p.Leu2689Ala variant [19]. These two variants are not in the C-terminal domain, usually involved in CCD, which could explain the need for both variants for the occurrence of the phenotype;
- **P2**, an 11-year-old girl, also has two distinct variants, in compound heterozygosis. One variant, p.Arg614Cys, was previously described [8] related to HM.

The second is a novel frameshift deletion in exon 104, p.(Thr4980Ala*fs), in the C-terminal region of the protein, inherited from the mother, is predicted to be pathogenic. The patient has a severe clinical picture of CCD, requiring ventilatory support at birth, delayed neuropsychomotor development in the first two years of life, and acquisition of gait after the age of 2 years old. At the age of 11, she presents myopathic facies, severe hypotonia and scoliosis. The pathogenic variant p.(Thr4980Ala*fs) alone could justify a clinical picture of CCD, as it is located in the C-terminal region of the protein and removes it from the reading frame. However, the mother - non affected – was carrier of the same mutation. On the other hand, the p.Arg614Cys variant was also described as pathogenic (HM susceptibility). Further studies would be necessary to assess whether the combination of the two variants present in the patient would have an additive effect and, in turn, to clarify the presentation of a more severe clinical picture;

- **Patient P4** has two variants, p.Thr2478Asn and p.Leu4568Pro in compound heterozygosis. The p.Leu4568Pro variant was previously described in a CCD patient [12]. The novel p.Thr2478Asn variant is in the central region of the protein and leads to an exchange in the threonine residue that is moderately conserved. This variant is present in low frequency in population databases (rs141298868, gnomAD MAF: 0.00002), but was not found in the literature in patients with RYR1-related disease. Algorithms of prediction are conflicting about the potential impact of this variant, ranging from benign (PolyPhen-2) to Disease causing (Mutation taster), and was classified as VUS. The clinical comparison between the patient described by Wu et al. [12] with our patient with biallelic mutations showed that the presence of the second variant does not seem to aggravate the clinical condition of our patients, since currently at 35 years of age, despite her weakness with difficulties to perform tasks like climbing stairs, she has a normal life and can even drive a car. Her muscle biopsy, on the other hand, is typical of CCD with large unique and structured cores in all muscle fibers;
- **P7** is a severely affected girl, which was unable to walk up to 5 years of age. She was a very hypotonic baby, requiring mechanical respiration support after birth. On muscle biopsy, she presented a very atypical dystrophic pattern of muscle degeneration, with scarce fibers surrounded by connective tissue. The isolated scarce fibers showed large and structured cores inside. She also presented two variants, p.Ala3774Val and p.Arg4861Cys in compound heterozygosis. The second variant was described [9] related to CCD in heterozygous state. The p.Ala3774Val still was not found in the literature in patients with RYR1-related disease, and showed very low frequency in the normal population. It was predicted to be potentially damaging. However, the presence of the first described mutation would be sufficient to cause the phenotype on this patient, since other patients with the same mutations presented also a severe phenotype with neonatal hypotonia, lordosis, severe weakness with inability to walk unassisted at the age of 11years old [20];
- **P8** is a 44-year-old female, with a 42-year-old affected brother, with clinically normal non-consanguineous parents. Both presented clinical history of slowly progressive weakness with frequent falls. In this family, two previously described pathogenic variants were found, involving the C-terminal region of the RYR1: p.Ala4558Gln in exon 94 [4] and p.Ala4846Val on exon 101 [21], inherited from the mother and father, respectively. These results corroborate the recessive biallelic pattern in this family. Therefore, according to the criteria used, both variants were classified as probably pathogenic.

Histopathological alterations

We could observe certain variability in our histopathological findings regarding the core pattern in the Brazilian CCD patients. Although a typical pattern of unique and well-structured core in practically all fibers was only found in patients with at least one mutation in the C-terminal region of the protein (P4, P7, P10 and P12), two patients with mutations in this region presented a pattern of few small and non-structured cores (P11 and P17), suggesting that it is not a mandatory pattern.

In summary, 20 families of patients with CCD were evaluated using NGS methods, and we identified 23 variants (7 novel) in the *RYR1* gene in 19 of them, confirming the pathogenicity in 16 cases. Most of these variants (22/23) were missense mutations and 20 of them were unique in families. Two variants were recurrent in two families. We also identified six families (five non-consanguineous) with biallelic variants resulting in 30% of the cases with a possible pattern of AR inheritance. In one family with AD inheritance, we identified two mutations in exon 102, segregating in cis, suggesting an additive effect of two pathogenic variants in the same allele.

This work highlights the importance of using Next-Generation Sequencing technology for the molecular diagnosis of genetic diseases when a very large gene is involved, associated to a broad distribution of the mutations along it. These data also influence the prevention through adequate genetic counseling for the families and cautions against malignant hyperthermia susceptibility.

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The changing spectrum of drug-induced myopathies

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Drug-induced myopathies are a group of disorders whose importance lies in the fact that they are potentially treatable and usually reversible if the causative agent is identified and withdrawn. A wide variety of medications used in many different branches of medicine have been recognised as causing muscle adverse effects, ranging from myalgia and asymptomatic hyperCKaemia to severe weakness and at times fatal rhabdomyolysis. There has been increased awareness of these complications since the introduction of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor group of drugs (statins) in the 1980s, and their subsequent association with a range of necrotising and immune-mediated inflammatory myopathies and muscle symptoms. More recently, since the introduction of the immune checkpoint inhibitors for the treatment of advanced malignancies, it has been increasingly recognised that these drugs also have a propensity to induce or exacerbate a variety of immune-mediated myopathies, neuropathies, myasthenic disorders and atypical overlap syndromes, and it is anticipated that these complications will become even more prevalent with increasing use of these medications in the future. This review focusses mainly on these two groups of drugs, and on cytokine-based therapies and VEGF inhibitors which have also been implicated in the induction of immune-mediated inflammatory myopathies.

Key words: drug-induced myopathies, statins, checkpoint inhibitors, immune-mediated

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Introduction

Drug-induced myopathies are an important group of iatrogenic disorders that may present in various ways, and are potentially reversible if the causative agent is identified and withdrawn. Many drugs used in different branches of medicine are known to have adverse effects on the skeletal muscles. These vary in severity, ranging from mild myalgia, cramps, and asymptomatic serum CK elevation, to a severe disabling myopathy with associated muscle pain, progressive muscle weakness and atrophy. While severe forms of drug-induced myopathy are uncommon, milder forms are probably more frequent than is appreciated. Some of the earliest drugs recognised to cause myopathy included corticosteroids, emetine, clofibrate, chloroquine, D-penicillamine, ε-aminocaproic acid and heroin [1]. With the continued expansion in the range of therapeutic agents, new myotoxic drug effects were recognised and the spectrum of drug-induced myopathies has continued to expand. Following the introduction of the first statin, lovastatin, in 1987, the myotoxic potential of this group of drugs was soon recognised, particularly after the introduction of cerivastatin in the late 1990s which caused over 100 fatal cases of acute rhabdomyolysis, leading to its withdrawal from the market in 2001. With the introduction of new statins, and their use by many millions of people for the primary and secondary prevention of vascular disease, the range of recognised myopathic effects associated with these drugs has grown, and they are now recognised as be-

ing the pre-eminent therapeutics associated with drug-induced myopathies in the modern era. More recently, other new groups of drugs have also been implicated, including cytokine-based therapies and anti-neoplastic therapies, in particular the group of immune checkpoint inhibitors which have been introduced for the immunotherapy of advanced malignancies.

Pathological mechanisms of drug-induced myopathies

As shown in Table I, the spectrum of pathological reactions induced by drugs in skeletal muscle is very broad, encompassing necrotising, inflammatory, mitochondrial, autophagic, myofibrillar and microtubular myopathies. The most basic reaction is myonecrosis, which typically occurs in statin myopathy, and less frequently with a variety of other therapeutic agents, as well as alcohol and drugs of addiction, and which in its most extreme form results in the syndrome of acute rhabdomyolysis. The underlying molecular mechanisms which lead to myonecrosis have yet to be fully investigated, but are thought to involve effects on the sarcolemma, intracellular calcium kinetics, mitochondrial function and muscle fibre bioenergetics [2,3]. Over the past two decades there has been increasing recognition of the potential of certain drug classes, in particular statins and a number of immune therapies to induce a necrotising autoimmune myopathy (NAM), as well as other types of inflammatory myopathy.

Statin-associated myopathies

Statins have been associated with a wide range of muscle disorders, as shown in Table II. The mildest symptoms include myalgia and muscle cramps, which have variably been reported to occur in as many as 10% of individuals taking a statin. However, because of the nonspecific nature of these symptoms, the frequency of a causative link with the statin has probably been over-estimated in many surveys. The incidence of severe necrotising myopathy and rhabdomyolysis requiring hospital admission is generally agreed to be very low (~1 in10,000 treated individuals) [4], and varies with different classes of statin drug. Risk factors for developing a myopathy include the type of statin, with the lipophilic drugs such as atorvastatin, lovastatin and simvastatin having a higher risk of myopathy than the hydrophilic compounds pravastatin and fluvastatin. Other risk factors include high drug doses, physical exercise, major surgery, co-administration of interacting drugs such as CYP3A4 enzyme inhibitors and fibrates, and other co-morbidities [2]. There is also evidence of a genetic predisposition to statin toxicity, with polymorphisms in the *SLCO1B1* and *COQ2* genes, and variants in the CYP enzyme system being associated with an increased risk of developing myopathy [2,5]. Asymptomatic carriers of mutations in the myophosphorylase, α-glucosidase, carnitine palmitoyltransferase-2, myoadenylate deaminase genes, and MELAS mutations, also have an increased risk of developing a myopathy when taking statins [2].

Statin myopathy is generally self-limiting if the causative drug is withdrawn as soon as possible after the onset of symptoms. The natural history of the myopathy is of improvement over a period of several weeks to months, depending on the initial severity of the muscle symptoms. However, recovery of full muscle strength is usually slow in cases of severe rhabdomyolysis and may take several months. When the symptoms fail to improve or continue to progress after withdrawal of the statin, the possibility of an immune-mediated myopathy initiated by the statin, or of an undiagnosed pre-existing myopathy need to be considered, and further investigations including a myositis autoantibody screen and a muscle biopsy are required.

Immune-mediated myopathies

A shown in Table III, a number of therapeutic agents have been associated with the development of an immune-mediated inflammatory myopathy. In particular, statins and more recently the immune checkpoint inhibitors have been implicated in causing multiple cases of necrotising autoimmune myopathy (NAM), as well as polymyositis and dermatomyositis and other autoimmune diseases. The underlying immunopathological mechanisms in such cases have yet to be fully elucidated, but

Table I. Pathological mechanisms of drug-induced myopathy.

• Necrotising myopathy/rhabdomyolysis (statins, fibrates, alcohol, heroin)
• Immune-inflammatory myopathies (statins, α-interferon, TNFα inhibitors, check-point inhibitors, bevacizumab)
• Mitochondrial myopathy (antiretrovirals, statins, clevudine)
• Lysosomal/autophagic myopathies (chloroquine, hydroxychloroquine, amiodarone)
• Microtubular myopathies (colchicine, vincristine)
• Myofibrillar myopathies (emetine, acute quadriplegic myopathy)
• Catabolic myopathy with type 2 fibre atrophy (corticosteroids)

Table II. Spectrum of statin-induced neuromuscular disorders.

• Necrotising myopathy/rhabdomyolysis
• Immune-inflammatory myopathies – Necrotising autoimmune myopathy (NAM) – Polymyositis/dermatomyositis
• Mitochondrial myopathy
• Unmasking of pre-existing metabolic myopathy
• Myasthenia gravis
• Axonal polyneuropathy

Table III. Drugs associated with induction of immune-mediated myopathies.

• HMGCR-inhibitors (statins)
• Immune checkpoint inhibitors
• Cytokine therapies (interferon-α/β. TNFα blockers)
• Bevacizumab
• D-penicillamine
• Others: tryptophan, procainamide, leflunomide

are thought to involve both humoral complement-dependent antibody mechanisms on the one hand, and T-cell mechanisms on the other.

Statin-associated necrotising autoimmune myopathy (NAM)

Necrotising autoimmune myopathy (NAM) is one of the most common forms of immune-mediated inflammatory myopathy and has multiple causative associations (Tab. IV). Statin-associated NAM is well-documented and is characterised clinically by progressive subacute muscle weakness, very high serum CK levels (> x10), and the presence of circulating autoantibodies to 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), which is the pharmacological target of the statin drugs [4]. Symptoms may develop shortly after commencement of a statin drug, but more often develop in patients who have been on a statin for a number of years. Muscle pathology studies usually show evidence of disseminated polyphasic myonecrosis and regeneration, with an absent or sparse inflammatory infiltrate, comprising predominantly macrophages and small numbers of CD4 and CD8 T-cells. Immunohistochemistry shows diffuse expression of MHC-I antigen in muscle fibres (Fig. 1), together with sarcolemmal deposition of the complement membrane-attack complex (C5b-9) on non-necrotic muscle fibres, in keeping with a complement-dependent antibody mediated mechanism of muscle injury [4,6].

Table IV. Associations of necrotising autoimmune myopathy (NAM).

• Anti-HMGCR antibodies – Statin therapy – Statin-naïve
• Anti-signal recognition antibodies
• Anti-synthetase antibodies
• Malignancy
• Viral infections

The condition is estimated to affect ~2-3 per 1000 individuals taking statins, but it is also recognised that about one-third of cases of NAM with anti-HMGCR antibodies have not had previous exposure to a statin [7]. Because of this, and because of the large number of individuals over the age of 50-65 years who are taking statins, some doubt has therefore been cast on the causative role of statins in NAM, and it has been suggested that the association could be coincidental [8]. The possibility that individuals affected by NAM may have a genetic predisposition is suggested by the finding that the Class II HLA allele DRB1*11:01 is strongly associated with the development of anti-HMGCR autoantibodies, even in the absence of previous statin exposure [4]. Further confirmation is required to support the hypothesis that statins can initiate an autoimmune myopathy in genetically susceptible individuals, by leading to over-expression of HMGCR in muscle fibres, and loss of immune tolerance [4].

The natural history of anti-HMGCR associated NAM is of continued progression even after withdrawal of the statin, although some milder cases may improve spontaneously. In the majority of cases, however, aggressive treatment with prednisone in combination with an immunosuppressive agent such as azathioprine, methotrexate or mycophenolate is required, and is often effective in controlling the disease. However, in cases with more severe weakness, or who are not responsive to corticosteroids and immunosuppressants, a third-line agent such as intravenous immunoglobulin or rituximab may need to be added to achieve control of the myopathy.

Immune checkpoint inhibitors

During the past 6 years there have been increasing reports of a spectrum of neuromuscular and other neurological complications of treatment with immune checkpoint inhibitors (ICPIs) in patients with advanced malignancies such as metastatic melanoma, non-small cell lung cancer, genitourinary and gastrointestinal malignancies, and Hodgkin's lymphoma [9-11]. These drugs work by blocking the co-stimulatory molecules on T cells (CTLA-4 and PD-1, or its ligand PD-L1), thereby allowing them to exert their cytotoxic effects on tumour cells. However, the resulting upregulation of the immune response can result in initiation of a variety of autoimmune disorders, or aggravation of pre-existing disorders such as myasthenia gravis or multiple sclerosis, which

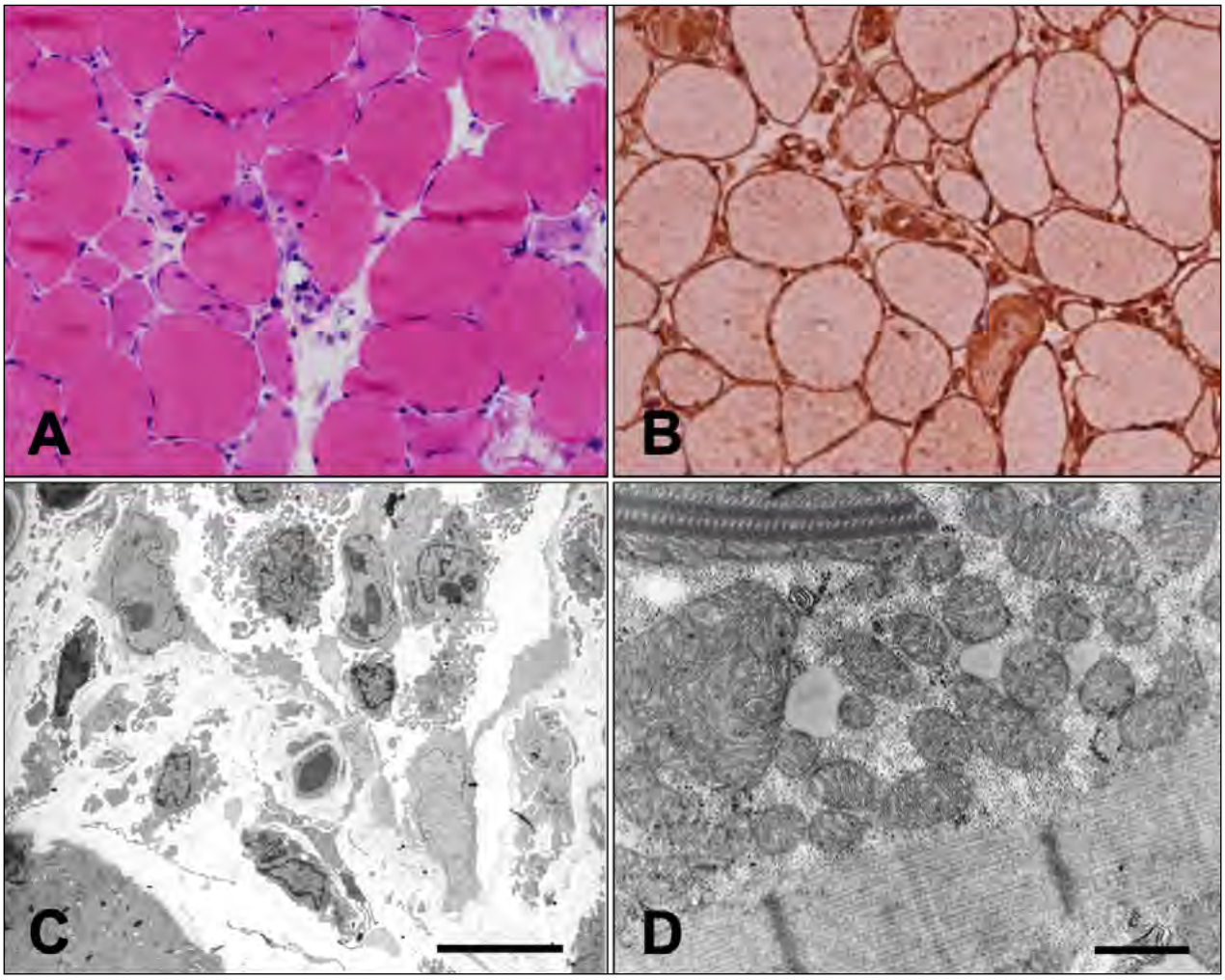

Figure 1. A) Statin-induced necrotising myopathy (H&E x40); B) Widespread sarcolemmal and sarcoplasmic MHC-I expression in case of statin-induced NAM (x40); C) Electron micrograph showing interstitial mononuclear cellular infiltrate in a case of statin-induced NAM (Bar 10 µm); D) Electron micrograph showing mitochondrial pleomorphism and paracrystalline inclusions in a case of statin-induced myalgia and exercise intolerance (Bar 1µm).

may previously have been subclinical and undiagnosed. A variety of ICPIs are now available to treat different malignancies, and include ipilimumab, which targets CTLA-4; pembrolizumab, novolumab and cemiplimab that block PD-1; and atezolizumab, avelumab and durvalumab that are PD-L1 inhibitors [11]. These drugs can be used either on an individual basis, or in combination to improve the chances of an effective response to the tumour.

The range of neuromuscular disorders which have been reported in patients treated with ICPIs is shown in Table V, and include myasthenia gravis, immune-mediated necrotising and inflammatory myopathies and polyneuropathies, as well as radiculopathies and mononeuropathies. The overall incidence of these complications is thought to be less than 1% with the ICPs in current use, and is higher when CTLA-4 and PD-1/PD-L1 inhibitors are administered in combination. Although the incidence of these disorders is low, it is still much higher than in the general population, and it is predicted that it will continue to rise with increasing use of ICPs for different types of malignancy [9].

A review of 22 reported cases of ICPI-associated myopathies by Puwanant and colleagues [10] in 2019, revealed that the onset was usually within the first 2-3 months after commencement of treatment, and that the clinical phenotype was quite variable. While the phenotype may resemble classical presentations such as NAM, polymyositis or dermatomyositis, atypical features such as oculo-bulbar

symptoms and co-existing myocarditis were quite common. In addition, overlap syndromes including myopathy with associated myasthenia or neuropathic features were also not uncommon. Orbital myositis has also been reported [12]. Over 50% of cases improved following discontinuation of ICPIs and treatment with varying combinations of corticosteroids, immunosuppressive agents, IVIG and plasma exchange, but there was a 27% mortality [10].

The immunopathological mechanisms which underlie these diverse autoimmune complications of ICPI therapy remain to be investigated, but are presumed to involve induction of autoreactive T-cells and stimulation of autoantibody production as a result of removal of co-stimulatory molecule control over the immune response. These effects are likely to be further enhanced in individuals with a pre-existing autoimmune disorder, with resulting deterioration or unmasking of the underlying condition. ICPIs are thought to enhance Th1 and Th17 cell responses and have effects on regulatory T-cell function, resulting in a shift in the T-reg/Th17 balance, favouring the development of autoimmunity [9]. The possibility that genetic variants in CTLA-4 may predispose certain individuals to develop autoimmune disorders when treated with an ICPI has also been suggested [9].

Cytokine-based therapies

It is important to be aware of the possibility that Type 1 interferons may induce an inflammatory myopathy or other autoimmune conditions when they are used therapeutically for the treatment of immune disorders, malignancies or chronic infections although this occurs only rarely [13]. There have been a number of reports of the onset of polymyositis or dermatomyositis, as well as other autoimmune disorders, in patients treated with interferon-α2 for hepatitis C or certain malignancies [12]. Moreover, the onset of severe dermatomyositis has been reported in a patient with multiple sclerosis who had been treated with interferon-β for 5 years [14].

A number of TNFα-blockers have also been associated rarely with the development of dermatomyositis or polymyositis when used for the treatment of rheumatoid arthritis or other types of arthropathy, or inflammatory bowel disease. In a review of 24 such cases by Zengin and colleagues [15], there were 12 cases of polymyositis and 12 of dermatomyositis, and the drugs implicated included etanercept (10), infliximab (5), adalimumab (5) and lenercept (2). In the majority of these patients the myositis developed in spite of the fact that they were on methotrexate as a disease-modifying therapy, and improved with immunotherapy.

VEGF inhibitors

Bevacizumab is a recombinant humanized monoclonal antibody that inhibits angiogenesis by binding circulating vascular endothelial growth factor (VEGF), thereby inhibiting its binding to cellular receptors, and is used in the treatment of non-small cell lung cancer, colon and renal cancer, glioblastoma multiforme and other malignancies. Although not identified in RCTs, there have been occasional reports of rhabdomyolysis developing after commencement of treatment with the drug [16], as well as reports of inflammatory myopathy [17] (Oflazer P, personal communication). There have also been reports of rhabdomyolysis associated with administration of sunitinib and erlotinib which are also VEGF inhibitors [18]. The mechanism of the myopathy with these drugs has not been investigated and it remains to be determined what role immune-mechanisms play.

Table V. Neuromuscular complications of immune checkpoint inhibitors.

• Myopathies – Necrotising myopathy – Polymyositis – Dermatomyositis – Nonspecific myopathy – Orbital myositis
• Myasthenia gravis
• Neuropathies – Guillain-Barré syndrome – Chronic inflammatory demyelinating polyneuropathy – Polyradiculopathy – Vasculitic neuropathy – Cranial neuropathies
• Overlap syndromes

Conclusions

The spectrum of drug-induced myopathies has changed quite markedly over the past 30 years, and is continuing to change with the introduction of newer therapeutic agents, particularly for the treatment of malignant disorders. It is likely that with the increasing use of ICPIs for the treatment of an increasing range of advanced malignancies, the prevalence of immune-mediated myopathies and other neuromuscular complications will continue to increase, and that, together with statins, ICPIs will become one of the major causes of drug-induced necrotising and immune-inflammatory myopathies and neuromyopathies. The underlying pathophysiology of these drug-induced disorders is still poorly understood, and further investigation is needed of the immunopathogenesis and mechanisms leading to the loss of immune tolerance.

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Inflammatory myopathies: update on diagnosis, pathogenesis and therapies, and COVID-19-related implications

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The inflammatory myopathies constitute a heterogeneous group of acquired myopathies that have in common the presence of endomysial inflammation. Based on steadily evolved clinical, histological and immunopathological features and some autoantibody associations, these disorders can now be classified in five characteristic subsets: Dermatomyositis (DM) Polymyositis (PM), Necrotizing Autoimmune Myositis (NAM), Anti-synthetase syndrome-overlap myositis (Anti-SS-OM), and Inclusion-Body-Myositis (IBM). Each inflammatory myopathy subset has distinct immunopathogenesis, prognosis and response to immunotherapies, necessitating the need to correctly identify each subtype from the outset to avoid disease mimics and proceed to early therapy initiation. The review presents the main clinicopathologic characteristics of each subset highlighting the importance of combining expertise in clinical neurological examination with muscle morphology and immunopathology to avoid erroneous diagnoses and therapeutic schemes. The main autoimmune markers related to autoreactive T cells, B cells, autoantibodies and cytokines are presented and the concomitant myodegenerative features seen in IBM muscles are pointed out. Most importantly, unsettled issues related to a role of autoantibodies and controversies with reference to possible triggering factors related to statins are clarified. The emerging effect SARS-CoV-2 as the cause of hyperCKemia and potentially NAM is addressed and practical guidelines on the best therapeutic approaches and concerns regarding immunotherapies during COVID-19 pandemic are summarized.

Key words: dermatomyositis, polymyositis, inflammatory myopathies, COVID-19

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Introduction

Inflammatory myopathies (IM) are a heterogeneous group of acquired myopathies that have in common the presence of inflammation in the muscle. Based on distinct clinical, histological, immunopathological and autoantibody features, they have evolved in five distinct subsets: Dermatomyositis (DM), Polymyositis (PM), Necrotizing Autoimmune Myositis (NAM), Anti-synthetase syndrome-overlap myositis (Anti-SS-OM), and Inclusion-Body-Myositis (IBM) [1-6]. Each subset has distinct clinical features, pathogenesis, response to therapies and different prognosis requiring careful clinicopathologic correlation with expertise in muscle histopathology for a correct diagnosis and distinction from disease mimics. The article describes the main clinicopathologic and immune features of all subtypes, highlights

how best to avoid erroneous diagnoses, and provides practical guidelines on therapeutic approaches.

Patients with all IM forms experience slow, subacute and rarely acute onset of difficulty performing tasks requiring the use of proximal muscles, such as climbing steps or getting up from a chair; patients with IBM however, may present first with weakness in the distal muscles of hands and feet and difficulties with buttoning, typing or raising toes and feet. Neck-extensor and pharyngeal muscles can be affected in all subsets resulting in difficulty holding up the head (head drop) and dysphagia. In advanced cases, respiratory muscles can be affected. Myalgia and muscle tenderness may also occur, most often in anti-SS-OM; if myalgia is prominent, a co-existent fasciitis should be considered. Extramuscular manifestations may occur in all IM, but rarely in IBM, and include arthralgia, Raynaud's phenomenon and pulmonary complications due to interstitial lung disease as seen in anti-SS-OM [1-6] or in amyopathic DM with anti-Melanoma Differentiation–Associated protein-5 [MDA-5] antibodies [1,7].

Clinical characteristics

Dermatomyositis (DM)

DM, seen in both children and adults, presents with characteristic skin manifestations accompanying or preceding muscle weakness. Periorbital heliotrope (blue-purple) rash with edema, erythematous rash on face, knees, elbows, malleoli, neck, anterior chest (in V-sign), back and shoulders (in shawl sign), and knuckles with a violaceous eruption (Gottron's rash) that evolves into a scaling discoloration, are typical skin lesions [1-8]. Dilated capillary loops at the base of the fingernails, irregular and thickened cuticles, and cracked palmar fingertips ("mechanic's hands") are also characteristic [1-4]. Subcutaneous calcifications, sometimes extruding to the surface, were common in our practice 20-30 years ago especially in children, as highlighted [8], but they are rarely seen today due to early initiation of effective immunotherapies. When DM is clinically limited to the skin (amyopathic dermatomyositis), the patients seem to have normal strength, but their muscle is always subclinically involved; based on our experience with a number of such patients we have biopsied, their muscle shows the typical features of DM described below but to a lesser degree [9]. In children, an early symptom is "misery," defined as an irritable child with red-flush on face, fatigue and reluctance to socialize [1-4]. Dermatomyositis may overlap with systemic sclerosis and mixed connective tissue disease and requires distinction from the anti-SS-OM subset. In adults with DM there is a malignancy risk in up to 15% of patients, especially in the first 3-5 years from the disease onset [1,8]. Common cancers are ovarian, breast, colon, melanoma, nasopharynx (in Asians) and non-Hodgkin lymphoma, necessitating a thorough annual work-up the first 3 years [10].

Polymyositis (PM)

PM is a very rare entity. In our experience most patients referred for PM have another disease most often IBM, NAM, or an inflammatory dystrophy [1-3]. Polymyositis does exist but remains a diagnosis of exclusion. It is best defined as a subacute proximal myopathy in adults who do not have rash, family history of neuromuscular disease, exposure to myotoxic drugs (d-penicillamine, zidovudine), involvement of facial and extraocular muscles, endocrinopathy, or the clinical phenotype of IBM [1-3].

Necrotizing Autoimmune Myositis (NAM)

NAM, also referred by some as Immune-mediated necrotizing myopathty (IMNM), has now evolved as the most common IM subtype [1]. It starts either acutely reaching its peak over days or weeks, or subacutely progressing steadily causing severe weakness and very high creatine kinase (CK) levels in the thousands [1]. NAM may also occur after viral infections and in association with cancer or immune check point inhibitors as discussed later. Unfortunately, very often NAM is erroneously attributed to statins or over-diagnosed as a "statin-myopathy" in patients on chronic statin administration [11], even though there is no convincing evidence as explained later. Acute rhabdomyolysis, like seen in NAM, can very rarely coincide with statin initiation and may be the causative factor in some cases of acute-onset NAM but there is no convincing evidence that statins play a triggering role in patients who develop a subacute NAM, *while taking statins for years* and their myopathy continues to worsen even after statin withdrawal [1,11,12]. Most NAM patients have antibodies against signal recognition particle (SRP) or 3hydroxy3-methylglutaryl-coenzyme A reductase (HMG-CR) [1,11,14] as discussed later.

Anti-synthetase syndrome-Overlap Myositis (Anti-SS-OM)

Anti-SS-OM, often presents with systemic sclerosis-like lesions, mild-to-moderate proximal muscle weakness, arthritis in the form of subluxation of the interphalangeal joints, "mechanic's hands", Raynaud phenomenon, and interstitial lung disease [1]. The syndrome is highlighted by the presence of anti-synthetase antibodies, primarily anti-Jo-1, hence the naming of *anti-Jo-1 syndrome,* and distinct histology with necrotizing features in the perimysium and perifascicular muscle fibers [1,11,16].

Inclusion Body Myositis (IBM)

This is the most common and disabling inflammatory myopathy above the age of 50 [1-5,17,18]. It starts

insidiously, over years, at times asymmetrically, and progresses steadily simulating a late-life muscular dystrophy or slowly progressive motor neuron disease [1-3]. Although IBM is commonly suspected when a patient with presumed PM did not respond to therapy [1-3], early involvement of distal muscles, especially foot extensors and finger flexors, atrophy of the forearms and quadriceps muscles, frequent falls due to quadriceps muscle weakness causing buckling of the knees, and mild facial muscle weakness, are clues to early clinical diagnosis [1-5,17-21]. Axial muscles may be affected resulting in camptocormia or head drop. Dysphagia occurs in more than 50% of the patients. [1-5,17-21] IBM is a progressive disease leading to disability.

Diagnosis and diagnostic work-up

The diagnosis of IM is based on the combination of clinical history including the pattern of muscle involvement and tempo of disease progression (as described above), combined with determination of serum muscle enzymes, muscle biopsy findings and at times auto-antibodies. Ancillary information is provided by electromyography, which can be useful to exclude neurogenic conditions or assess disease activity. Muscle MRI with contrast can reveal edema and inflammation in muscle and fascia and is mainly useful to define and assess the distribution of atrophic muscles[1]. The usefulness of muscle MRI has been excessively overestimated because the findings are not diagnostic for an IM and, contrary to suggestions that it can help selecting the specific muscle to biopsy, it does not provide more than a careful neurological examination because the surgeon can still obtain tissue from a very atrophic muscle fascicle since the biopsy is not MRI-or CT-guided and within the seemingly viable muscle tissue there are long atrophic fascicles (Dalakas unpublished observations).

Serum muscle enzymes

Creatine Kinase is elevated in all subtypes with active disease but can be normal when the disease has become chronic. Very high levels point to NAM, while normal levels from the outset can be seen in DM and anti-SS-OM reflecting predominant pathology in the interstitial tissues. Aldolase may be also elevated especially if the fascia is involved [1,24-26].

Muscle biopsy findings

It shows features distinct for each subset and, although not always typical, remains the most reliable diagnostic tool when interpreted in the context of the clinical findings and processed in the clinician's expert laboratory that performs enzyme histochemistry and immunocytochemistry. Findings for each subtype are:

a) in dermatomyositis, there is inflammation predominantly perivascularly, in the interfascicular septae or at the periphery of the fascicles. The muscle fibers undergo necrosis and phagocytosis, often in a portion of a muscle fasciculus or the periphery of the fascicle, due to microinfarcts leading to hypoperfusion and perifascicular atrophy [1-5,25]. Perifascicular atrophy, characterized by layers of atrophic fibers at the periphery of the fascicles, often with perivascular infiltrates, is diagnostic of dermatomyositis even without skin manifestations [1-5, 24,25,26];
b) in anti-synthetase syndrome-Overlap Myositis the histology may overlap with that of DM but this entity predominantly affects the perimysium with necrotizing features of the perimysial and perifascicular areas along with actin myonuclear inclusions [1,16,17,25,26];
c) in polymyositis there is inflammation perivascularly and in multiple foci within the endomysium consisting predominantly of $CD8^+$ T cells invading healthy, non-necrotic, muscle fibers expressing MHC-I antigen (normal muscle fibers do not express MHC-I antigen) [1-5].
   The MHC/CD8 complex is useful to confirm the diagnosis and exclude disorders with non-immune inflammation, as seen in some muscular dystrophies [1-5,17,25];
d) in Inclusion Body Myositis (IBM), in addition to the same inflammatory pattern described for PM, there are chronic myopathic changes with increased connective tissue and fiber-size variability; autophagic vacuoles with bluish-red material "ragged-red" or cytochrome oxidase–negative fibers due to abnormal mitochondria; and congophilic amyloid deposits next to the vacuoles best visualized with crystal violet or fluorescent optics [1-5,17,18, 20,21]. In up to 30% of IBM patients with the typical clinical IBM- phenotype, the biopsy does not show vacuoles or amyloid deposits but only inflammation, leading to erroneous diagnosis of polymyositis [1,17]. Such patients have *clinical IBM* diagnosed on clinicopathologic correlations [1,17,27];
e) in *necrotizing autoimmune myositis* there are abundant necrotic fibers invaded or surrounded by macrophages. Lymphocytic infiltrates are sparse and MHC-I upregulation mostly in the necrotic fibers[1-5,17,25]. In a number of patients, the muscle biopsies show deposition of complement on blood vessels and, as expected, on necrotic fibers. Up to 65% of the patients have specific, albeit non-pathogenic, antibodies[1,12-14,17].

Autoantibodies

Directed against nuclear RNAs or cytoplasmic antigens, autoantibodies are detected in up to 75% of all IM patients depending on methodology [1]. Although their pathogenic role is unclear, some antibodies appear specific for distinct clinical phenotypes. They include:

a) anti-*aminoacyl-tRNA synthetases*, detected in 20-30% of the patients [1,11,28,29]. Among the eight different anti-synthetases, the antibodies directed against the histidyl-transfer RNA synthetase (anti-Jo-1), is the most common accounting for 75% of all anti-synthetases and defines the "anti-synthetase- syndrome" described above;
b) *necrotizing autoimmune myositis-specific antibodies*, against the translational transport protein SRP (Signal Recognition Particle) or against a 100-kd autoantigen identified as HMGCR (3hydroxy3-methylglutaryl-coenzyme A reductase). Because HMGCR is the pharmacological target of statins [1,11-14], these antibodies have been thought to be associated with a prior statin use. These antibodies however are more often seen in statin-naive patients, and they are detected in up to 65% of all NAM cases from any cause [1,13,17]. Most importantly, anti-HMGCR may be more often associated with malignancies rather than statins. They are disease markers and, contrary to some publications, they do not have a pathogenic role as explained below;
c) *dermatomyositis-associated antibodies* that include: i) Mi-2, highlighting the typical skin lesions; ii) melanoma differentiation–associated protein-5 (MDA-5) mostly connected with amyopathic dermatomyositis or interstitial lung disease [1,30]; and iii) transcriptional intermediary factor-1 (TIF-1) and nuclear matrix protein NXP-2, highly connected with cancer-associated adult DM [30]; and
d) *anti-cytosolic 5'-nucleotidase- 1A (cN1A*), detected in 33-51% of IBM patients [31]. These antibodies have no pathogenic significance, and they can be also seen in patients with other types of myositis or rheumatic diseases. Their presence in IBM highlights however the immune dysregulation and B-cell activation.

Triggering factors and associations

Malignancies

Two IM subtypes are associated with malignancies, DM and NAM. In DM with malignancy a common antibody is the one against transcriptional intermediary factor-1 (TIF-1), while in NAM antibodies against HMGCR, especially in patients above the age of 50, are most frequent. Among 349 patients with IM, 75 (21%) had cancer manifested usually within a year; among those patients, 48% had DM with anti-TIF-1 antibodies and the other half had NAM with HMGCR [13].

Immune check-point inhibitors (ICPI's)

An increasing number of patients with advanced malignancies treated with ICPI's can develop immune-related neurological complications including inflammatory myopathies [32,33]. The neurological events can evolve rapidly, necessitating the need for vigilance at all stages of treatments, even after completion, because early immunotherapeutic interventions with steroids and IVIg are effective. The main ICPIs currently on the market are directed against a) CTLA-4: *Ipilimumab*; b) PD-1: *Pembrolizumab and Nivolumab*; and *c)* PD-L1: *Atezollizumab, Avelumab, and Durvalumab.* The process by which ICPI's trigger autoimmunity has been discussed elsewhere [32]. Briefly, tumors, like other antigen presenting cells, express on their cell surface the inhibitory ligands PD-L1/PDL-2 and B7-1/B7-2 which are respectively engaged with PD-1 and CTLA-4 on T cells, downregulating T cell responses. These receptor/ligand interactions essentially act as an *off switch*, like "telling the T cells to leave the tumor cells alone" so T cells do not attack the tumor[3 2]. The ICPI's prevent the CTLA-4 or PD-1 from binding to their respective receptors CD80/86 and PDL-1 and, by doing so, inhibit the inherent "inhibitory" costimulatory interactions between T cells and tumor cells, resulting in positive signals. What ICPI's essentially do is turning the *switch back on* resulting in positive costimulation and strong cell activation, like taking the *brakes off* the immune system [32]. This blockade allows the T cells to kill tumor cells, but at the same time the resulting enhanced co-stimulation causes an uncontrolled T cell activation that disrupts immune tolerance resulting in immune-related events against muscle.

Among all the inflammatory myopathy subtypes, the most frequent autoimmune myopathies triggered by pembrolizumab, ipilimumab and nivolumab are DM and especially NAM. In some patients, NAM may co-exist with myasthenia gravis presenting with head drop, proximal muscle weakness, myalgia, dyspnea, ophthalmoparesis or bulbar weakness. Among 654 patients receiving ICPI's (pembrolizumab: 389; nivolumab: 264; both: 1), 5 on pembrolizumab had biopsy proven myopathies (2 NAM, 1 dermatomyositis, and 2 nonspecific myopathy) 33. Patients respond to steroids and IVIg especially if treated promptly.

Viruses, including SARS-CoV-2

Among potential triggers, except of the Immune checkpoint inhibitors discussed above, viruses have clearly the potential to break tolerance and trigger an im-

mune inflammatory myopathy. Although IM have been seen during or after a viral infection, attempts to amplify viruses from the muscles, including coxsackieviruses, influenza, paramyxoviruses, mumps, cytomegalovirus and Epstein-Barr virus, have failed [1-5]. The best studied viral connection until now has been with retroviruses. Patients infected with HIV or human-T-cell-lymphotropic virus-I develop polymyositis or inclusion-body myositis [1-3, 34-35] with retroviral antigens detected not within the muscle parenchyma but within some endomysial macrophages (Trojan-horse mode). The autoinvasive T cells are however clonally driven and some are retroviral-specific [35].

During the present COVID-19 pandemic**,** there is evidence that more than 10% of COVID-19-infected patients develop myopathic symptoms with myalgia, weakness and elevated CK sometimes at very high CK levels > 10,000 suggestive of Necrotizing Autoimmune Myositis (NAM) [36]. Although COVID-19-associated myositis has not yet been studied but only characterized as "skeletal muscle injury" or "rhabdomyolysis", two just published cases suggest an autoimmune COVID-19-triggered NAM as summarized [36]. One, an 88-year old man from New York presented with acute bilateral thigh weakness and inability to get up from the toilet, without fever or other systemic symptoms, and very high CK level (13,581 U/L) [36]. He was found COVID-19-positive, given hydroxychloroquine and a week later his painful weakness improved with CK reduction. The other, a 60-year-old man from Wuhan had a 6-day history of fever, cough and COVID-19-positive pneumonia with normal strength and CK; seven days later, although systemically had improved, his CRP doubled and developed painful muscle weakness with very high CK (11,842 U/L) [36]. He was given IVIg and his strength improved while became COVID-19-negative.

In a recent retrospective study, patients hospitalized for a flue also had elevated CK level as high as those seen in a large series of patients with COVID-19 [37] confirming the long-term notion that hyperCKemia can frequently occur in sick patients with an acute viral illness [1-3]. However, an acute onset of severe muscle weakness with increased inflammatory markers and very high CK levels in the thousands, as noted in the two cases above, is highly suggestive of an autoimmune inflammatory myopathy within the spectrum of NAM triggered by the virus, similar to what we first reported with HIV early in that epidemic [35,36]. Considering that very high CK level and painful muscle weakness were seen in 10% of COVID-19-positive patients [36], a potentially treatable autoimmune myopathy might have been likely overlooked. This notion however requires a great deal of caution because without muscle biopsy confirmation and antibody screening, the diagnosis of COVID-19-NAM remains still undocumented because myopathic symptoms in a severe systemic viral disease are multifactorial [37]. The need to study COVID-19 muscle invasion is therefore needed and will be highly interesting because ACE2, the SARS-CoV receptor, is reportedly expressed in skeletal muscle [summarized in [37]]. If this is confirmed, COVID-19 may represent the first virus directly capable of infecting muscle fibers. None of the viruses implicated as possible myositis triggers has been shown to directly infect the muscle fiber and our molecular studies have so far failed to detect any of them [38]; instead, viruses induce an immune T cell-mediated with clonal expansion of viral-specific T cells, or macrophage-mediated, muscle fiber autoinvasion with abundant pro-inflammatory cytokines [1-3, 35,36].

Statin exposure

A very small number of patients early on statin initiation may experience transient myalgia, and some others transient CK elevation but no muscle weakness. In some patients, myalgia persists demonstrating statin intolerance. Very rarely, patients may develop rhabdomyolysis soon after statin initiation. The implication however that *chronic* statin administration can, all of a sudden, trigger " statin-myopathy" in the form of NAM [11,14] with antibodies against HMGCR, a ubiquitous and non-muscle-specific antigen within the endoplasmic reticulum, has never been substantiated. Statins can upregulate HMGCR in cultured cells in vitro, and HMGCR is the target of action of statins, but studies from many centers throughout the world have consistently shown that anti-HMGCR autoantibodies are more often seen in statin-naïve NAM patients and more often connected with cancer [13,39,40]. Since NAM is now the commonest inflammatory myopathy and more than 25% of Americans above 40 years take statins, the association between statins and NAM is likely a chance phenomenon [1,17,41,42]. Some authors correctly proposed that the term "statin myopathy" should not be used [40] because only a minority of NAM patients had statin exposure and, even in those patients, NAM appears many years after statin initiation making a causative role dubious if not impossible.

Immunopathogenesis

Although the causes of inflammatory myopathies are unknown, an autoimmune pathogenesis is strongly implicated, and seems to be specific for each subset.

Dermatomyositis

In DM, early activation of complement C5b-9 membranolytic-attack-complex is deposited on the endothelial cells [1-5,43], leading to capillary necrosis, reduction of endo-

mysial capillaries, ischemia, and muscle-fiber destruction resembling microinfarcts [1-5]; the remaining capillaries have dilated lumens to compensate for the ischemia [1-5]. The residual perifascicular atrophy reflects the endofascicular hypoperfusion, which is most prominent at the periphery of the fascicles. The membrane attack complex activation is triggered by binding of C1q to endothelial cells and releases proinflammatory cytokines, upregulates the adhesion molecules on endothelial cells, and facilitates the migration of activated lymphocytes including B cells, $CD4^+$T cells and plasmacytoid-dendritic cells to perimysial and endomysial spaces. Innate immunity also plays a role based on increased expression of type-I interferon-inducible proteins in the perifascicular regions [44]; this effect appears secondary to ischemic damage which is probably sensed by the retinoic acid-inducible gene-1 signaling leading to auto-amplification of local inflammation by activating β-interferon and MHC-1 [45].

Necrotizing autoimmune myositis and the misconception of statin association or pathogenicity of antibody markers

Within the necrotic fibers of NAM, there are macrophages, MHC-I expression and deposition of complement; these findings have been loosely interpreted to suggest that in NAM there is complement- mediated cytotoxicity and the recruitment of macrophages invading the muscle fibers represent an antibody-dependent cell-mediated cytotoxicity (ADCC) process [11,14,46]. There is no convincing evidence however supporting a pathogenic role of these antibodies in causing or triggering muscle fiber necrosis via an ADCC mechanism [41,42]. Both, SRP and HMGCR antibodies, are against ubiquitous and non-muscle-specific antigens firmly localized in the endoplasmic reticulum and there is no explanation how antibodies against such cytoplasmic targets can selectively cause muscle fiber cell necrosis, as discussed [41]. Further, MHC-1-expression and C5b-9 complement deposits are *always* observed in necrotic and regenerating fibers from any cause, such as commonly in muscular dystrophies [47,48], and lack specificity for NAM. Classic work of AG Engel et al dictates that *all* necrotic fibers in non-immune myopathies, such as muscular dystrophies, unambiguously activate complement which in turn stimulates cellular infiltrates and macrophages [47,48]. Further, claims that these antibodies can cause muscle fiber atrophy or affect regeneration in vitro[49] are irrelevant to the cause of NAM where a macrophage-mediated muscle fiber necrosis causes devastating muscle destruction, not muscle fiber atrophy. Although not pathogenic, anti-SRP and anti-HMGCR antibodies remain important disease markers of diagnostic value because they are detected in up to 65% of NAM patients [1].

Polymyositis and Inclusion-Body Myositis

In PM and IBM, $CD8^+$cytotoxic T cells surround and invade healthy, non-necrotic muscle fibers that aberrantly express MHC-I [1-3,50-53]. MHC-I expression, absent from the sarcolemma of normal muscle fibers, is probably induced by cytokines secreted by activated T cells. The CD8/MHC-I complex is characteristic of polymyositis and inclusion-body myositis and its detection aids in confirming the histologic diagnosis [2-5,50-53]. The $CD8^+$ T cells contain perforin granules directed towards the surface of the muscle fibers, resulting in myonecrosis upon release [54,55]. Analysis of T-cell receptor molecules expressed by the infiltrating $CD8^+$ T cells reveals clonal expansion of T-cell receptor chains and conserved sequences in the antigen-binding region, suggesting an antigen-driven T-cell response [56-58]. This is further supported by the expression of co-stimulatory molecules and upregulation of adhesion molecules, chemokines, and cytokines [59-61]. Chemokines and cytokines, including interleukin-6, 8,10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1a (MIP-1a), or IP-10 and its receptors, are expressed in the endomysial inflammatory cells and the neighboring extracellular matrix and may enhance leukocyte recruitment, trafficking and activation [62]. Adhesion of lymphocytes to muscle may be facilitated by metalloproteinases, which are expressed on the autoinvasive CD8+ T cells and make cell-to-cell contact with muscle fibers [1,17,63,64]. There is also B-cell activation, most prominent in IBM [65] as supported by the presence of *anti-cytoplasmic 5'-nucleotidase 1A (cN1A; NT5C1A)* autoantibodies directed against the cN1A nuclear protein involved in RNA processing [31]. These antibodies are not however pathogenic or IBM-specific but simply denote the autoimmune dysregulation in IBM muscles. Plasma cells and myeloid dendritic cells, potent antigen-presenting cells, are also seen among the endomysial infiltrates of patients with PM, DM, and IBM [66] but their significance is still unknown.

Non-immune factors in Inclusion Body Myositis and cross-talk between inflammation degeneration and muscle autophagy

Inclusion-body myositis is a complex disorder because, in addition to the afore-mentioned autoimmunity, there co-exists an important degenerative component, highlighted by the presence of congophilic amyloid deposits within some fibers [18,20,66,67]. Similar to Alzheimer's disease, these deposits immunoreact against amyloid precursor protein (APP), β-amyloid, apolipoprotein-E, α-synuclein, presenilin, ubiquitin, and phosphorylated-tau attesting to protein aggregation [18,20,66]. Immunostaining for the ubiquitin or tau components, TDP43 and p62, has been even advocated as

Table I. A step-by step approach in the treatment of inflammatory myopathies: 2020 and beyond.

Dermatomyosits (DM) [1-3,72-83]
1. High-dose prednisone (oral or intermittent intravenous in acute cases) 2. In steroid-responsive patients add an immunosuppressant [mycophenolate, (most preferable) azathioprine, or methotrexate] 3. High-dose intravenous immunoglobulin (IVIg) if steps 1-2 fail 4. Rituximab, if IVIg is not sufficiently effective 5. Consider new biologics including eculizumab, other anti-B cell agents or JAK inhibitors 6. Most promising future: anti-complement agents such as eculizumab, ravulizumab (ultomiris), zilucoplan
Polymyositis (PM) [1-3,72-83]
1. High-dose prednisone (oral or intermittent intravenous in acute cases) 2. In steroid-responsive patients add an immunosuppressant [mycophenolate, (most preferable) azathioprine, or methotrexate] 3. High-dose intravenous immunoglobulin (IVIg), if steps 1-2 fail 4. Rituximab, if IVIg is not sufficiently effective 5. If above unsatisfactory, reconsider the diagnosis and explore it with a new muscle biopsy
Necrotizing Autoimmune Myositis (NAM) [1-3,72-83]
1. High-dose prednisone (intravenously 1g/daily for 5 days may be needed in acute cases) 2. High-dose intravenous immunoglobulin (IVIg) 3. Rituximab, if IVIg not sufficiently effective 4. Consider new biologics, including eculizumab, other anti-B cell agents or JAK inhibitors 5. Most promising future: anti-complement agents, such as eculizumab, ravulizumab (ultomiris), zilucoplan
Anti-synthetase syndrome-Overlap Myositis (Anti-SS-OM) [1-3,72-83]
1. High-dose prednisone (oral or intermittent intravenous in acute cases) 2. In steroid-responsive patients add an immunosuppressant [mycophenolate, (most preferable) azathioprine, or methotrexate] 3. High-dose intravenous immunoglobulin (IVIg) if steps 1-2 fail 4. Rituximab, if IVIg is not sufficiently effective 5. If interstitial lung disease, may also consider cyclophosphamide
Inclusion Body Myositis [1-3,84-92]
1. Physical therapy; CoQ10; encourage participation in a controlled study 2. If dysphagia is prominent, IVIg 3. All trials with immunosuppressants, immunomodulating agents, muscle growth factors TGF-β inhibitors have failed. Among them, most promising was alemtuzumab in an uncontrolled study

diagnostic markers [1,18,67]. It remains however unclear, how these proteinacious aggregates, which are also seen in other vacuolar myopathies, induce an inflammatory myopathy and what triggers disease, inflammation or protein aggregation [1,20]. Laser microdissection of T-cell-invaded fibers, compared to non-invaded or vacuolated ones, has revealed differential upregulation of inflammatory signaling such as interferon-γ-receptor [68]. Compelling evidence suggests that aging, abnormal proteostasis (the network controlling proteins) [1,28,20], cell stress induced by MHC-1 or nitric oxide, long-standing inflammation and proinflammatory cytokines like interferon-γ and IL1-β [69-70], may cumulatively trigger or enhance degeneration leading to further accumulation of stressor molecules and misfolded proteins [1,69-71].

Treatment of DM, PM and NAM (Tab. I)

Oral prednisone 1 mg/kg, or up to 100 mg per day, as a single daily dose is the first-line drug based on experience, but not controlled trials [1-6,17,72,73]. Some clinicians prefer adding an immunosuppressant from the outset. In patients with severe or rapidly worsening disease, intravenous methylprednisolone 1 gm/kg for 3-5 days is preferable before starting oral glucocorticoids. After 3-4 weeks, prednisone is tapered as dictated by the patient's response, preferably by switching the daily dose to alternate-days [1-3]. If by then objective signs of increased strength and activities in daily living are absent, tapering is accelerated to start the next in-line agent. A tactical error is the practice of "chasing" the CK level as a sign of response, especially in patients reporting a sense of "feeling better" but not necessarily stronger. When the strength improves, the serum CK drops, but fall in CK alone is not a sign of improvement [1-3].

In glucocorticoid-responsive patients, *azathioprine*, *mycophenolate mofetil, methotrexate* or *cyclosporine* are empirically used for "steroid-sparing" [1-3, 17,72,73]. When

interstitial lung disease co-exists, *cyclophosphamide* or *tacrolimus* may be helpful [74]. When glucocorticoids fail to induce remission or in rapidly progressive cases, *intravenous immunoglobulin* (IVIg) 2 gm/kg is appropriate [1-3;69,70,73]. In a double-blind study, IVIg was effective in refractory dermatomyositis [75]; monthly infusions may be required to maintain remission. In open-label trials, IVIg also seems effective in polymyositis and necrotizing autoimmune myositis[1-3,17,75]. Subcutaneous Ig appears to sustain remission (Tab. I) [76].

If glucocorticoids and IVIg have not helped, the diagnosis should be revisited, and a repeat muscle biopsy might be considered. If the diagnosis is re-confirmed, biologics approved for other immune diseases are further options [1-3,70,73]. Among those, the first is *rituximab* (anti-CD20 antibody), which at 2 gm (divided in two bi-weekly infusions) seems effective in several dermatomyositis, polymyositis, and necrotizing autoimmune myositis patients. A placebo-controlled study in 200 patients however, did not meet the primary end-point largely because of study design; although at week 8 there was no difference between placebo and rituximab, at week 44 when all patients had received rituximab, 83% met the definition of improvement [77,78]. Patients with anti-Jo-1, Mi-2, or anti-SRP antibodies are also likely to respond [78,79,80]. *TNF inhibitors* (infliximab, adalimumab, etanercept) are ineffective and may worsen or trigger disease [1,17]. Tocilizumab and IL-1b inhibitors may be of help in small case series [81,82]. Among the new biologics, *anti-complement C5 (eculizumab),* should be very promising especially in dermatomyositis where complement plays a major role in microangiopathy and muscle fiber necrosis. Eculizumab may be also effective in NAM but controlled studies have not been done. Overall, the long-term outcome of treatment for inflammatory myopathies has substantially improved, with a 10-year survival at > 90% [83]. A step-by-step therapeutic approach in all IM subsets is provided in Table I.

Immunotherapies for IM during COVID-19

Patients with IM have been justifiably concerned as to whether their disease status adds an additional risk placing them into an "immunosuppressed or immunocompromised" category. As discussed previously [36], there is no evidence that the inflammatory myopathy itself makes them more susceptible to COVID-19 or the immunosuppressive therapies they are receiving have such a potential. If clinically stable and not lymphopenic, there is no data-driven reasons to change anything and disturb clinical stability. For patients on monthly IVIg, there may be even a theoretical advantage that IVIg offers additional protection due to natural autoantibodies [36]; if IVIg is not infused as home-infusion, switching to self-administered subcutaneous IgG might be an option to diminish exposure. For patients on rituximab, the infusion intervals can be prolonged to more than 6 months, because both, B-cell reduction and clinical benefit, can persist longer [36].

Treatment of Inclusion-Body Myositis

Because of T-cell-mediated cytotoxicity and the enhancement of amyloid aggregates by pro-inflammatory cytokines as outlined earlier, immunosuppressive agents have been tried in IBM but all failed probably because the disease starts long before patients seek medical advice, when the degenerative cascade is already advanced and inflammatory mediators have enhanced degeneration and autophagy [1-3,17,84-86]. Glucocorticoids, methotrexate, cyclosporine, azathioprine or mycophenolate are ineffective and, although some patients initially experience mild improvements, there is no long-term benefit [1,17,84]. IVIg is ineffective in controlled trials but may transiently help some patients, especially those with life-threatening dysphagia where is the treatment of choice based on statistically significant changes in the controlled trial [87,88]. Alemtuzumab may provide short-term stabilization [89] but a controlled study is needed. A*nti- IL1-receptor (Anakinra)* [90] and IL1 receptor antagonist (Ilaris) also failed [91]. Trials targeting muscle-inhibiting TGF-β molecules or muscle growth factors are also disappointing and doubleblind studies have been clearly negative[92]. Although life expectancy seems normal, most patients with end-stage disease require assistive devices such as cane, walker, or wheelchair [22]. Dysphagia can be life threatening if IVIg has not helped.

Evolution of the IM field in the context of the Mediterranean Society of Myology (MSM) with a personal tribute to G. Nigro

Inflammatory myopathies have been discovered and subsequently studied by Neurology scholars with expertise in neuromuscular pathology fostering progress in muscle immunopathology, disease recognition, subset subtyping and pathogenesis. Over the last 30 years the very best minds and Neurology scholars in this field with leaders like W King Engel, Valerie Askanas, Andrew Engel, George Karpati, Victor Dubovitz and many others from USA, Italy, France, Australia, Israel etc. have participated on a regular basis in the MSM meetings. I have had the chance to be there every year almost since the creation of the MSM and have hosted two such events in Greece, one in Corfu and another one in Athens. Writing

this piece in the memory of Giovani Nigro brings back a blend of unique pleasantries of good science and humour in a relaxing and friendly atmosphere of picturesque environments and scholarly, formal, and informal, discussions about inflammatory myopathies. Being honoured by Giovanni in his unique style at the gala dinner among the best of friends and neuromuscular colleagues was the epitomy of the MSM that I will never forget.

This opportunity in honouring the memory of Giovanni Nigro and the unique meetings he has organized and overseen, is also an introspective on the future of the IM field as it is now moving from the neuromuscular clinicians/scientists that splendidly served it for years and advanced the field, to other subspecialties with different training backgrounds. We have all witnessed the last few years that neurologists with muscle pathology and immunopathology training are becoming increasingly scarce as very few of us continue to keep an active muscle pathology laboratory. Muscle biopsies are mostly performed now by surgeons, read by general pathologists either on paraffin sections with just the very basic – if any – immunopathology or enzyme histochemistry stains on fresh-frozen sections, and without knowledge of the clinical neuromuscular evaluation. The lack of clinicopathologic correlation, a fundamental principle of a neuromuscular neurologist for the diagnosis of myopathies, as pioneered by WK Engel and taught all of us, may be impacting on the identification of the correct inflammatory myopathy subtype and the distinction from dystrophies. We had been proud of our unique expertise to precisely assess and quantify the patient's muscle strength, being aware on how best to distinguish the contribution of functional weakness or pains from true muscle weakness, and bring this to diagnostic fruition by personally performing muscle biopsy, selecting the muscle to biopsy, looking at the slides and, after combining clinical with histology, initiate proper therapy. Concurrently, research on expanding the diagnostic muscle histopathology, immunopathology or molecular muscle pathology had flourished. Today, most clinicians involved in the diagnosis and care of patients with IM are of different subspecialties with different training backgrounds, such as rheumatologists, rheumatoneurologists or neurologists/elctromyographers. The prior focus on myopathology and molecular muscle immunopathology is slowly being shifted to serology, circulating humoral factors and antibodies, and muscle imaging. Whether will prove more fruitful remains to be seen.

Serving for more than 40 years as head of Neuromuscular service with still a fully functioning laboratory and having trained more than a hundred neuromuscular fellows around the world, I am also witnessing the directional shift of our neuromuscular trainees who are mostly centered around electromyography. We are not however to blame; it is economics that has prevented the maintenance of active neuromuscular pathology laboratories in many Universities. As a result, previously flourishing regional myology meetings, such as the MSM under Dr Giovanni Nigro's leadership, have vanished as if there is not need to have them; electromyographers go to electrophysiology meetings, rheumatologists to rheumatology meetings and general neurologists to neurology meetings.

Writing this in honouring of Giovanni Nigro' memory, I remain with the pleasant memories of blending the many years of myology progress with innovative discussions about culture and civilization with stimulating leaders in the clinical and basic science of muscle diseases. These unforgettable memories in the middle of the COVID-19 pandemic bring me back to the sad reality that the wonderful Giovanni Nigro's era of the MSM may never return; yet at the same time, as the sun comes after a storm, these memories also bring shining hopes on how Giovanni's legacy will build a bright future for our field. After the COVID-19 pandemic ends, we should be all armed with enthusiasm, determination and organizational to re-build the society from where it started, teach the new generation of neuromuscular experts what we have all learnt, and provide them with the stimulus on how best to combine the excellence in the clinic with histopathology, immunology, immungenetics and molecular biology to advance the filed towards effective target-specific therapies. Afterall, the advances in molecular science and means of communication are on our side. This will be Giovani's best legacy.

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Giant cell myositis and myocarditis revisited

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Giant cell myositis (GCMm) and giant cell myocarditis (GCMc) are two rare autoimmune conditions. Among these, GCMc is a life-threatening disease with a 1-year mortality rate of 70%. Lethal ventricular arrhythmias, rapid evolution to heart failure and sudden death risk makes GCMc an emergency condition. It is thought to be mediated by T-cells and characterized by the presence of myofiber necrosis and giant cells in biopsies. Most commonly co-manifesting conditions with GCMm and/or GCMc are thymoma, myasthenia gravis and orbital myositis, all of which are treatable. As suspicion is the key approach in diagnosis, the physician following patients with thymoma with or without myasthenia gravis and with orbital myositis should always be alert. The fatal nature of GCMc associated with these relatively benign diseases deserves a special emergency attention with prompt institution of combined immunosuppressive treatment and very early inclusion of heart failure teams.

Key words: giant cell myositis, giant cell myocarditis, thymoma/myasthenia gravis, orbital myositis

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Giant cell myositis/giant cell myocarditis

Giant cell myositis and myocarditis, diseases of skeletal and cardiac muscles are both abbreviated as GCM. As both conditions are the subjects of this report abbreviations will be GCMmuscle (GCMm) for giant cell myositis and GCMcardiac (GCMc) for giant cell myocarditis.

Myositis with giant cells is a rare but treatable acquired inflammatory disease of the skeletal muscle. As cardiac involvement rapidly leads to lethal ventricular arrhythmias, heart failure or sudden death, combination of GCMm with GCMc can develop into a fulminant and fatal condition. Without any treatment, the 1-year mortality approaches 70% when GCMc takes the stage [1]. Diagnosing and differentiating the entity from other more frequent cardiomyopathies and inflammatory myopathies depend on demonstrating the presence of a non-granuloma forming, diffuse giant cells in biopsy specimens [2]. Therefore, if not suspected it is arduous to make a prompt and accurate diagnosis. Furthermore, the importance lies in its recognition not only by cardiologists but also by other disciplines such as neurologists, rheumatologists, and ophthalmologists for rapid involvement of necessary emergency teams to start an aggressive treatment [3].

First description of GCM cases was on fatal idiopathic GCMc and was made by Saltykow in 1905 [4]. The main pathological findings in this report were widespread inflammation and myocyte necrosis with abundant giant cells. The condition was considered in tandem with sarcoidosis until the 1950s when received recognition as a separate entity with the diffuse nature of inflammation and necrosis versus granuloma and fibrosis formation in sarcoidosis (Veia A, 2014). From 1905 to 1980s, all reported GCMc

cases were diagnosed at autopsy [1]. The development of diagnostic tools such as endomyocardial biopsy (single or repeated), imaging-guided endomyocardial biopsy, apical vedge-sectioning during placement of ventricular assist devices, cardiac gadolinium-enhanced MRI, FDG-PET, has allowed diagnosis and treatment in living patients only after mid 1980s, prolonging transplant-free survival and life expectancy [5,6]. Tissue histopathology is still the gold standard of diagnosis and almost all studies on the inflammation of muscle tissue with giant cells is performed in GCMc. Due to the rarity of the condition, a multicenter Giant Cell Myocarditis Registry was established in the early 1990s in the United States [7].

The first description of GCMm was appended to GCMc and appeared in the report of Giordano and Haymond in 1944. This patient also manifested thymoma and myasthenia gravis [8-10]. From 1944 to 1974, only 10 patients with idiopathic GCMm were found to be reported in the literature with additional 3 patients with GCMc. All the reported GCMc patients were associated with thymoma [11]. As GCMc is a more serious manifestation of the GCMm/GCMc, the combination and diagnosis is established by myocardial histopathology and there is usually no time to evaluate any skeletal muscle pathology unless the patient presents relative symptoms. Thus, as it is not known how much latent GCMc patients also have GCMm it could be way more common than reported [13]. On the other hand, there are also patients with GCMm without GCMc [14,15].

GCMc is a very rare condition that affects otherwise healthy young adults. Incidence rate is 0.007% in Japan and 0,051% in India [16]. Review of 377,841 autopsies over a 20-year period found myocarditis in 0.11% but giant cell myocarditis in only 0.007% of the cases [17]. Considering that patients passed away before reaching the hospital, this incidence rate might even be higher.

GCMc can be idiopathic or occur in various conditions, such as infection (tuberculosis, syphilis, pneumocystis jirovecii parvovirus, coxsackie), inflammation, drug sensitivity, hypersensitivity and vasculitis [7]. At least one group of the condition is considered as an autoimmune disease in 19% of patients in some registries that co-manifest with other autoimmune diseases such as inflammatory bowel disease, cryofibrinogenemia, optic neuritis, Hashimoto thyroiditis, rheumatoid arthritis, Takayasu arteritis, temporal arteritis, myasthenia gravis (MG), alopecia totalis, vitiligo, orbital myositis, autoimmune hepatitis, Guillain-Barré syndrome, systemic lupus erythematosus, Sjögren's and pernicious anemia [16]. Furthermore, the inducibility of a similar idiopathic myocarditis in Lewis rats by cardiac myosin strongly suggests that GCMm and GCMc are both autoimmune conditions [16,18]. Combined immunosuppression retards the progression of the disease and results in a partial remission both in humans and experimental rat models. This fact, together with the up-regulation of genes involved in T-cell response are other evidences of autoimmunity [7]. Tumors of the immune cells such as thymoma and lymphoma – thymoma being the most common – are the main tumours accompanying GCMm and GCMc, either separately or in combination [7]. Considering that other autoimmune diseases also accompany thymoma, this feature also suggests autoimmune or dysimmune mechanisms in GCMm/GCMc. When looked at from GCMm point of view, almost – if not all- cases reported are present in patients with myasthenia gravis with or without thymoma.

Although clinically presents similar to other myocarditis conditions GCMc is more severe and has a unique pathology [11]. Histopathological hallmark of GCMm or GCMc adheres to the presence of diffuse inflammation including giant cells without well-formed granuloma formation. Muscle fiber destruction in the muscle biopsy specimens from heart and skeletal muscles, CD8(+) lymphocytes, plasma cells and eosinophils all accompany giant cells at different proportions [2,16]. Giant cells generally embrace necrosis areas diffusely but they don't form granulomas. Origin of these giant cells are reported to be histiocytic and/or myogenic. It was immunohistochemically demonstrated in one patient that myogenic giant cells express increased lysosome-proteasome system (cathepsins A, B and D) and late endosomal system (LAMP-2) proteins, whereas histiocytic giant cells express only lysosome-protein system proteins (cathepsins A, B, D, L and S) but not the late endosomal marker LAMP-2. They were both negative for ubiquitin-proteasome system, autophagosome and aggresome systems [19]. Thus, it is assumed that the digestion of the same endocytotic material by more than one macrophage engenders these macrophages to fuse and form a giant cell [19].

GCMm/GCMc and thymoma

Approximately 1% of patients with thymoma exhibit GCMc and/or GCMm [10,20]. Being an autoantigen expressing tumor which results in production of antibodies mainly against muscle and nerve tissues such as acetylcholine receptors (AChr), voltage gated calcium channels (VGCC), voltage gated potassium channels (VGKC/Kv1.4), and striational proteins called ryanodine (RyR) and titin, thymoma is the most common tumor associated with GCMc or GCMm [21]. These antibodies are either clinically silent or they cause MG, myositis, neuro-myotonia, encephalitis, autonomic neuropathy, subacute hearing loss and even encephalitis [22]. Most of the cases with co-manifestation of thymoma and GCMm and/or GCMc bear Kv1.4, RyR and/or titin antibodies [19]. The defective immunity found in patients with thymoma is thought to

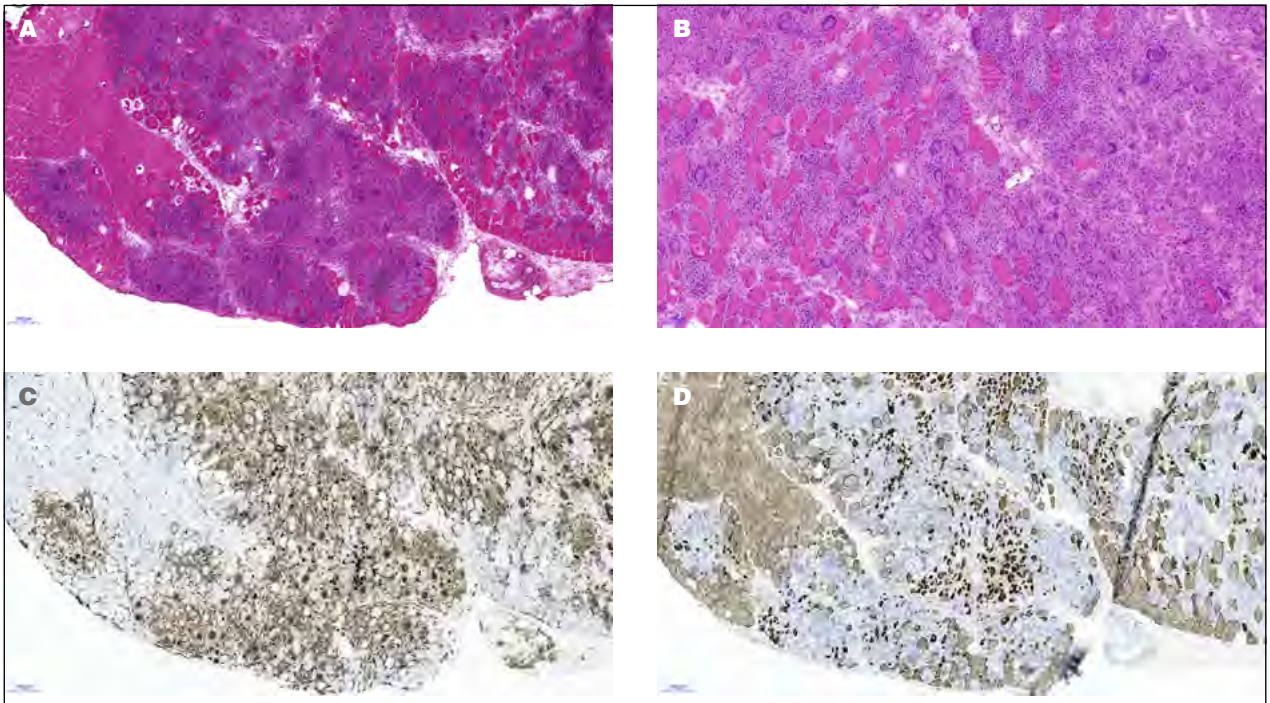

Figure 1. Muscle biopsy of a 58-year old patient with GCMm, who had been followed up for AChR and Titin antibodies positive generalized MG under corticosteroid therapy. He also had had metastatic colon cancer and received anti-VEGF therapy 1 year before the development of GCMm. He did not develop GCMc and the GCMm was controlled by increasing the corticosteroid dose. A) H&E (320x): diffuse myonecrosis with inflammatory cells including many giant cells; B) H&E (640x): abundance of giant cells; C) CD68 positive macrophages (320x): macrophages diffusely invade the necrosis area. Giant cells are also positive for macrophage marker CD68; D) Desmin (320x): only the normal areas and redundant, atrophic myofibers are positive for desmin.

play a role in a giant cell immune reaction against heart and skeletal tissues (Fig. 1) [10].

GCMm/GCMc and MG (with or without thymoma)

Although rare, presence of polymyositis with lymphocytic inflammation in autoimmune myasthenia with or without thymoma has been a well-known entity. Myositis-MG combination has gained more importance lately due to the employment of immune checkpoint inhibitors (ICI) in cancer therapy with widespread uncontrolled T-cell response attacking different tissues [23,24]. Myocarditis is also discernible in ICI myositis and may co-manifest with skeletal myositis and myasthenia gravis [20]. However, the description of GCMm, GCMc or the combination of them in patients with MG with or without thymoma is an extremely rare but a more important situation as GCMm has a very rapid course and fatal outcome [22]. In the report of Evoli, among 50/207 patients 3 of whom died without any previous cardiac disease, had sudden death, giving the suspicion that they may have held GCMc [25]. Uchio et al reported 8/889 (9%) myositis cases with MG, only one of them had GCMm [26].

Case reports on GCMm are almost exclusively in myasthenic and/or thymomatous patients, whereas only some of GCMc cases do occur in the same patient population [22]. Therefore, idiopathic GCMc cases without an autoimmune etiology do exist and these cases are much more frequent (around 80%) than the autoimmune disease related group (19%) [15]. In general patients with MG and myositis are more likely to express striational antibodies Titin, RyR, Kv1.4. This is the same for GCMm or GCMc in myasthenic patients that express Kv1.4, titin or RyR antibodies [19,27]. Heart and skeletal muscles may be the autoimmune targets in some patients with MG [27].

GCMm/GCMc and orbital myositis

Orbital myositis is a self limited condition under immunosuppressive treatment. This self-limited disease be-

comes life-threatening when co-manifests with GCMc. Presence of GCMc in orbital myositis was first described in 1989 by Klein BR [29]. In this report, authors suggested that this combination could be regarded as a separate entity [29]. To date, less than 10 cases of GCMc and/or GCMm with orbital myositis are reported, most of which have died due to cardiac involvement [3,29-34]. It has also been described in a pediatric patient [33]. In all reported cases cardiac disease manifested later in the course with a time lapse of days, months or even years [34]. Therefore, the possible development of GCMc and/or GCMm should be kept in mind in the follow-up of patients with orbital myositis [34,35].

Management of GCMm and GCMc

Immunosuppressive treatment either with corticosteroids or in combination with others is sufficient for GCMm only, like in other inflammatory myopathies, and it responds well. Long term maintenance therapy is necessary to prevent relapses and also for its potential to develop GCMc. However, the potential of developing GCMc in the course of GCMm is a factor that alerts the physician than in other inflammatory myopathies. Therefore, it is of vital importance to start an immunosuppressive treatment immediately upon suspicion or diagnosis and include cardiology dicipline for supportive therapy right from the beginning in the follow-up [22,34]. As GCMm may start simultaneously or before the diagnosis of thymoma or MG and also most GCMc patients carry striational antibodies, every patient with GCMm should be checked for thymoma and thymoma-related striational antibodies. Presentation of GCMm and/or GCMc in all reported orbital myositis cases manifest later in the course of orbital myositis. Therefore, orbital myositis itself should raise the suspicion of potential emanation of GCMcm or GCMc.

As mentioned before, most of the research on GCM has been performed in GCMc patients and Lewis rat models. It has been shown that the GCMm transferred to rats were prevented by cyclosporine or anti-T-lymphocyte antibodies but not by corticosteroids alone [6,9,36-38]. Although there are no controlled large scale trials, due to the positive response in anecdotal cases or small patient groups of this fulminant disease, different combinations of immunosuppression with corticosteroids, azathioprine, cyclosporine-A and anti-T-lymphocyte antibodies are used [6, 8,11,39]. In a multicentric retrospective study of 63 biopsy- proven GCMc patients showed that the median survival was 3 months in patients who did not receive any immunosuppression, 3,8 months in the ones receiving corticosteroids only, but was 12.6 months in the patients who received cyclosporine with different combinations (corticosteroids, azathioprine, muromonab-CD3) [8]. As fast tapering and cessation of immunosuppression is associated with the recurrence of GCMc even up to 8 years after the initial diagnosis careful tapering of the drug doses without discontinuation is recommended [40].

Supportive treatment has vital importance in GCMc. If the diagnosis is prompt or GCMc is suspected in a living patient, left ventricular assist devices, medical therapy of heart failure and timely heart transplantation is life-saving when needed. However, it should be noted that and although the cause is unknown, a transplanted heart may also develop GCMc as well. To avoid this probability,, immunosuppression should be maintained in tolerable doses [6,8,39].

Conclusions

The consequences of developing GCMc during the course of some neurological diseases which are treatable and have more benign courses such as GCMm, thymoma, MG or orbital myositis are devastating. Neurologists should be alert in prompt diagnosis of the condition and should immediately include cardiology teams into the diagnostic and treatment process.

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The impact of SARS-CoV-2 on skeletal muscles

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In 2019-2020, the SARS-CoV-2 pandemic has shocked the world and most health care systems, and a "second wave" of the viral spread is ongoing in Europe and in Italy too. While, at the initial outbreak, the treatment of patients had focused on the respiratory symptoms, many diverse clinical manifestations of the disease have to date been reported. However, the complete course of the disease has not yet been fully clarified. In particular, several reports from the real-world clinical practice have highlighted the noxious effects of SARS-CoV-2 on skeletal muscles. In this brief review, we summarized the main current findings about muscular and neuromuscular damages that may be triggered by the virus or by the drugs used to treat COVID-19. Moreover, we underlined the need of attentive care and vigilance for patients with neuro-muscular disorders, who may be particularly susceptible to infection and at increased risk for severe COVID-19.

Key words: SARS-CoV-2, COVID-19, skeletal muscles

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel beta-coronavirus that causes a diverse range of symptoms in patients, now defined as coronavirus disease (COVID-19). Declared as a pandemic on 11 March 2020 by the World Health Organization (WHO), it has currently affected (as of October 2020) more 37 million people worldwide and caused more than 1 million of deaths [1]. So far, the fatality rate due to COVID-19 varies from 1% to more than 7% and the main cause of death remains a respiratory failure. However, the complete course of the disease has not yet been fully clarified and little is presently known about the long-term effects of the infection on physiology and health conditions. At the initial outbreak of the pandemic, the treatment of patients focused on the respiratory symptoms, and thus on the management of fever, cough, shortness of breath, and respiratory failure. With the months and virus worldwide spread following the initial outbreak, it has become increasingly evident that SARS-CoV-2 infection may cause a large variety of other symptoms, including significant central and peripheral neurological manifestations of the disease [2].

This brief review aims at highlighting the main findings about the effects of SARS-CoV-2 infection and the potential damages directly and indirectly caused by COVID-19 on skeletal muscles. The so far suggested patho-physiology mechanisms are also mentioned. We analyzed the relevant reports on the topics published on PubMed until 12th October 2020. Additionally, we performed a focused literature search in the same data-

base for articles on drugs used to treat COVID-19 and drugs affecting muscle functions. We finally searched on clinicaltrials.gov for ongoing clinical trials on the topic.

Biology and pathophysiology of neurological and muscular damages caused by SARS-CoV-2

The binding of SARS-CoV-2 to angiotensin converting enzyme 2 (ACE2) has a pivotal role in the pathophysiology of clinical manifestations in patients with COVID-19 [3]. ACE2 is widely expressed on multiple organs including nose, lungs, kidneys, liver, blood vessels, immune system, skeletal muscles and brain. Skeletal muscles in particular, and other cells in the muscles like satellite cells, leukocytes, fibroblasts, and endothelial cells, express ACE-2 [3,4]. Therefore, it is postulated that skeletal muscles are also susceptible to direct muscle invasion by SARSCoV-2. Moreover, after binding ACE2 and invading cells, SARS-CoV-2 may cause a "cytokine storm", with marked elevation in levels of interleukin-1, interleukin-6, and tumor necrosis factor. High levels of these cytokines increase vascular permeability, edema, and widespread inflammation with consequent damage in multiple organs, including nerves and muscles. The cytokine storm also triggers hypercoagulation cascades to cause small and large blood clots [5]. Combined hyperactivation of inflammatory markers, vascular injury, and coagulation factors contributes to Acute Respiratory Distress Syndrome (ARDS), kidney failure, liver injury, heart failure, myocardial infarction, as well as multiple neurological conditions [2,5]. A direct entry of SARS-CoV-2 into the brain has been described for other coronaviruses and may play a role in SARS-CoV-2's possible contribution to demyelination or neurodegeneration. Furthermore, the cytokines activated by SARS-CoV-2 can also trigger vasculitis in and around nerves and muscles, with or without a molecular mimicry, intended as cross-reactivity of immunoglobulins (formed in response to the viral antigens) with specific proteins on the myelin, axon, or neuro-muscular junction[4]. A direct invasion by the virus to the peripheral nerves may also potentially occur, however a prevalent immune-mediated etiology for peripheral and cranial neuropathies as well as damages to muscles in patients with COVID-19 represents a realistic hypothesis [4].

Neurological, muscular, and rheumatic skeletal muscle clinical manifestations of COVID-19 (Tab. I)

As SARS-CoV-2 presents neurotropic properties, neurological disease manifestations may occur in both symptomatic and asymptomatic patients [2]. In particular, various neurological manifestations have been described in COVID-19 patients, involving the central nervous system, peripheral nervous system, and skeletal muscles. Importantly, neurological manifestations could appear alone and might present as non-specific symptoms [6]. According to several published studies, patients with severe COVID-19 are more likely to develop neurological dysfunctions, among which acute cerebrovascular disease, consciousness disturbance, encephalopathy, prominent agitation and confusion, ischemic strokes of acute onset, or corticospinal tract signs. Some patients manifest only neurological symptoms, including headache, tiredness, malaise and signs of cerebral hemorrhage, or cerebral infarction. Cases of encephalitis, necrotizing hemorrhagic encephalopathy, strokes, epileptic seizures, or rhabdomyolysis associated with SARS-CoV-2 infection have also been described [2,4,6]. Facial weakness, difficulty breathing, being unable to stand or walk, or having difficulty weaning off respiratory ventilators may be in part due to Guillain-Barre syndrome (GBS) caused by COVID-19, as described in some reports [7]. Roman et al., on behalf of the World Federation of Neurology, have recently stressed the urgent need for international neuro-epidemiological collaboration in order to create local registries of cases with neurological manifestations and better define the short-term and long-term neurology of COVID-19 [8].

SARS-CoV-2, similar to SARS-CoV-1, can cause serious injury to cranial nerves, peripheral nerves, and muscles. Muscle weakness, fatigue or myalgia, and muscle atrophy are among the most commonly reported symptoms by patients with COVID-19 [2,9]. For instance, the prevalence of myalgia among currently published reports may range from 21% to more than 50% of affected patients. Moreover, myalgia tends to persist after cessation of viral shedding for a median time of 23 days [10]. In a retrospective study by Zhang et al., muscle ache was one of the independent predictors for worsening of symptoms and disease status in patients with COVID-19 [11]. In a Chinese retrospective case series by Mao et al., one of the first reports, conducted on 214 COVID-19 patients hospitalized in Wuhan, 8.9% presented peripheral nerve disease, and 7% had muscular injuries. Moreover, among patients with severe COVID-19, 19.3% had evidence of muscle injury. Similar findings have been reported for COVID-19 patients in other ICU settings [12]. Furthermore, hematologic biomarkers of inflammation, cardiac and muscle injury were found to be significantly elevated in patients with both severe and fatal COVID-19 [13]. Consistently, some reports have described patients with COVID-19-related myositis and rhabdomyolysis. All these patients presented elevated serum CK levels, as well as high serum levels of CRP, LDH and ferritin. In addition to myositis and rhabdomyolysis, critical-ill-

Table I. Main reported neurological, muscular and rheumatic clinical signs and symptoms associated with COVID-19 [2, 4-15].

Classification	Clinical manifestations and symptoms of COVID-19
Neurological	Acute cerebrovascular disease, consciousness disturbance, encephalopathy, prominent agitation and confusion, acute ischemic strokes, headache, tiredness, malaise, cerebral hemorrhage, cerebral infarction, encephalitis, necrotizing hemorrhagic encephalopathy, strokes, epileptic seizures
Neuromuscular	Muscle weakness, fatigue or myalgia, and muscle atrophy, peripheral nerve disease, muscle injury, myositis and rhabdomyolisis, exacerbations of myasthenia gravis
Rheumatic	Cytokine storm/Secondary Hemophagocytic lymphohistiocytosis (sHLH), Guillain-Barré syndrome (GBS), Kawasaki-like disease

ness myopathies, cachexia and sarcopenia have also been described in patients with COVID-19 [2,9]. While there are no current reports of de-novo cases of myasthenia gravis caused by COVID-19, episodes of SARS-CoV-2-related exacerbation of pre-existing myasthenia gravis have been recently reported [9,14].

On the other hand, the immune dysregulation caused by COVID-19 may trigger or worsen auto-immunity and rheumatic disorders in genetically susceptible subjects. Several atypical clinical and laboratory manifestations of the disease mimicking rheumatic skeletal muscle diseases (RMDs) have been reported [15], including musculoskeletal and cardiovascular manifestations, as well as multisystem auto-inflammatory/auto-immunitary syndromes. In addition, laboratory reports of positive antinuclear antibodies (ANA), antiphospholipid antibodies, lupus anti-coagulant assay and increased level of D-dimer have been reported with COVID-19, suggesting the risk for persisting intermediate to long-term immune dysregulation [4,15]. Furthermore, the potential adverse effects of antiviral or immune-modulating therapies used to treat COVID-19 should be attentively monitored and analyzed, as several findings of musculoskeletal adverse reactions following the use of these drugs have been reported [15].

Impact of drugs with known iatrogenic effects on skeletal muscle in COVID-19 patients

As known, several drugs used for diverse therapeutic interventions may cause adverse effects and toxicities in skeletal muscle tissues. A drug-induced, also defined as "toxic", myopathy is the acute or subacute manifestation of myopathic symptoms such as muscle weakness, myalgia, creatine kinase (CK) elevation, or myoglobinuria in patients with no pre-existing muscle diseases when exposed to certain classes of drugs [16,17]. Many of these symptoms have been previously mentioned in this article as potential COVID-19 clinical signs. Drugs can cause muscle tissue toxicity through different mechanisms, for instance by directly affecting muscle organelles such as mitochondria, lysosomes, or myofibrillar proteins; or by triggering immunologic or inflammatory reactions; or by disrupting of electrolyte or nutritional balance, thus compromising the muscle physiologic functions [16,17]. The medications most commonly associated with toxic myopathy include statins, amiodarone, chloroquine, hydroxychloroquine, colchicine, certain antivirals, and corticosteroids [18,19]. Some of these drugs have been used and are being currently used to treat patients with COVID-19, more often at an advanced stage of the disease. For instance, as also shown in Table II, a long-term treatment with chloroquine and hydroxychloroquine may cause myopathy and neuromyopathy [15], while arthralgia, back pain, osteonecrosis, and vasculitis may occur during lopinavir-ritonavir therapy; musculoskeletal pain and myalgia may follow interferon therapy, which rarely can also lead to drug-induced RMDs, such as rheumatoid arthritis, lupus, Sjogren syndrome and myositis, sarcoidosis, and vasculitis. On the other hand, the current safety profile of remdesivir, one of the anti-virals mainly used to treat COVID-19 worldwide, is still incomplete and, to date, has shown no adverse reactions on muscles [20]. We suggest it is important for clinicians to be aware of iatrogenic effects of certain classes of drugs on skeletal muscle, especially for the following reasons: 1) some of the COVID-19 symptoms on muscles may shadow the toxicities caused by COVID-19 treating drugs and thus let them unrecognized, causing irreversible damages; 2) the noxious effects on skeletal muscles of COVID-19 and drugs used to treat COVID-19 might add up and worsen the symptoms and injuries caused; 3) the effects on muscles of all drugs currently used to treat COVID-19 on large populations of patients, such as remdesivir and corticosteroids, should be attentively monitored and signaled to pharmacovigilance authorities; 4) the use of COVID-19 drugs with potential adverse effects on muscles should be attentively considered and weighted in patients who already receive other agents known to be toxic on muscle tissues, such as statins; 5) the use of COVID-19 drugs with potential adverse effects on muscles should be attentively considered and weighted in patients with pre-existing neuromuscular disorders.

Table II. Potential expected adverse effects on skeletal muscle of main drugs currently used in Italy to treat COVID-19.

Main drugs used to treat COVID-19	Potential musculoskelatal adverse event
Hydroxychloroquine	Myopathy and neuromyopathy
Lopinavir-ritonavir	Musculoskeletal pain, arthralgia, osteonecrosis, vasculitis
Darunavir/cobicistat	Muscle weakness, muscle pain, muscle injury, osteonecrosis
Azythromicin	Muscle weakness or difficulty with muscle control
Heparin	Back pain, joint pain, stiffness, or swelling
Dexamethasone	Muscle weakness, muscle pain or tenderness, muscle atrophy, myopathy, osteonecrosis, neuropathic arthralgia
Ribavirin	Arthralgia, musculoskeletal pain, backache, myositis
Canakinumab	Arthralgia, musculoskeletal pain, backache
Remdesivir	Not reported
Convalescent plasma	Not reported; risk of hyperimmune reactions

List of drugs used in the clinical practice for the treatment of COVID-19 in Italy, based on AIFA website (last consulted on 14th October 2020) (https://www.aifa.gov.it/aggiornamento-sui-farmaci-utilizzabili- per-il-trattamento-della-malattia-covid19 ; https://www.aifa.gov.it/programmi-di-uso-compassionevole-covid-19).

COVID-19 in patients with neuromuscular disorders

Given the neurotropic properties and neuro-invasive potential of SARS-CoV-2, a special attention should be addressed to patients with pre-existing neuromuscular disorders, including muscle disorders (e.g., muscular dystrophies, congenital myopathies, metabolic myopathies, inflammatory myopathies, and muscle channelopathies), diseases of the neuromuscular junction (e.g., either acquired or congenital myasthenic syndromes), peripheral nerve disorders (e.g., dysimmune neuropathies, familial amyloid neuropathies, and Charcot-Marie-Tooth disease), and spinal muscular atrophies, in order to prevent and early recognize neuromuscular complications that may be – directly or indirectly – related to the viral infections [21]. These diseases constitute a group of very heterogeneous conditions, most often of genetic or autoimmune origin, and can affect both children and adults to a degree that varies widely from one individual to another. A few reports on the topic have been recently published and stressed the need for clinical research to develop evidence-based guidelines to minimize morbidity and mortality due to COVID-19 in patients with neuromuscular disorders [21,22]. As explained in a complete review by Guidon A and Amato A, the risks for these patients depend on several factors, including the specific neuromuscular disease, other co-morbidities, age, and type of immuno-therapy they receive [22]. In highly susceptible patients, it is hypothesized that novel disorders such as Guillain-Barré Syndrome, myopathies, myositis and polyneuropathies may occur. Case reports have recently been published proving this risk, which is related to immune dysregulation and molecular mimicry between specific viral antigens and proteins expressed on peripheral nerves, ultimately causing auto-immune damages on myelin or axon of peripheral nerves. A probably more significant risk resided in the exacerbation or disease progression of pre-existing rare neuromuscular disorders, or the unmasking of previously unrecognized ones, both inherited and immune-mediated. As mentioned, according to a recent retrospective study, COVID-19 has caused the exacerbation of myasthenia gravis [14,21]. Experts are therefore expecting increased rates of disease worsening and incidence of novel diagnoses during the pandemic, and in March 2020 the Association of British Neurologists had already published a "guidance on COVID-19 for people with neurological conditions, their doctors and carers" [22]. A guidance has also been released in June by the French Rare Health Care for Neuromuscular Diseases Network [23]. These guidelines suggest that patients with motor neuron diseases (e.g., amyotrophic lateral sclerosis, spinal muscular atrophy) and hereditary neuropathies, and patients with various muscular dystrophies, including myotonic dystrophy, and metabolic diseases (e.g., Pompe disease), who present ventilatory muscle involvement or cardiomyopathy may be particularly susceptible to infection and at increased risk for severe COVID-19. Moreover, patients with metabolic myopathies (e.g., lipid storage diseases and mitochondrial disorders) are at increased risk of rhabdomyolysis. It is also postulated that patients who develop COVID-19 may not return to their prior baseline. Furthermore, use of immunosuppressive therapies may put patients with neuromuscular disorders at increased risk of contracting COVID-19 and developing more severe symptoms [22,23]. In general, experts recommend patients to continue their treatments with steroid/immunosuppressants in the absence of any manifestations suggestive of COVID-19, and avoid sudden interruptions that may trigger a disease flare. In case of COVID-19 symptoms, patients may temporarily hold the therapy based on their neurologist advice [22]. Interestingly,

a recent Italian survey conducted in specialized neuromuscular centers has pointed out a significant disruption of clinical and support services for patients with neuromuscular diseases in the acute phase of the pandemic, particularly in terms of rehabilitative services and on-site outpatient visits [24]. The expected (or actually ongoing) "second wave" of the virus spread should not find our systems unprepared but instead accelerate the adoption of novel modalities, including tele-medicine services, for ensuring quality care and thorough monitoring to patients with neuromuscular disorders.

Conclusions

The SARS-CoV-2 pandemic has shocked the world and most health care systems, even in wealthy nations with advanced and renowned systems. Following the initial outbreak and the extensive efforts in all fields of clinical research, more and more evidence is being brought out and shared in order to tackle and, with effective treatments and vaccines, eradicate the virus and the disease. Many reports have highlighted the clinical manifestations caused by the effects of SARS-CoV-2 on muscles. Several research teams are currently investigating the muscular and neuromuscular damages that may be triggered by the virus or by the drugs used to treat COVID-19. In this review, we summarized the state-of-the-art on the topic and suggest a more thorough attention on symptoms and laboratory markers of muscle damages, especially considering that some of the therapies used to treat COVID-19 may be toxic on muscles. Finally, patients with RMDs and patients with auto-immune or genetic neuromuscular disorders should be attentively monitored for exacerbation of symptoms or disease flares. Based on a recent search on clinicaltrials.gov (as of October 12th 2020), we only found 5 clinical studies about COVID-19 and muscle, 4 of which are observational studies or disease registries in special populations of patients (patients with neuromuscular disorders and patients with inflammatory rheumatic diseases). While real-world evidence is essential for the understanding of the disease, especially considering the long-term effects of the viral infection, pharmacovigilance registries and experimental trials are also needed. It is about time for governments to substantially finance independent rigorous clinical research and encourage research networks and public sharing of findings.

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The correlation between cardiac and skeletal muscle pathology in animal models of idiopathic inflammatory myopathies

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Idiopathic inflammatory myopathies (IIMs) represent a heterogeneous group of disorders in which skeletal muscle is inappropriately targeted by the immune system. IIMs are characterized by inflammation of muscle and varying degrees of muscle dysfunction. Extra-muscular manifestations may involve heart, skin, joints, lungs, and gastrointestinal tract. Cardiovascular involvement is a feared event because is one of the leading causes of mortality in IIM patients. As the myocardium shares many features with the skeletal muscle, it is supposed that it can be affected by the same inflammatory processes, which take place during the different forms of IIMs. However, the full extent of this link and the mechanisms behind it are still not fully understood. Animal models have greatly improved our understanding of IIM pathomechanisms and have proven to be a useful tool for discovering therapeutic drug targets. Here we report the evidence of heart muscle involvement in different animal models of spontaneous IIMs, assuming a common autoimmune mechanism and presenting them as study models for human pathology.

Key words: neuromuscular disorders, myocarditis, myositis, idiopathic inflammatory myopathy, animal models

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Introduction

Professor Giovanni Nigro (Fig. 1) in his research and diagnostic activity has strongly supported the relationship between skeletal muscle and cardiac muscle diseases [1]. Skeletal and cardiac muscle share many structural and functional features; what affects one type of muscle is often associated with damage to the other as well [2]. This assumption is today well defined [3-6], but often still underestimated. Moreover, the treatment usually focuses on one of these tissue without addressing the other tissue involved in the same disease process; this can make general treat- ment less effective [2].

The study of cardiac pathology in animal with idiopathic inflammatory myopathies (IIMs) is an input that came from Prof. Giovanni Nigro and his determination to understand the correlation between heart and muscle pathology in both dystrophies and inflammatory diseases.

IIMs represent a heterogeneous group of disorders in which skeletal muscle is inappropriately targeted by the immune system [7]. The mean global prevalence of the IIMs in humans range from 4.27 to 7.89/100,000 [8].

Figure 1. From the left side: Lucia Ines Comi, Serenella Papparella, Valerie Askanas, Orlando Paciello, Giovanni Nigro and Corrado Angelini, at the XI International Congress on Neuromuscular Diseases in Istanbul (Turkey), 2006.

This group of diseases was typically divided into several subtypes such as dermatomyositis (DM), polymyositis (PM), inclusion body myositis (IBM), immune-mediated necrotizing myopathy (IMNM) and overlapping syndromes, a common feature of which is muscle inflammation leading to their progressive weakness; however, the skin and internal organs can also be affected [8]. The autoimmune origin is often regarded as the autoantibodies specific for myositis are detected in the serum of 50-70% patients [8].

Cardiac involvement in IIM was first reported by Oppenheim in 1899 [9]. Currently, more and more evidence has accumulated in support of a link between IIMs and cardiac involvement [10]. Since the myocardium shares many features with skeletal muscle, it is assumed that it can be affected by the same inflammatory process, which occur during the different forms of IIMs. However, the full extent of this link and the mechanisms behind it are still not fully understood [11].

Cardiac involvement is more commonly reported in patients with PM/DM, while patients with IBM have a lower risk [10]. Morphologically, in PM/DM the myocardium shows an inflammation similar to that of skeletal muscle [10]. An increased risk of myocardial infarction and venous thromboembolism has also been reported in patients with IIMs [4]. However, cardiac disease in IIM is most commonly subclinical and data in the literature suggest that rhythm disturbances are the most common subclinical cardiac manifestation of PM/DM, while congestive heart failure is the most frequently reported cardiac complication, and occurs in 10-15% of patients [10]. Congestive heart failure can develop at any time in the course of skeletal muscle disease, and even in remission state [10].

Cardiac involvement in IIMs is a feared event because it is one of the most common cause of patient death [4,10].

Increasing evidence suggests that the IIMs result from certain environmental exposures in genetically susceptible individuals [12,13]. The HLA 8.1 ancestral haplotype is a key risk factor for IIM in humans, and several genetic variants associated with other autoimmune diseases have been identified as IIM risk factors. Environmental risk factors are less well studied than genetic factors but could include viruses, bacteria, ultraviolet radiation, smoking, occupational and perinatal exposures and a growing list of drugs (including biologic agents) and dietary supplements [13].

Investigations have shown that a variety of infections not only cause infectious myopathies but could also be

possible triggers for IIM [12,13]. The link between infectious agents and IIM development has been determined through case reports, epidemiological investigations and animal models [12,13]. Examples include hepatitis B virus in PM and DM; hepatitis C virus in IBM; retroviruses, in particular human immunodeficiency virus (HIV) and human T-lymphotropic virus-1, in PM, DM and IBM; Toxoplasma spp. and Borrelia spp. in PM and DM; and influenza, picornaviruses and echoviruses in PM, DM and juvenile dermatomyositis [12,13].

Here we report the evidences of cardiac muscle involvement in different animal models of spontaneous IIMs, assuming a common autoimmune mechanism and presenting them as study models for human pathology.

Inflammatory myopathy and cardiomyopathy in cats associated with Feline Immunodeficiency Virus (FIV) infection

FIV infection is associated with an inflammatory myopathy (IM) and myocarditis in adult cats [14,15]. HIV is similarly implicated in a form of IM and myocarditis in humans [15-17]. This model has so far been poorly characterized and there is little information in the literature, however, the characteristics of this myopathy are comparable to human PM [14,15].

No clinical signs have been associated with this IM, however, an increase in serum CK values has been reported [14]. Needle electromyography may be characterized by mild to moderate abnormal spontaneous activity. Furthermore, a mixture of positive acute waves and fibrillation potentials can be detected in a multifocal pattern [14].

The pelvic limb muscles are more frequently affected than the thoracic limb muscles. The vastus lateralis is the most frequently affected muscle while the brachial triceps is the least affected muscle [14].

Histologically, this IM is characterized by perivascular and endomysial multifocal infiltration of CD8+ T lymphocyte (Fig. 2A). Sometimes, these lymphocyte infiltrate non-necrotic myofibers. Myofiber necrosis and phagocytosis has also been reported [14].

FIV infection in adult cats has also been associated with myocarditis characterized by a coalescing multifocal inflammatory infiltrate mainly composed of lymphocytes and, to a lesser extent, of macrophages, neutrophils, and plasma cells (Fig. 2B). A variable degree of interstitial fibrosis has also been reported [15]. Hypertrophic cardiomyopathy (HCM) is also described in cats associated with FIV infection. Clinical manifestations included dyspnea, lethargy, anorexia and vomiting [15].

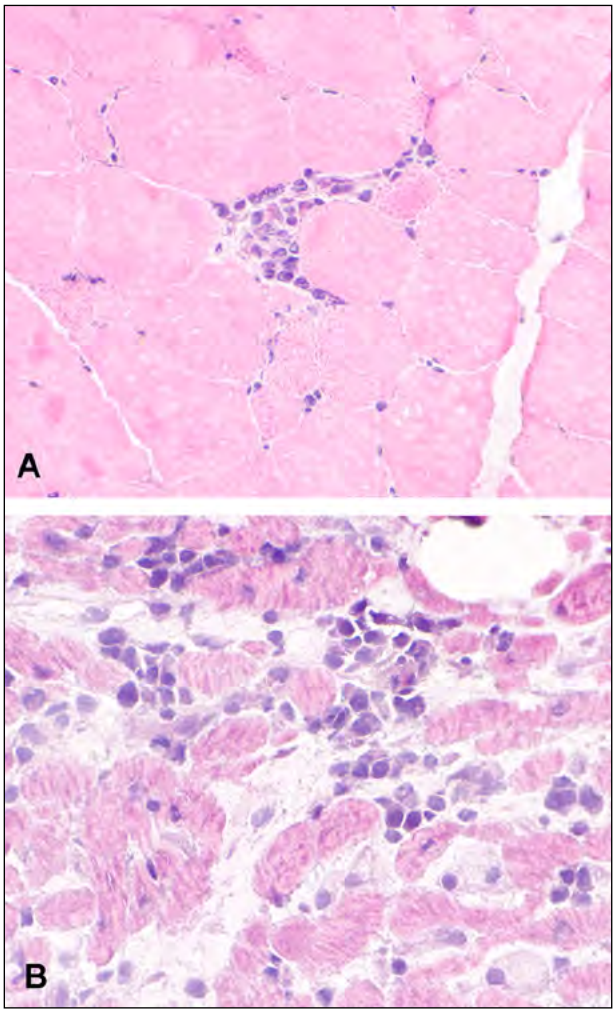

Figure 2. Histopathological findings in feline immunodeficiency virus (FIV) associated myositis and myocarditis, cat, hematoxylin and eosin (magnification 400 X). A) Skeletal muscle: the endomysium of the skeletal muscle is expanded by a lymphocytic infiltrate; B) Myocardium: the myocardium is infiltrated by numerous lymphocytes and plasma cells.

Canine inflammatory myopathy and cardiomyopathy associated with Leishmania infantum infection

In dogs, *Leishmania infantum* infection is associated with an IM with many similarities with the human PM (Fig. 3A-B) [18,19].

IM in these dogs is often subclinical [20]. The most common clinical signs of leishmaniasis are skin lesions, lymphadenopathy, hepatosplenomegaly, weight loss, onychogryphosis and ocular lesions. Rarely, these signs can be associated with clinically evident neuromuscular signs, such as paraparesis [20]. With electromyography,

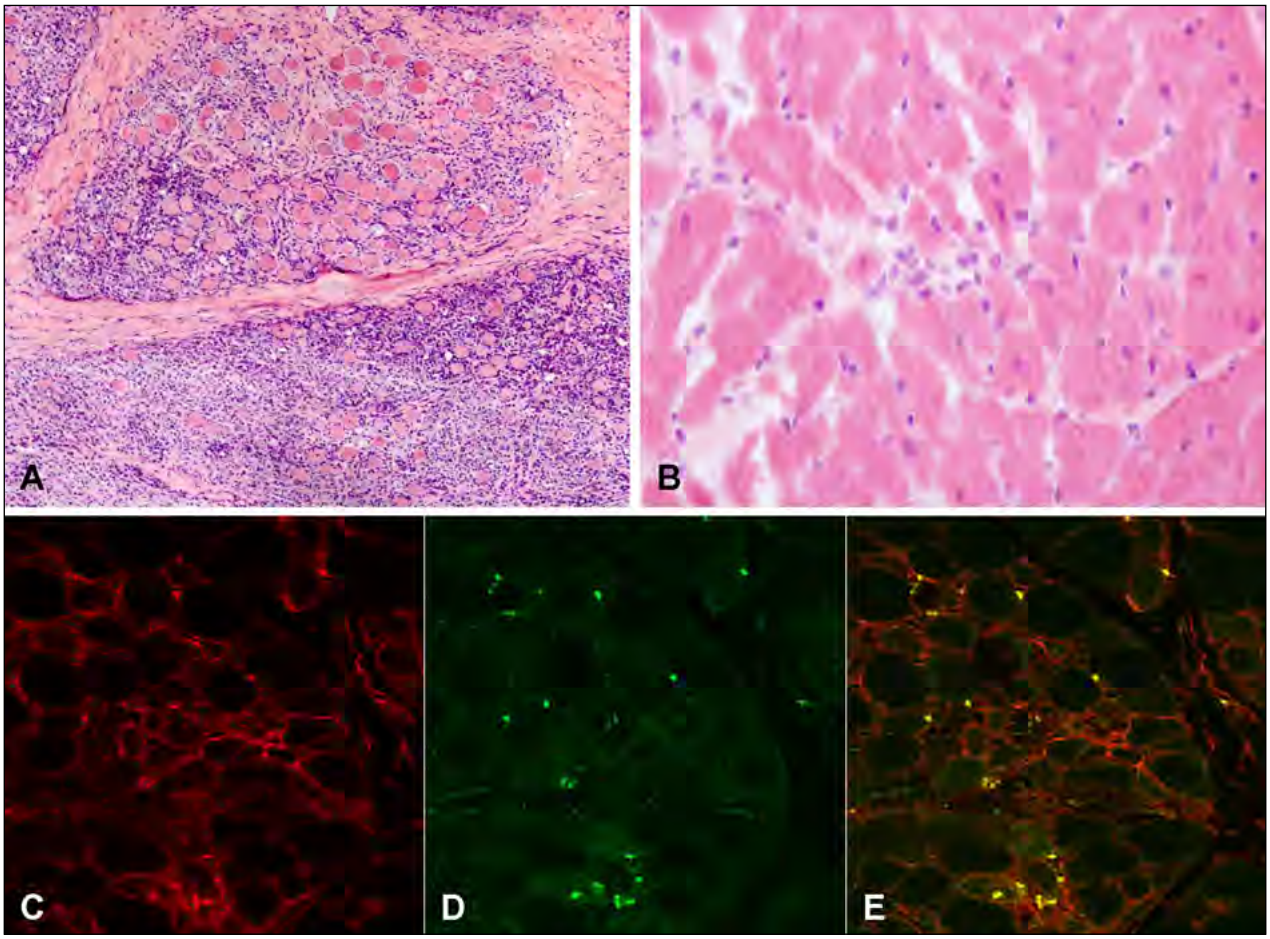

Figure 3. Histopathological findings in *Leishmania infantum* associated myositis and myocarditis, dog, hematoxylin and eosin. A) Skeletal muscle is effaced by a severe and diffuse lymphocytic and macrophagic infiltrate (magnification 100 X); B) Myocardium focally infiltrated by lymphocytes (magnification 400 X); C) Immunofluorescence detection of MHC I (red, TRIC, magnification 400 X); D: Immunofluorescence detection of *Leishmania spp.* (green, FITC, magnification 400 X); E: Colocalization of MHC I and *Leishmania spp.* (magnification 400 X).

fibrillation potentials, positive sharp waves and complex repetitive discharges can generally be detected even in asymptomatic dogs [20].

Morphologically, the inflammatory infiltrate in skeletal muscle is mainly perivascular in the perimysium and multifocally surrounds the muscle fibers in the endomysium (Fig. 3A). The inflammatory cells are mainly CD8+ T lymphocytes and macrophages with fewer CD4+ T lymphocytes [18,20]. Marked variation in fiber size, including atrophic and hypertrophic fibers, and different disseminated necrotic muscle fibers can usually be observed. Aspects of muscle regeneration can also be observed, including myotubes and type 2C fibers [18]. In the chronic stages, endomysial and perimysial thickening due to fibrosis can be observed. Usually, many muscle fibers show immunohistochemical sarcolemmal expression of Major Histocompatibility Complex (MHC) class I (Fig. 3C-D) and class II [18]. Moreover, CD8+ T lymphocytes invade histologically normal muscle fibers expressing MHC class I antigens (CD8/MHC-I complexes) supporting an immune-mediated pathogenetic hypothesis [18].

Dogs infected with *Leishmania infantum* also show myocarditis characterized by an inflammatory infiltrate similar the that reported in skeletal muscle (Fig. 3B). Interstitial fibrosis and sarcolemmal expression of MHC class I and MHC class II antigens have also been reported [19].

Several pathogenetic mechanisms have been hypothesized [18]; however, the most supported is an antibody-mediated autoimmune mechanism [21]. We hypothesize that the autoantibodies produced by dogs with leishmaniasis may be directed against one or more proteins shared by skeletal and cardiac muscle, triggering immune-mediated damage in both tissue [18,19].

Inflammatory myopathy in Syrian hamster associated with Leishmania infantum infection

Syrian hamster infected with *Leishmania infantum* develop an IM that shows close similarities to human PM [22].

Clinically, during the chronic phase of the disease (> 3 months after infection) infected hamsters have lost weight and are weaker. They are usually asthenic, have reduced activity and peeling in the extremities. Skeletal muscles usually appear moderately atrophic. In addition, an increase in serum muscle enzymes has been reported, with an increase in LDH and AST values more than 5 times and an increase in CK even more than 50 times [22].

This IM is histologically characterized by a multifocal inflammatory endomysial infiltrate composed mainly of CD8+ T lymphocytes. In addition, numerous perivascular aggregates of macrophages and CD4+ T lymphocytes have also been reported. Numerous muscle fibers with class I and II MHC sarcolemmal positivity has also been reported. Furthermore, the presence of some CD8+ lymphocytes invading histologically healthy muscle fibers expressing MHC class I antigens (CD8/MHC I complex) support an autoimmune hypothesis [22].

The Syrian hamster infected with *Leishmania infantum* also develops myocarditis characterized by an inflammatory infiltrate similar the that reported in skeletal muscles. (Paciello et al., personal observation).

Inflammatory myopathy in sheep associated with *Sarcocystis tenella* infection

For years, it has been argued that Sarcocystis infection almost always did not cause injury to the ruminant's muscle or is associated with an eosinophilic myositis in the case of cyst rupture. We have defined that *Sarcocystis tenella* infection is associated with an IM in sheep [23].

In intermediate hosts, such as sheep, infection is commonly asymptomatic and the presence of muscle cysts is considered an incidental finding [23]. However more that 95% of infected animals show subclinical myopathy [23].

Histologically, this myopathy is characterized by a multifocal inflammatory endomysial infiltrate mainly composed of CD8+ lymphocytes, occasionally centered around parasitic and non-parasitic fibers and rarely arranged in perivascular cuffs (Fig. 4A). Variability of fiber diameter and different disseminated necrotic muscle fibers invaded by macrophages are reported [23].

We also reported widespread sarcolemmal immunopositivity for MHC I and MHC II in almost all cases and variable expression of MHC I antigen on the cyst wall [23]. Moreover, occasionally CD8+ cells invade non-necrotic parasitized and non-parasitized fibers [23].

Sheep infected with *Sarcocystis tenella* also show myocarditis characterized by an inflammatory infiltrate similar the that found in skeletal muscle (Fig. 4B).

Inflammatory CD8+ T lymphocytes infiltrate, sarcolemmal immunopositivity to MHC I and CD8+ T lymphocytes invading non-necrotic muscle fibers suggest that parasitized muscle fibers might play an active role in antigen presentation and stimulating inflammatory response [23].

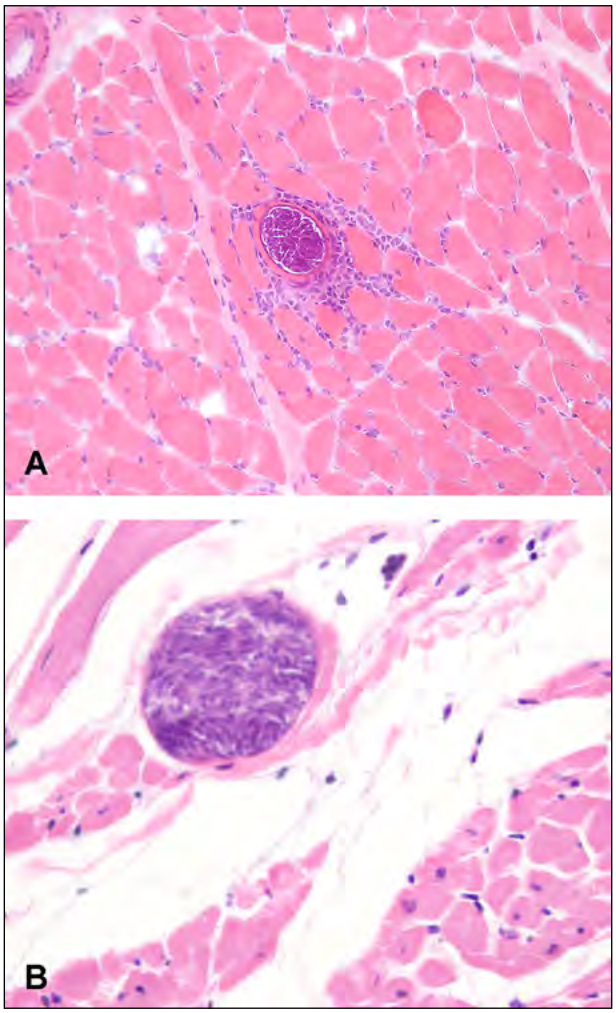

Figure 4. Histopathological findings in *Sarcocystis spp.* associated myositis and myocarditis, sheep, hematoxylin and eosin (magnification 200 X). A) Skeletal muscle: a lymphocytic infiltrate surrounds and infiltrate a parasitized muscle fiber; B) Myocardium: thin-walled septate sarcocyst containing myriad of banana-shaped bradyzoites in the myocardium with scattered inflammatory cells.

Inflammatory myopathy in horses associated with piroplasmosis

Equine piroplasmosis is a protozoal disease caused in horses by two apicomplexan hemoprotozoa, *Theileria equi* and *Babesia caballi* [24]. Equine piroplasmosis has been associated with an IM characterized by circulation of anti-muscle antibodies [24].

Clinically, horses with chronic piroplasmosis can develop poor performance and muscle atrophy. The serum activity of CK, AST and LDH is usually slightly elevated [24].

Histologically, the main myopathic change is a multifocal lymphocytic infiltrate often organized in cuffs around the perimysial and endomysial blood vessels and less frequently expanding multifocally into the endomysium. The inflammatory infiltrate is mainly composed of both CD8+ and CD4+ T lymphocyte, fewer macrophages and rare scattered CD79α+ B lymphocytes [24].

Various degrees of atrophy of nonangular fibers, necrotic fibers invaded by macrophages, mild perimysial fibrosis were also observed. Mitochondrial abnormalities include ragged blue fibers with SDH and fibers with a moth-eaten appearance with COX and NADH stains [24].

Muscle fibers immunohistochemically overexpress MHC I and MHC II [24].

Increased mRNA levels of IL-12, TNF-α, and IFN-γ were found in the muscles of affected animals, while no changes in IL-10 mRNA levels were observed. Moreover, DNA from *Theileria equi* or *Babesia caballi* was not detected in muscle samples affected, by RT-PCR [24].

Involvement of the heart during piroplasmosis has been well established in dogs, but there is little sporadic information in the literature on heart involvement during piroplasmosis in horses [25,26]. Further studies are needed to better clarify the involvement of the heart in this species.

Conclusions and perspectives

In this review we have summarized our studies and observations on the heart muscle involvement during inflammatory myopathies. We have shown in previous papers that various pathogens such as viruses and protozoa responsible for myositis in animals can also be responsible for myocarditis in the same animals. This possibility should always be considered during the diagnostic process and in the therapy of myositis in animal and humans [4,10].

The pathogenesis of most immune-mediated diseases is related to chronic organ inflammation that can be caused by specific interactions between genetic and environmental risk factors. In these diseases, immune activation often involves both innate and adaptive and non-immune mechanisms; however, the details and interactions of the various pathways are generally unclear and new animal models can be very valuable in elucidating these mechanisms [7,13].

Infections are the main actors in the environmental factors which modulate the development of autoimmune diseases. The underlying mechanisms are multiple and complex, probably different according to pathogens [27]. In these cases, as in the studies we reported, the most interesting observation is that even in the absence of the pathogen, the damage to the muscle persisted as immune-mediated damage [7]. We hypothesized that the pathogenetic mechanism underlying this form of IMs, due to the numerous shared structural features, may also involve the myocardium.

Therefore, these animals may be useful study models for IIM and myocarditis in humans. Furthermore, these observations lead to the hypothesis that in IIM it must always be considered that an etiological agent may have been involved as a primary or secondary cause of the disease. These pathogenetic mechanisms could be common to animals and humans and should be considered during both the diagnostic and therapeutic process for patients suffering from myositis and myocarditis.

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Cutaneous and metabolic defects associated with nuclear abnormalities in a transgenic mouse model expressing R527H lamin A mutation causing mandibuloacral dysplasia type A (MADA) syndrome

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Conflict of interest
The Authors declare no conflict of interest

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***LMNA* gene encodes for lamin A/C, attractive proteins linked to nuclear structure and functions. When mutated, it causes different rare diseases called laminopathies. In particular, an Arginine change in Histidine in position 527 (p.Arg527His) falling in the C-terminal domain of lamin A precursor form (prelamin A) causes mandibuloacral dysplasia Type A (MADA), a segmental progeroid syndrome characterized by skin, bone and metabolic anomalies. The well-characterized cellular models made difficult to assess the tissue-specific functions of 527His prelamin A. Here, we describe the generation and characterization of a MADA transgenic mouse overexpressing 527His *LMNA* gene, encoding mutated prelamin A. Bodyweight is slightly affected, while no difference in lifespan was observed in transgenic animals. Mild metabolic anomalies and thinning and loss of hairs from the back were the other observed phenotypic MADA manifestations. Histological analysis of tissues relevant for MADA syndrome revealed slight increase in adipose tissue inflammatory cells and a reduction of hypodermis due to a loss of subcutaneous adipose tissue. At cellular levels, transgenic cutaneous fibroblasts displayed nuclear envelope aberrations, presence of prelamin A, proliferation, and senescence rate defects. Gene transcriptional pattern was found differentially modulated between transgenic and wildtype animals, too. In conclusion, the presence of 527His Prelamin A accumulation is further linked to the appearance of mild progeroid features and metabolic disorder without lifespan reduction.**

Key words: mandibuloacral dysplasia type A, p.Arg527His pathogenic variant, transgenic mouse model, prelamin A

Introduction

Lamins A/C are major components of the nuclear lamina, playing a fundamental role in the maintenance of the size and shape of the nucleus and in several nuclear processes such as transcription, chromatin organization and DNA replication [1]. Lamins are encoded by *LMNA* gene, located on 1q.21.1 chromosome region. Pathogenetic variants in *LMNA* gene cause a group of heterogeneous genetic disorders, called laminopathies ranging from muscle-skeletal, cardiac, and peripheral nervous diseases to progeroid diseases. In particular, homozygous or compound heterozygous variants in *LMNA* gene have been associated to the first identified progeroid laminopathy, known as Mandibuloacral dysplasia type A (MADA; OMIM #248370) [2]. This rare autosomal recessive disorder is characterized by the development of mild growth retardation, craniofacial anomalies with mandibular hypoplasia and prominent appearance of the eyes, generalized osteoporosis, osteolysis of terminal phalanges and clavicles, overcrowded teeth and delayed closure of cranial suture usually from the first decade of life. Patients present with lipodystrophy pattern type A, characterized by loss of subcutaneous fat in the extremities and normal or heightened presence of fatty tissue in the neck and trunk. These clinical features are often accompanied by metabolic syndrome including insulin resistance, impaired glucose tolerance and diabetes. In the second decade of life, mild progeroid features become visible, such as thin and sparse nose, thin hair (in both sexes; alopecia is described in males, but is generally less evident e less precocious compared to other progeroid syndromes); the skin appears thin, wrinkled and atrophic over the acral region, with visible veins, and with patchy brown hyperpigmentation area (Acanthosis nigricans) [3-6]. Skeletal and cardiac muscle are not affected in most MADA patients. However, muscle weakness overlapping with other laminopathies have been described in few patients [7].

The most common causative pathogenic variant of MADA disease is the homozygous transition c.1580G>A, mapping in the exon 9 of *LMNA* gene, which changes Arginine 527 in Histidine (p.Arg527His) in the C-terminal domain of lamins A/C [2]. This domain presents a carboxyterminal CAAX (cysteine-aliphatic-aliphatic-any aminoacid) motif involved in a complex post-translational processing to produce mature lamin A from the precursor protein, prelamin A. After farnesylation of the cysteine residue, -AAX cleavage, and cysteine methylation, prelamin A undergoes a second proteolytic cleavage removing an additional 15 C-terminal amino acids, producing the mature lamin A protein [8]. Pathogenic variants at C-terminus of lamins A/C, as in MADA, cause an accumulation in the nucleus of prelamin A having deleterious consequences on many cells and tissues, and disease severity is often related to prelamin A abundance [6]. Thus, the first pathogenic event in MADA is the toxic accumulation of mutated prelamin A, provoking abnormal nucleus morphology and a disruption of nuclear envelope organization as demonstrated by anomalous distribution of emerin, SUN1 e SUN2, main nucleoskeleton component, shown in cultured fibroblasts from affected individuals [9-11]. Moreover, accumulation of prelamin A has deleterious consequences on cellular differentiation in specific tissues, explaining thus some MADA clinical features. Noteworthy, lipodystrophy can be explained by impaired preadipocytes differentiation; in fact, accumulation of prelamin A in these cells can provoke a reduction of SREBP1, the adipocytes transcription factor, due to the binding of prelamin A to SREBP1 and its subsequent admission in the nuclear rim; retention of SREBP1 causes the down-regulation of PPARγ expression reducing thus the rate of preadipocytes differentiation [12].

Accumulation of prelamin A is involved in impairment of bone tissue turnover causing an excessive production of TGF-β2 levels, a cytokine acting on monocytes to commit them to osteoclastogenesis, from osteoblasts. Increased TGF-β2 levels trigger elevated secretion of osteoprogerin (OPG) and catepsin K, activating a non-canonical pathway of osteoclast differentiation and increasing resorption activity [13-15]. Moreover, previous studies found elevated serum levels of matrix metalloproteinase 9 (MMP-9) in MADA patients; such evidence suggests a role of this enzyme in the regulation of bone remodeling, bone resorption and cartilage damage [16]. In addition, accelerated aging in MADA resembles cellular aspects of physiological aging, as nuclear enlargement, and heterochromatin loss. Prelamin accumulation is a trigger of chromatin reorganization, likely mediated by different anchorage or activity of epigenetic factors in the presence of diverse levels of prelamin A. Thus, epigenetic enzymes, such as HDAC2 or SIRT1, are affected in MADA cells, and an increased solubility of heterochromatin protein 1 beta (HP1β) is observed, causing increased histone H4K16 and H3K9 acetylation and decreased H3K9 trimethylation, all age-associated epigenetic marks [7,10,17]. Moreover, similarly to other progeroid disorders, MADA cells expressing p.Arg527His show endogenous DNA damage, genomic instability and persistence of unrepaired damage DNA features, probably caused by prelamin A accumulation and, consequently, impaired recruitment of DNA repair protein to the DNA lesion [18].

The complexity of phenotype in MADA disease could be also explained by a tissue-specific gene expression pattern. MADA fibroblasts present specific up- and downregulation of expression of genes involved in many cellular processes, such as lipid metabolism, cell cycle

checkpoint, cell adhesion, electron transport and transcription [19]. These data could confirm the main role of lamin A in nuclear transit of transcription factors and, consequently, in transcriptional regulation.

To further provide insights about the consequences of p.Arg527His on the prelamin A accumulation affecting mechanical integrity of the nucleus as well as signaling pathways, we generated a transgenic mouse line overexpressing the most frequent human MADA mutation in *LMNA* gene in order to understand its contribution to the pathogenesis of the disease.

Materials and methods

Construct of 527His LMNA plasmid

Human *LMNA* coding sequence (NCBI RefSeq NM_170707) containing the homozygous c.1580G>A substitution has been amplified from mRNA obtained by a MADA patient using the following primers pairs containing the BamH1 and EcoRI restriction sites: R527H-Fw GGATCCATGGAGACCCCGTCCCAG and R527H-Rv CTTAAGTTACATGATGCTGCAGTTC. After Sanger sequencing analysis, mutated human *LMNA* cDNA was inserted between BamH1 and EcoRI restriction sites of the pcDNA3.1 expression vector (ThermoFisher Scientific, Waltham, MA USA) (Fig. 1A).

Generation of 527His LMNA transgenic mice

Transgenic mice were generated at SEAT 44 CNRS Transgenic Mouse Facility at Villejuif (France). PvuI was used to linearize the recombinant 527His *LMNA* plasmid. Six to seven-week-old C57BL/6J female mice were superovulated by pregnant mare serum gonadotropin and human chorionic gonadotropin. Females were

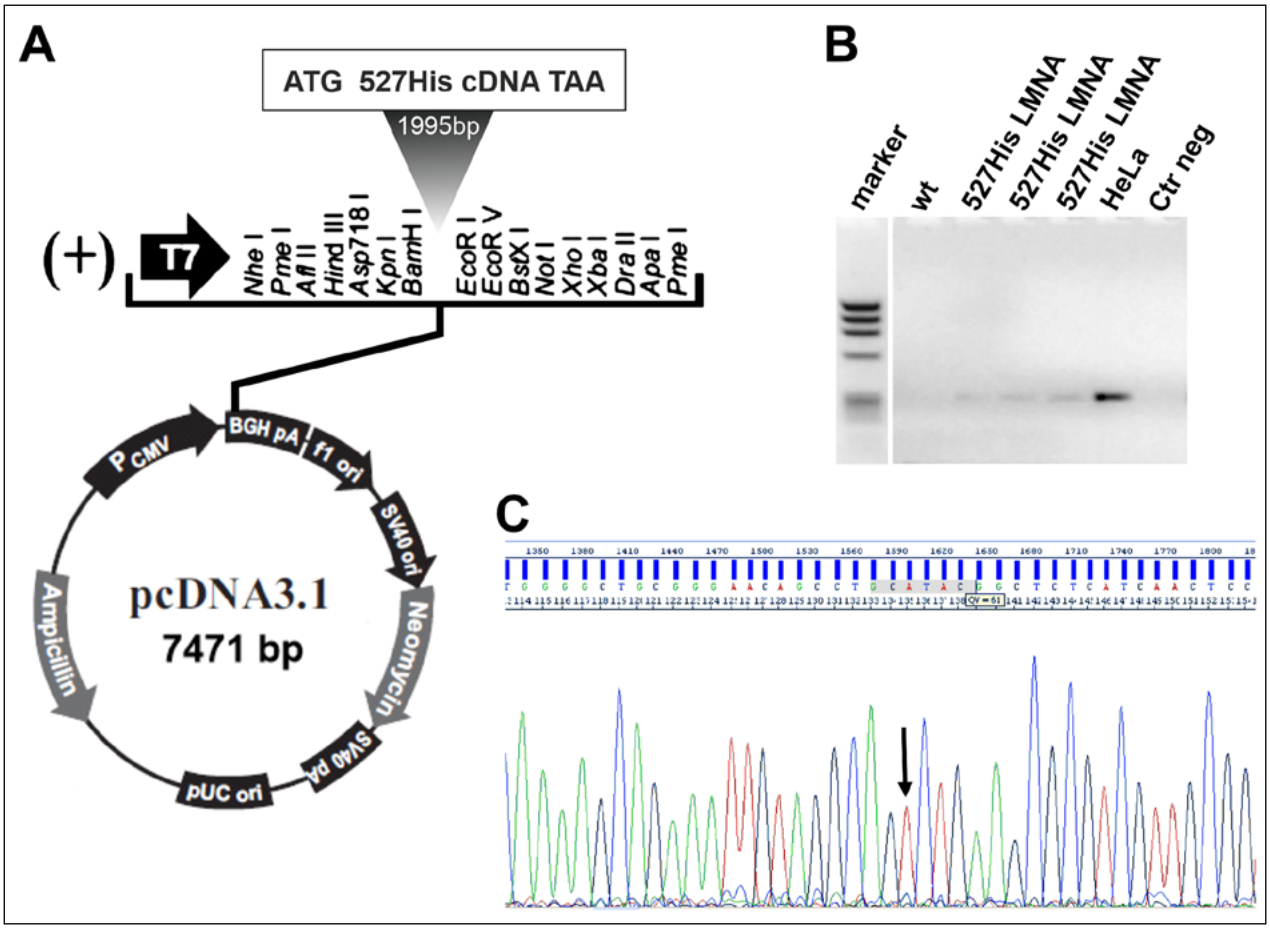

Figure 1. Generation of 527H *LMNA* transgenic mice. Schematic representation of gene construct (A) containing a CMV promoter followed by full length cDNA encoding 527His prelamin A. BamH1 and EcoRI restriction endonuclease sites are indicated. The 7.4 kb fragment generated by digestion with PvuI was used for microinjection of pronuclei of fertilized mouse oocytes. 527H *LMNA* gene expression analysis (B) from three F1 transgenic mice, compared to WT littermates. Sanger sequencing analysis (C) shows the c.1580G > A substitution (arrow).

firstly placed with males for mating, and then sacrificed in the following morning. The ovum was taken for microinjection with the depurated recombinant plasmid. On the next day, the fertilized ovum was put back into the oviduct of pseudopregnant female mice. Newborn mice were obtained after 20 days. Founder transgenic positive mice carrying human c.1580A *LMNA* gene were identified by PCR and Sanger sequencing analysis and crossed with WT C57BL/6J mice to generate F1 mice for breeding. Phenotypic analyses were performed in parallel with age – and sex – matched littermates. All mice were genotyped 2 weeks after birth, amplifying genomic DNA (gDNA) with specific human *LMNA* primers ghLMNA F GTGAGTGGCAGGGCGCTTGG and ghLMNA R GCATCTTTTGGTTTGCCTACTGGG. Animals were housed according to their gender after weaning in a light – and temperature -controlled facility (12-h light/12-h dark cycle, 21 degree), and allowed free access to food and water. General phenotype characterization of mice (body weight and lifespan) was carried out weekly.

Copy number assay

For copy number determination assays of 527His *LMNA* transgene, we used gDNA isolated from the murine tail. We used TaqMan™ Copy Number Reference Assay RNase P as the standard reference assay for copy number analysis, following manufacturer's instructions (Applied Biosystems).

Human LMNA gene expression analysis

Total mRNA from 527His *LMNA* fibroblasts was extracted and purified using TRIzol reagent (Invitrogen). 527His *LMNA* cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and gene expression was evaluated by RT-PCR using the following human specific primers: hLMNA F GAGATGATCCCTTGCTGACTTAC and hLMNA R TGGATCTCCATCCTCATCCTCGTC.

Mice tissue collection and histological analysis

Animals were euthanized and all *in vivo* studies were carried out in accordance with European Economic Community Council Directive 86/609, OJ L 358, 1, Dec.18, 1986 and with the NIH-used Guide for the Care and Use of Laboratory Animals [20].

For histological analysis, cutaneous biopsies obtained from the dorsal skin was collected and fixed in 4% paraformaldehyde overnight and embedded in paraffin blocks. The blocks were sectioned into 5 um-thick slices and place on slides. Hematoxylin and eosin (H&E) staining were performed according to standard protocols. White adipose tissue (WAT) and liver samples were obtained from 4 months old mice, specimens were fixed in 10% paraformaldehyde, and embedded in paraffin. 10 mm consecutive WAT sections were then mounted on slides and stained with H&E. Formalin-fixed liver tissue was processed, and 5-µm-thick paraffin sections were stained with H&E for histological analysis.

Metabolic assays

Metabolic testing procedures have been previously described [21,22]. Briefly, for oral glucose tolerance tests (OGTT) animals were fasted for 16 hours and injected with 2 g/kg body weight of glucose into the peritoneal cavity; insulin tolerance tests (ITT) were performed by injection of 0.75 units/kg body weight of human regular insulin (Novo Nordisk) into the peritoneal cavity of animals fasted for 6 hours. Blood Glucose concentrations were determined by using an automated Onetouch Lifescan Glucometer. Insulin levels were measured using a commercial kit (Mercodia). Cholesterol and Triglycerides were measured using a Roche Modular T analyzer. Mice were fed a High Fat Diet (HFD, 60% of calories from fat, code D12492 from Research Diets, NY) or standard chow (SC, 10% calories from fat, code 4RF18 GLP Mucedola, Italy) for 20 weeks after weaning as indicated. Studies were performed only in male mice.

Cell culture

WT and 527His *LMNA* fibroblast cultures had been obtained from skin biopsies, using standard procedure. Immediately after collection, the sample was rendered sterile by 3 consecutive washes in PBS (DPBS-Dulbecco's Phosphate-Buffered Saline; w/o calcium, w/o magnesium; Thermo Fisher Scientific) and antibiotic-antifungal (PAA, The Cell Culture Company), then it was placed in a solution of Dispase (2 mg/mL; Gibco) all night at 4°C, in order to clivate the components of the extracellular matrix. The following day, pieces were incubated with Collagenase I for 4h at 37°C and then they were transferred into Tissue Culture Plates pre-treated with gelatin, in DMEM High Glucose (Gibco) media, containing 10% FBS (Gibco), 1% L-Glutamine (PAA, The Cell Culture Company), 1% Penicillin / Streptomycin (PAA, The Cell Culture Company), 1% NEAA (Gibco) and 0.1% β-mercaptoethanol (Gibco) in a 5% CO_2 humidified atmosphere at 37°C.

Immunofluorescence staining and imaging analysis

WT and 527His *LMNA* fibroblasts were fixed and incubated with the appropriate primary antibodies against Prelamin A (C-20; 1:100, Santa Cruz Biotechnology, INC) and Lamin A/C (N-18; 1:100, Santa Cruz Biotechnology, INC). Appropriate Alexa Fluor 488- or 568-la-

beled secondary antibodies were incubated for 1 h at room temperature (Invitrogen, Carlsbad, CA, USA). Cellular senescence was performed using a SA-β-gal staining kit (Cell Signaling, #9860) according to the manufacturer's instructions. For the proliferation assay, cells were grown on glass coverslips and cultured 24 h. BrdU was added at a concentration of 10 μM for the last 6h after which proliferation assay was performed according to the manufacturer's instructions (Roche Applied Science). Cell nuclei were labeled with 4,6-Diamidino-2-phenylindole (DAPI-Sigma Aldrich). Images are acquired using a Zeiss (Zeiss, Thornwood, NY, USA) Axioplan 2 microscope. Immunofluorescence analyses were conducted from cellular passage 11 to 17.

Western blot

Whole-cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). After incubation with 5% milk in TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20 [pH 8.0]) for 1 hour, the membrane was incubated with indicated antibodies overnight at 4°C. Membranes were washed with TBST three times and incubated with a 1:5,000 dilution of horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies for 1 hour. Blots were washed with TBST three times and developed with the ECL system (Bio-Rad).

Microarray analysis and processing

Total RNA from dermal fibroblasts derived from adult (1 year) mice overexpressing 527His *LMNA* was extracted and purified using TRIzol reagent (Invitrogen); its quality and quantity was assessed using a Nanodrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. Synthesis of the labelled first strand cDNA was conducted according to manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis, Agilent) with starting material of 1 μg of total RNA. The labeled cDNAs were co-hybridized to the Whole Mouse Genome Oligo Microarray (G4122A, Agilent) in duplicate, with one dye swap. Whole Mouse Genome Oligo Microarray Kit slides contained about 44,290 oligonucleotides corresponding to 41,174 genes and transcript (www.agilent.com/chem/dna). Detailed methods for sample processing and microarray experiments have been previously described [23]. Image analysis and processing were performed as described in Tiano et al., 2020 [24].

Validation of relative gene expression by real-time RT-PCR

1 μg of total murine RNA has been retrotranscribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to evaluate the expression levels of selected differentially expressed genes (DEGs). We analysed the following genes: Igf2, Fgf10, Epyc, Zic1, Fst, Pdgfc, Tnfrsf21, and Chi3l1. Real time PCR (qRT-PCR) has been performed using ABI7500 Fast Real-time PCR System (Life Technologies) and murine Taqman assays (Applied Biosystems, USA). All samples were run in triplicate and average values were calculated. Two independent reverse transcriptions were tested for each gene. Relative quantification of gene expression among each sample was achieved by normalization against Gapdh as endogenous control using the ΔΔCt method of quantification [23].

Functional analysis and pathway enrichment analysis

We used KEGG pathway enrichment analysis for the DEGs analysis. KEGG (http://www.genome.ad.jp/kegg/) is a comprehensive database resource, which consists of chemical information, genomic information and systems information (REF). Enrichment analysis of KEGG pathways of DEGs was done by "clusterProfiler" R package to explore the most likely gene function [25]. $P < 0.05$ was used as the cut-off criterion.

Statistical analysis

All data were expressed as means ± SD. For *in vivo* studies, three to six mice per genotype per assay were used. For *in vitro* cell studies, each experiment was repeated at least three times. Data were analyzed by Student t test, two-way ANOVA, and post hoc test (GraphPad Prism 8). The significance level was set at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$).

Results

Generation of 527His LMNA transgenic mouse and disease phenotype

The general structure of the 527His *LMNA* gene construct is shown in Figure 1A. 527His *LMNA* gene expression assessment and Sanger sequencing analysis were determined in studied F1 generation mice (Fig. 1B-C), after copy number assay (data not shown). Transgenic progeny of F1 and subsequent generations was born at the expected ratio of approximately 1:1 when compared with nontransgenic littermates. At birth, the macroscopic appearance of MADA transgenic mice was indistinguishable from their WT siblings. All mice were lively, active, explorative, and eating, drinking and interacting with cage mates. By the 2nd month of age, most animals were smaller than wild type littermates (Fig 2A). 527His *LMNA* mice grow slightly less and gain weight slowly than their littermates until 30th week of age for the males and until 44th week of age for the females (Fig. 2B). At fifth month, we observed loss of hair in transgenic animals, compared with

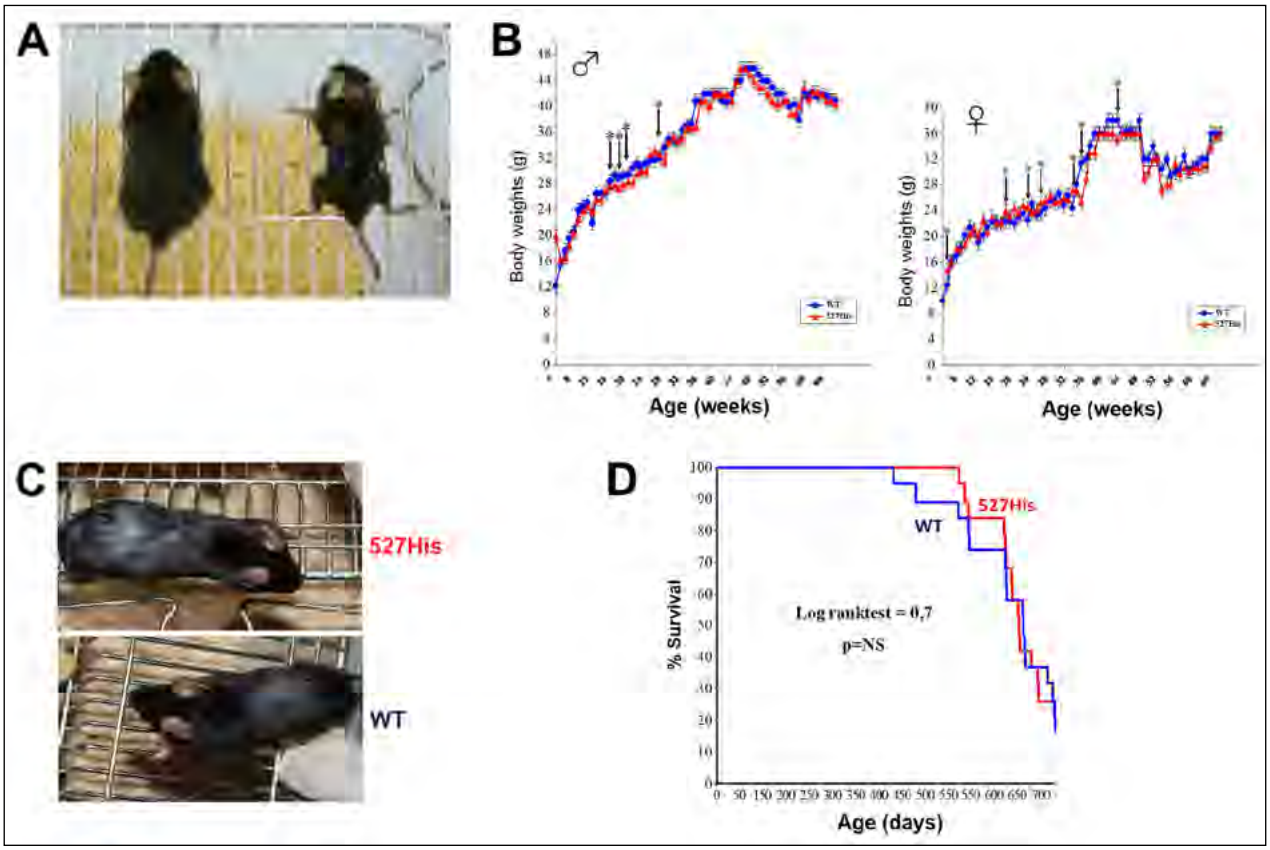

Figure 2. Photograph (A) of 527His *LMNA* and WT littermate progeny, at 3 month-old. Cumulative plot of body weight versus age (B) in male and female animals. Dots represent mean values (mice = 6) and error bars indicate SD. Photographs (C) of a 527His and a WT animal at 3-month-old. Note the hair thinning and loss, especially evident in the dorsal region. Kaplan-Meier graph (D) showing a no significant differences in life span 527His *LMNA* mice (n = 15) compared with WT mice (n = 13).

WT mice (Fig. 2C). Kaplan-Meier survival curves did not reveal any differences in the lifespan (Fig. 2D).

Metabolism

Glucose tolerance and insulin sensitivity were comparable in WT and 527His *LMNA* mice fed regular chow for 12 weeks (not shown) or 24 weeks (Fig. 3A-B). When mice were challenged with a diet rich in fat (HFD) we did not observe differences in body weight (Fig. 3C). Moreover, 527His *LMNA* animals revealed no differences in serum lipids levels (Fig. 3D), a mild significant glucose intolerance and insulin resistance (Fig. 3A-B) and slightly higher insulin levels in the fed state (Fig. 3E). Histological analysis of tissues relevant for metabolic homeostasis such as white adipose tissue and liver revealed no gross differences between WT (Fig. 4A,C) and 527His *LMNA* (Fig. 4B,D) littermates apart mild increase in inflammatory cells in adipose tissue from transgenic animals.

Skin

Microscopic analysis of dorsal skin sections obtained by cutaneous biopsy from transgenic mice at 24 weeks of age, showed a reduction of hypodermal thickness compared with WT littermates (Fig. 4E,F), due to the loss of subcutaneous adipose tissue.

Cellular results

Once established primary cell lines from murine dermal fibroblasts, cells were morphologically characterized *in vitro*. Irregularly shaped nuclei with intra/transnuclear membrane invaginations, large protrusions ("buds" or "blebs") or doughnut-shaped nuclei, and independent nucleus-like structures, were detected in 20% of all MADA cells and in 12% of WT ones (Fig. 5A,B, p-value < 0.05). In order to analyze the nuclear envelope, immunofluorescence analyses were conducted both for the detection of prelamin A and mature lamin A. Prelamin A showed an abnormal accumulation in about 66% 527His *LMNA* nu-

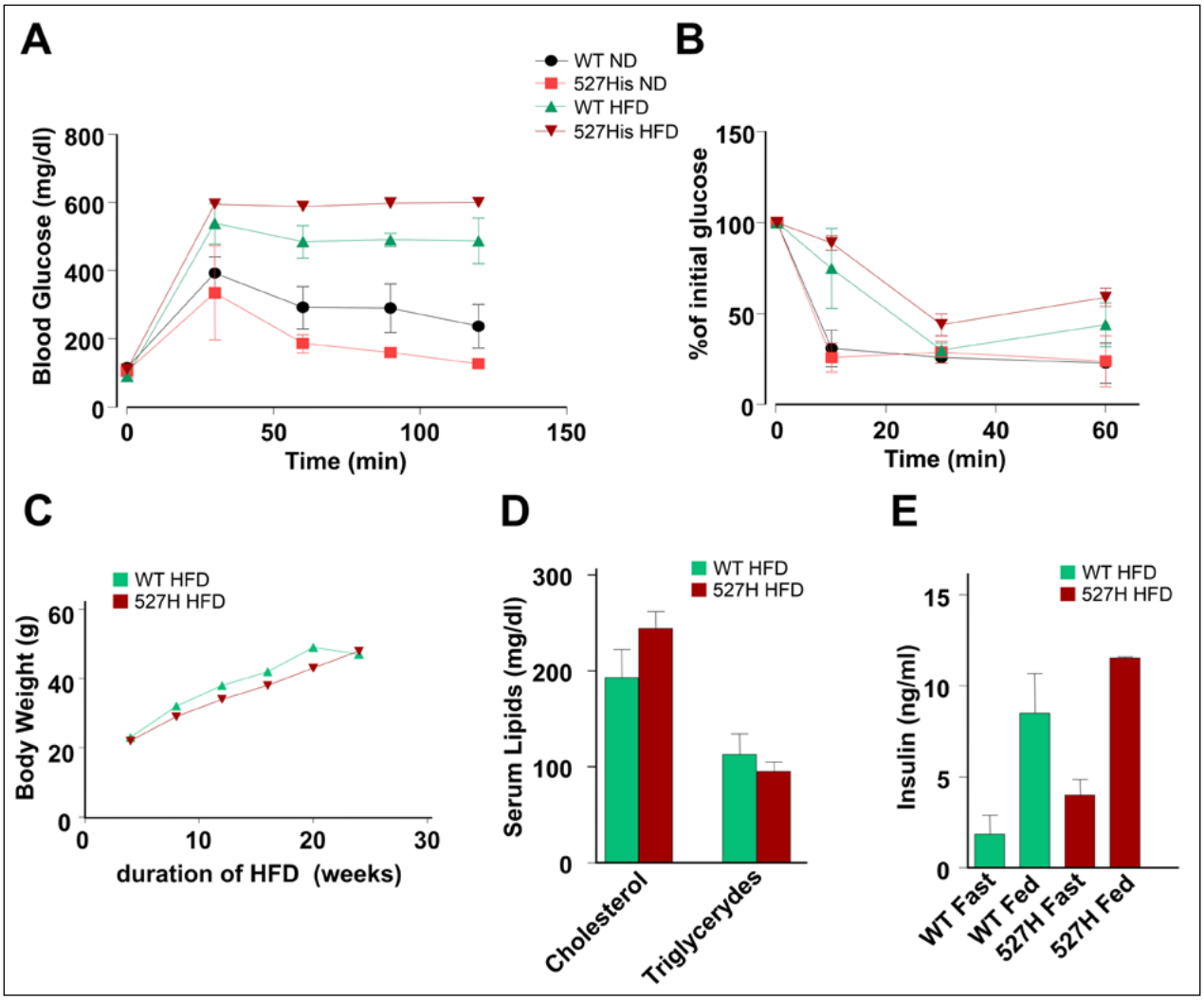

Figure 3. Metabolic assessment of WT *vs* 527His *LMNA* mice at regular chow and high fat diets (20 weeks). Measurement of glucose tolerance (A) and insulin sensitivity (B) in both diet conditions. No differences were observed in body weight (C) and serum lipids (D) during the HFD diet. Slight higher insulin levels (E) were showed for 527His *LMNA vs* WT mice in the fed state.

clei mostly located at the nuclear rim, within membrane invaginations and occasionally in intranuclear structures (Fig. 5A). As expected, prelamin A rarely detected (9%) in WT cells (p-value < 0.01) which don't show any nuclear alterations (Fig. 5A). Meanwhile, lamin A is expressed in all nuclei both in WT and 527His *LMNA* fibroblasts with the same rim nuclear distribution (Fig. 5B). The data have been reported in a histogram (Fig. 5C). We also performed a Western blot analysis showing the presence of Prelamin A in primary cellular lines from two different 527His *LMNA* mice while no appreciable signal was visible in WT. As expected, the lamin A and C is present in all samples. Hela cells accumulating Prelamin A after treatment with Farnesyltransferase inhibitors (FTI) were used as control (Fig. 5D).

In addition, 527His *LMNA* fibroblasts proliferated at lower rate than WT as underlined by BrdU assay especially at high culture passages, (i.e 15 and 17) (Fig. 5E), ($p < 0.01$). Moreover, under normal growth conditions, 527His cells showed an increased percentage of senescence associated β-galactosidase staining especially at passage 13 (12% in WT and 34% in 527His *LMNA* cells) (Fig. 5F, p-value < 0.05).

Analysis of differentially expressed genes (DEGs)

After filtering out unreliable probe sets with expression at background level, 222 out of 41,174 murine genes and transcript were considered as significant expressed in adult fibroblasts derived from mice overexpressing 527His

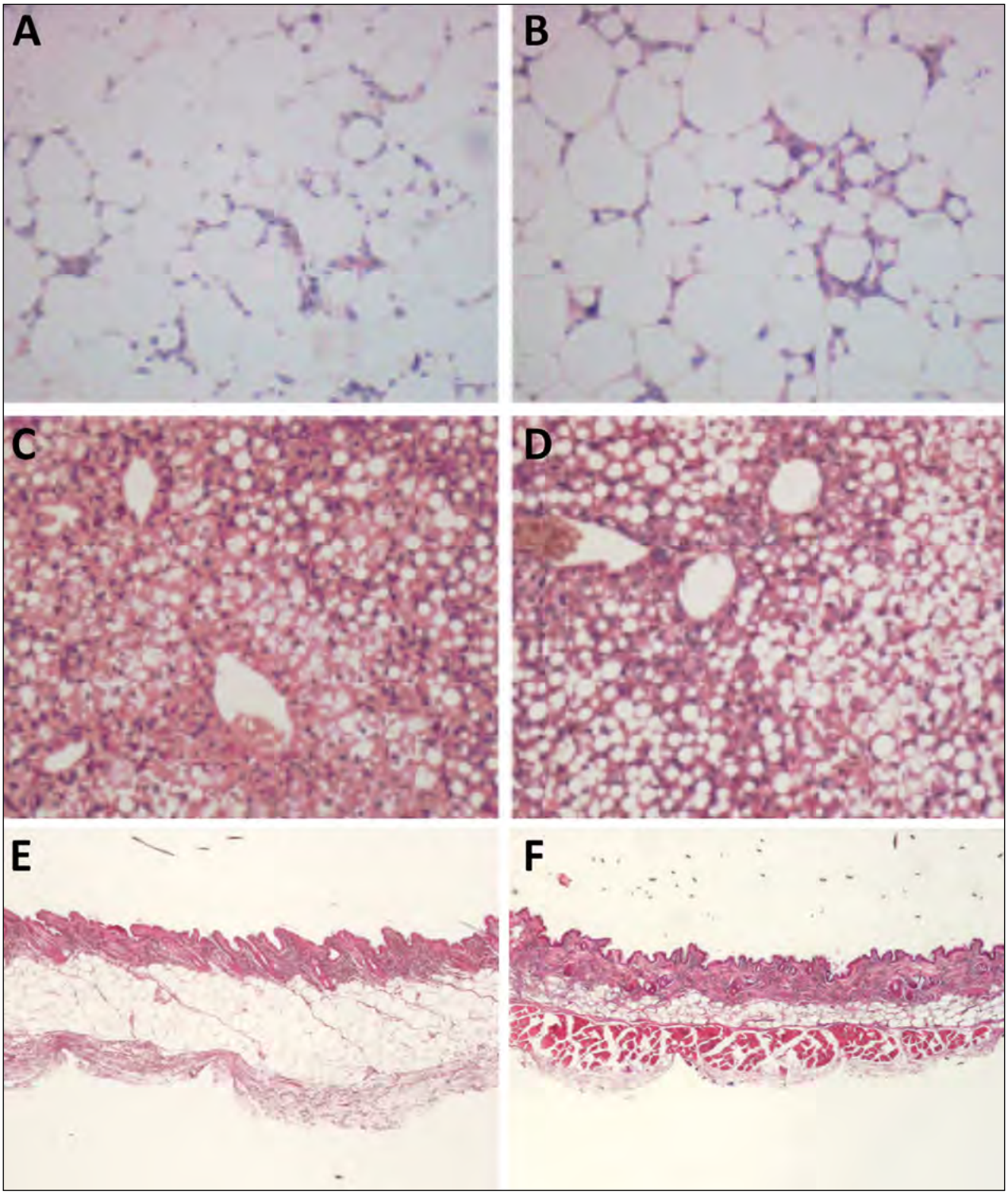

Figure 4. Representative images of histological analysis of white adipose tissue, liver and skin tissues in WT (A, C, E) *vs* 527His LMNA mice (B, D, F). Mild increase in adipose tissue inflammatory cells was noted in 527His *LMNA* mice. No differences were observed in liver. Reduction of hypodermal thickness was viewable in 527His *LMNA* mice. Haematoxylin and eosin (H&E) staining.

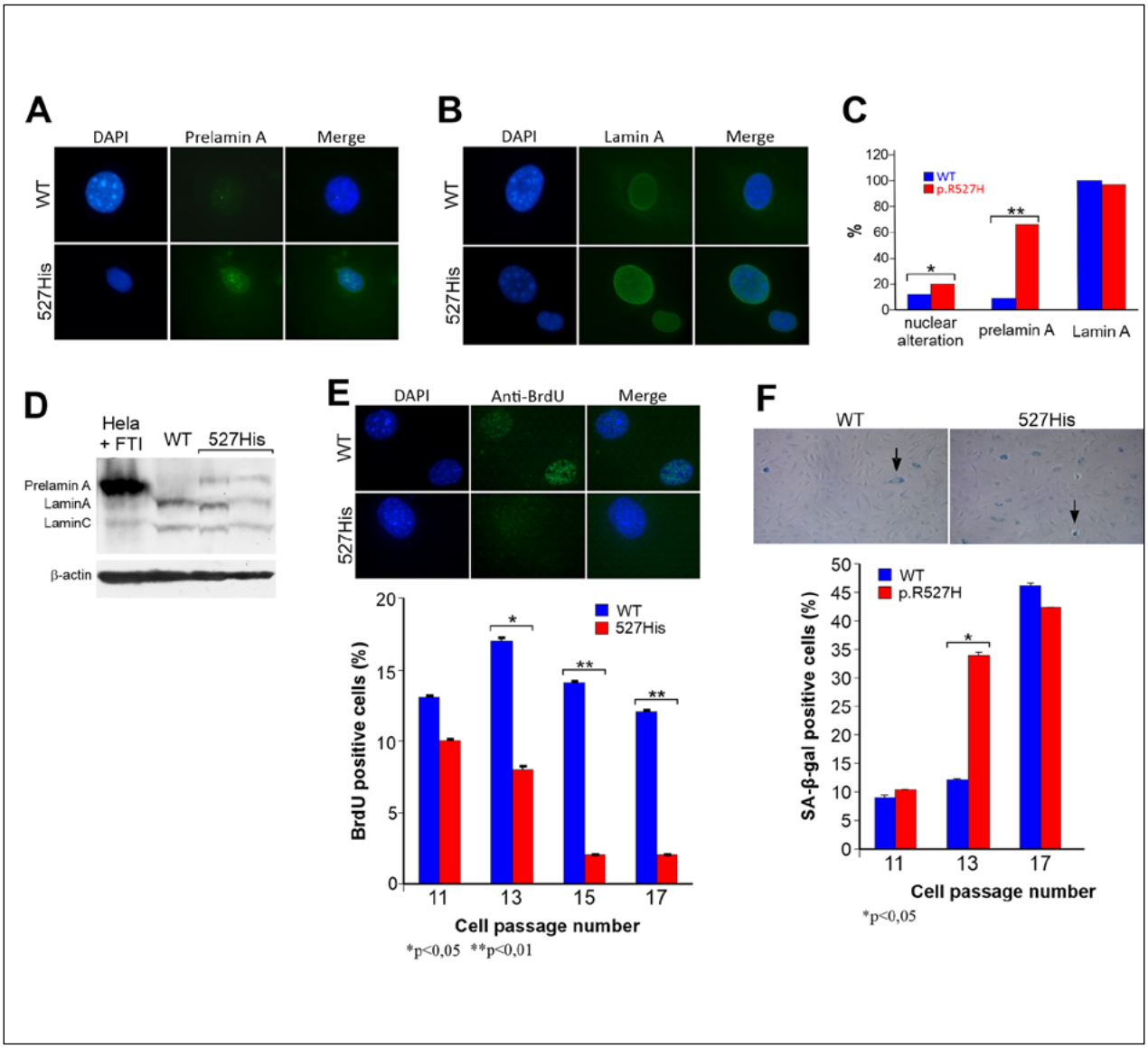

Figure 5. Representative image of immunofluorescence staining showing the abnormal presence of prelamin A (A) in 527His *LMNA* nuclei and aberrant nuclear envelope conformations, while control cells (WT) show regular nuclear envelope shape and a rarely detection of prelamin A staining. Meanwhile, lamin A is expressed in all nuclei both in WT and 527His-dermal fibroblasts with the same rim nuclear distribution (B). DAPI nuclear staining (blue). Scale bar 100 µm. Histogram (C) represents the percentage of aberrant nuclear conformations, Prelamin A and lamin A positive nuclei. Error bars represent the SD from the analysis of 100 cells from three independent experiments and WT values are displayed as the average percentages of 2 different controls (**p < 0.01). Representative Western blot analysis (D) of Prelamin A, Lamin A and C of equal amount of total proteins from 527His *LMNA* (n = 4) and WT mice fibroblasts (n = 5). Protein extracts of Hela cells treated with FTI were used as positive control. β-actin was used as control. Data are presented as means ± SD. Representative immunofluorescence images (E) showing the presence of BrdU positive cells in 527His and WT mice cells. DAPI nuclear staining (blue). Scale bar 100µm. The histogram shows the percentages related to BrdU positive cells in 527His cells at passages 11,13,15,17. Error bars represent the SD from the analysis of 100 cells from three independent experiments. WT and MADA values are displayed as the average percentages of 5 different mice respectively (*p < 0.05, **p < 0.01). Representative image (F) of senescence-associated β-galactosidase assay at passage 13. A greater amount of intensely positive blue cells are displayed in 527His-fibroblasts than WT controls. The histogram shows the average percentage of β-galactosidase-positive cells in WT and 527His *LMNA* fibroblasts at passage 11,13,17. Error bars represent the SD from the analysis of 100 cells from three independent experiments. WT and MADA values are displayed as the average percentages of 5 different mice respectively (*p < 0.05).

LMNA. Twenty-nine up-regulated and 37 down-regulated transcripts out of 66 DEGs were detected according to the criteria of "Benjamini adjusted p-value < 0.05" and "FC≥ ± 1.5" (Tab. I). The expression levels of 11 DEGs were confirmed by QRT-PCR analysis.

Pathway analysis

KEGG pathway analysis identified the main molecular pathways altered in adult fibroblasts derived from mice overexpressing 527His *LMNA* (Tab. II). KEGG analysis recognizes that the 66 DEGs were significantly enriched in multiple pathways including cell signaling, pathways in cancer, immune system, human diseases. The pathway "Environmental Information Processing" that comprehends membrane transport, signal transduction and signaling molecules and interaction pathways, resulted the most significant enriched pathway in mutant fibroblasts ($p < 0.005$; Tab. II). Noteworthy, inside this general pathway, "Signaling molecules and interaction" pathway was significant enriched too ($p < 0.0001$, Tabs. II-III). Nine DEGs belongs to this pathway: Il6,

Table I. DEGs (FC≥ ± 1.5) in adult mice fibroblasts.

	Gene name	Accession number	FC	P-value	Gene position
1	Pdlim3	NM_016798	8.63	1.13E-07	chr8
2	Onecut2	NM_194268	3.45	1.87E-07	chr18
3	Epyc	NM_007884	3.08	5.59E-06	chr10
4	Tmeff2	NM_019790	2.86	9.12E-07	chr1
5	Agtr1a	NM_177322	2.41	4.53E-05	chr13
6	Zic1	NM_009573	2.41	0.000150879	chr9
7	Gria4	NM_019691	2.29	3.89E-06	chr9
8	Cpz	NM_153107	2.21	7.14E-05	chr5
9	Spon2	NM_133903	2.16	2.33E-05	chr5
10	Fndc5	NM_027402	2.13	0.000158579	chr4
11	Il1r2	NM_010555	1.97	1.14E-05	chr1
12	Rpl39l	NM_026594	1.91	2.81E-05	chr16
13	Osr1	NM_011859	1.89	6.07E-05	chr12
14	Trib3	NM_175093	1.86	0.00023456	chr2
15	Olfml2b	NM_177068	1.85	9.26E-06	chr1
16	Aldh1l2	NM_153543	1.79	0.000183763	chr10
17	Angptl2	NM_011923	1.75	0.000169423	chr2
18	Lama2	U12147	1.72	1.03E-19	chr10
19	Emilin2	NM_145158	1.69	0.000202906	chr17
20	2210409E12Rik	AK008869	1.68	5.07E-05	chr11
21	Tnfrsf21	NM_178589	1.60	0.000112189	chr17
22	Lpl	NM_008509	1.60	0.000169284	chr8
23	C2	NM_013484	1.59	0.00031931	chr17
24	Il15ra	NM_008358	1.57	0.000182628	chr2
25	Sh3bp5	NM_011894	1.56	0.000201258	chr14
26	D0H4S114	NM_053078	1.56	0.00012852	chr18
27	Meox2	NM_008584	1.56	3.20E-06	chr12
28	S1pr3	NM_010101	1.55	0.000151178	chr13
29	Cebpa	NM_007678	1.51	4.19E-05	chr7
30	Cd28	NM_007642	-1.52	9.62E-13	chr1
31	Tspan6	NM_019656	-1.53	0.000171921	chrX
32	Lxn	NM_016753	-1.54	0.000113059	chr3
33	Saa1	NM_009117	-1.54	0.000289162	chr7
34	Kctd12	NM_177715	-1.55	0.00015173	chr14
35	Ccbe1	NM_178793	-1.57	3.78E-05	chr18
36	Fgf10	NM_008002	-1.57	3.39E-15	chr13
37	Tnfsf11	NM_011613	-1.60	3.95E-05	chr14

Table I. *continue*

	Gene name	Accession number	FC	P-value	Gene position
38	Bambi	NM_026505	-1.62	1.44E-13	chr18
39	Il6	NM_031168	-1.64	1.11E-14	chr5
40	Fxyd6	NM_022004	-1.65	0.000280392	chr9
41	Cfh	NM_009888	-1.66	1.46E-06	chr1
42	Steap4	NM_054098	-1.68	5.13E-05	chr5
43	Pdgfa	NM_008808	-1.70	8.61E-06	chr5
44	Igfbp5	NM_010518	-1.73	0.000111058	chr1
45	Hs6st2	NM_015819	-1.74	0.000205243	chrX
46	4930550L24Rik	NM_023774	-1.82	3.22E-05	chrX
47	Rprm	NM_023396	-1.82	0.000114862	chr2
48	Mtss1	AK046628	-1.83	1.87E-05	chr15
49	Zdhhc2	NM_178395	-1.84	0.000117462	chr8
50	Gpr88	NM_022427	-1.85	0.000226715	chr3
51	Fst	NM_008046	-1.86	5.64E-05	chr13
52	Cspg4	NM_139001	-1.87	0.000199449	chr9
53	9930013L23Rik	AK018112	-1.88	2.25E-05	chr7
54	Hpgd	NM_008278	-1.89	3.86E-05	chr8
55	Lnx1	NM_010727	-1.89	6.37E-07	chr5
56	Btbd3	NM_145534	-1.91	0.000226342	chr2
57	Igf2	NM_010514	-1.93	1.08E-22	chr7
58	Mme	NM_008604	-1.95	4.46E-05	chr3
59	Camk4	NM_009793	-2.15	7.41E-06	chr18
60	Frmd5	NM_172673	-2.22	4.24E-06	chr2
61	Angptl7	NM_001039554	-2.25	0.000144913	chr4
62	Pdgfc	NM_019971	-2.28	4.21E-06	chr3
63	Ahr	NM_013464	-2.30	3.68E-22	chr12
64	Mia1	NM_019394	-2.67	3.20E-08	chr7
65	Penk	NM_001002927	-3.83	8.06E-06	chr4
66	Chi3l1	NM_007695	-4.55	0.000223198	chr1

Tnfsf11, Pdgfc, Pdgfa, Il1r2, Il15ra, Tnfrsf21, Lama2, S1pr3 (Tab. III). Seven genes out of nine are in "Cytokine-cytokine receptor interaction" (mmu04060) pathway (Tab. III).

In addition, the pathway named "Human diseases" showed a significant enrichment ($p < 0.05$, Tab. II). Inside this large pathway, two specific pathways are significant enriched in mutant fibroblast: Cancer ($p < 0.005$) and Neurodegenerative diseases ($p < 0.05$). Five DEGs are part of "Cancer" pathway, while three DEGs of "Neurodegenerative diseases" (Tab. III). Finally, also the "Immune system" pathway revealed significant enriched ($p < 0.05$) with five DEGs (Tab. III).

Discussion

Studies during the past 15 years have established that several progeroid syndromes are caused by genetic defects that interfere with the processing of prelamin A to mature lamin A. It is known that the balance between these two proteins triggers the severity of ageing.

MADA is caused by point mutations in C-terminal domain of *LMNA* gene that through an unknown process produce a mutated prelamin A. MADA patients show a mild accelerate aging compared to patients with Hutchinson-Gilford progeria syndrome (HGPS), caused by the presence of truncated prelamin A (progerin), that lacks the endoproteolytic cleavage site domain that would normally release mature lamin A. The absence of mature lamin A causes a more severe progeroid disorder, restrictive dermopathy (RD), caused by homozygous loss of ZMPSTE24 enzyme involved in the cleavage of C-terminus of prelamin A. Partial loss of ZMPSTE24 activity has been associated Mandibulocral Dysplasia type B (MADB) with severe metabolic syndrome, abnormal fat accumulation and dilated cardiomyopathy [26-28].

The toxic accumulation of mutated prelamin A provokes alterations of nuclear morphology, perturbations of cell cycle, defects in cellular replication, senescence rate,

Table II. KEGG enriched pathways analysis results.

Pathway	Genes on slides/pathway	DEG/pathway	P-value
Signaling molecules and interaction	683	9	0.000035
Cancers	335	5	0.002
Environmental information processing	1472	10	0.0029
Human diseases	1054	7	0.02
Immune system	714	5	0.045
Neurodegenerative diseases	287	3	0.046
Cellular processes	1101	4	n.s
Genetic information processing	1094	1	n.s.
Metabolism	1358	2	n.s.
Organismal systems	2421	7	n.s.
Cardiovascular diseases	187	2	n.s.
Cell communication	391	3	n.s.
Cell growth and death	291	1	n.s.
Cell motility	198	2	n.s.
Development	180	1	n.s.
Endocrine system	433	2	n.s.
Glycan biosynthesis and metabolism	202	1	n.s.
Immune system diseases	185	1	n.s.
Infectious diseases	270	1	n.s.
Lipid metabolism	335	1	n.s.
Signal transduction	961	5	n.s.
Translation	431	1	n.s.

n.s.: not significative

Table III. DEGs in different pathways.

Gene name	Accession number	FC	P-value	Pathway	C1	C2	C3
Fst	NM_008046	-1.86	5.64E-05	mmu04350	Environmental information processing	Signal transduction	TGF-beta signaling pathway
Il15ra	NM_008358	1.57	0.0002	mmu04060	Environmental information processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction
Il15ra	NM_008358	1.57	0.0002	mmu04630	Environmental information processing	Signal transduction	Jack/STAT signalling pathway
Il15ra	NM_008358	1.57	0.000183	mmu04672	Organismal system	Immune system	Intestinal immune network for IgA production
Il1r2	NM_010555	1.97	1.14E-05	mmu04060	Environmental information processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction
Il1r2	NM_010555	1.97	1.14E-05	mmu04010	Environmental information processing	Signal transduction	MAPK signalling pathway
Il6	NM_031168	-1.64	1.11E-14	mmu04060	Environmental information processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction
Il6	NM_031168	-1.64	1.11E-14	mmu04630	Environmental information processing	Signal transduction	Jack/STAT signalling pathway

Table III. *continue*

Gene name	Accession number	FC	P-value	Pathway	C1	C2	C3
Il6	NM_031168	-1.64	1.11E-14	mmu05200	Human disease	Cancers	Pathways in cancer
Il6	NM_031168	-1.64	1.11E-14	mmu05020	Human disease	Neurodegenerative diseases	Prion diseases
Il6	NM_031168	-1.64	1.11E-14	mmu04623	Organismal system	Immune system	Cytosolic DNA-sensing pathway; hematopoietic cell lineage; intestinal immune network for IgA production; NOD-like receptor signaling pathway; toll-like receptor signaling pathway
Lama2	U12147	1.72	1.03E-19	mmu04512	Environmental information processing	Signaling molecules and interaction	ECM-receptor interaction
Lama2	U12147	1.72	1.03E-19	mmu05200	Human disease	Cancers	Small cell lung cancer; Pathways in cancer
Pdgfa	NM_008808	-1.70	8.61E-06	mmu04060	Environmental information processing	Signaling Molecules and Interaction	Cytokine-cytokine receptor interaction
Pdgfa	NM_008808	-1.70	8.61E-06	mmu04010	Environmental information processing	Signal transduction	MAPK signalling pathway
Pdgfa	NM_008808	-1.70	8.61E-06	mmu05214	Human disease	Cancers	Glioma; melanoma; prostate cancer; renal cell carcinoma; pathways in cancer
Pdgfc	NM_019971	-2.28	4.21E-06	mmu04060	Environmental information processing	Signaling Molecules and Interaction	Cytokine-cytokine receptor interaction
Pdgfc	NM_019971	-2.28	4.21E-06	mmu05218	Human disease	Cancers	Melanoma; prostate cancer
S1pr3	NM_010101	1.55	0.00015	mmu04080	Environmental information processing	Signaling molecules and interaction	Neuroactive ligand-receptor interaction
Tnfrsf21	NM_178589	1.60	0.0001	mmu04060	Environmental information processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction
Tnfsf11	NM_011613	-1.60	3.95E-05	mmu04060	Environmental information processing	Signaling molecules and interaction	Cytokine-cytokine receptor interaction
Cebpa	NM_007678	1.51	4.19E-05	mmu05221	Human disease	Cancers	Acute myeloid leukemia, pathways in cancer

Table III. *continue*

Gene name	Accession number	FC	P-value	Pathway	C1	C2	C3
Lpl	NM_008509	1.60	0.0002	mmu05010	Human disease	Neurodegenerative diseases	Alzheimer's disease
Mme	NM_008604	-1.95	4.46E-05	mmu05010	Human disease	Neurodegenerative diseases	Alzheimer's disease
Mme	NM_008604	-1.95	4.46E-05	mmu04640	Organismal system	Immune system	Hematopoietic cell lineage
Cfh	NM_009888	-1.66	1.46E-06	mmu04610	Organismal system	Immune system	Complement and coagulation cascades

and changes of chromatin organization affecting gene transcriptional processes. In particular, these effects on nuclear dynamics may account for many of the clinical features and tissue-specific alterations observed in human progeroid laminopathies [29,30]. The characterization of 527His *LMNA* transgenic mice confirms and extends these evidences. The transgenic MADA mice generated overexpressing 527His prelamin A showed a significative percentage of nuclei with morphological alterations of envelope shape and a reduction of cellular proliferation with an increase of senescence rate.

According to numerous studies in MADA and HGPS animal and cellular models supporting that accumulation of prelamin A affects changes in gene expression levels, we explored the transcriptional pattern in 527His *LMNA* transgenic fibroblasts compared to WT cells [19]. Sixty-six DEGs are implicated in distinct pathways. The most significant enriched pathways in mutant fibroblasts comprehend signal transduction, cytokine-cytokine and extracellular matrix (ECM)-receptor interaction pathways. These changes are reminiscent of the effect of the Senescence-associated Secretory phenotype and suggest that MADA effects on gene expression might affect tissue integrity or regeneration via systemic inflammation. In fact, in experimental models overexpressing 527His *LMNA* mutation or in MADA patients' serum it has been observed an alteration of cytokine secretion and ECM enzymes release and activity [13,16]. This aspect has to been further investigated in order to clarify the pathogenic aspect and to develop therapeutic strategies for MADA and other ageing-related disorders.

Muscle phenotype is apparently normal in our transgenic mice, reflecting the human p.Arg527His MADA phenotype. On the contrary, MADB or RD patients with mutations responsible for a partial or complete abolishment of the catalytic function of the ZPMPSTE24 enzyme, develop muscle weakness and cardiovascular disease. Zmpste24 deficiency in murine models determines progeroid features with muscle weakness or cardiomyopathies and muscular dystrophy [31,32]. These data link high levels of prelamin A with altered structure of the nuclear lamina that could affect mechanically stressed tissues such as the muscle fibers of the heart and skeleton. The relative low levels of prelamin A in 527His *LMNA* transgenic mouse and MADA patients may be not sufficient to determine a muscle damage.

Notably, in this study, transgenic MADA mice described showed a mild phenotype, with a minimal change in body weight and a normal rate of survival compared with WT animals. Glucose metabolism and insulin sensitivity were comparable in WT and 527His *LMNA* mice fed regular chow and just a mild significant glucose intolerance and insulin resistance in 527His mice is observed when animals were challenged with a diet rich in fat. Nevertheless, we observed typical cutaneous alterations of MADA patients, such as loss of hair and decrease of subcutaneous adipose tissue. A possible explanation of the observed mild phenotype could be the known correlation between the efficiency levels of prelamin A maturation process and disease severity.

In conclusion, our 527His *LMNA* transgenic model resembles the mild phenotype observed in individuals with hereditary MADA laminopathy and may provide additional evidence about the role of nuclear integrity, specific biological pathways and transcriptional changes in order to in depth understand the pathological and physiological aging.

Acknowledgments

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25 years of the SMN genes: the Copernican revolution of spinal muscular atrophy

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The new era of advanced therapies has influenced and changed the views and perspectives of a neuromuscular disease such as spinal muscular atrophy (SMA). Being an autosomal recessive motor neuron disorder, characterized by different degrees of muscle weakness, after 25 years of the discovery of the determinant and modifier genes (*SMN1* and *SMN2*, respectively) three SMN-dependent specific therapies are already approved by FDA (two by EMA), so that worldwide patients are currently under clinical investigation and treatment. This success was the combined effort mainly of patients and families, physician and researchers, advocacy groups and several Institutions together with the support of pharmaceutical companies. Progression trajectories, phenotypes, follow-up and care of the patients are continously evolving. Clinical investigations are currently demonstrating that early diagnosis and intervention are essential for better and more effective response to treatment, consistently improving prognosis. This scenario has created the need for awareness, early diagnosis and even implementation of of newborn screening programs. New views and perspectives of patient and family expectations, genetic counselling and multidisciplinary care: a truly Copernican revolution in neuromuscular and genetic diseases.

Key words: spinal muscular atrophy, early diagnosis and intervention, advanced therapies, genetic counselling, antisense oligonucleotides, gene therapy

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Introduction/overview

Spinal muscular atrophy (SMA) linked to 5q is an autosomal recessive neuromuscular disorder caused by the degeneration of alpha motor neurons of the spinal cord anterior horns. The main manifestation of the disease is muscle weakness by denervation followed by respiratory failure and infant death in the most severe cases. However, the experience of patients is dominated by the downstream complications such as compromised respiration, impaired nutrition, deformities (i.e. scoliosis and contractures) and limited functional ability. SMA is one of the commonest severe hereditary disorders of infancy and early childhood, with an incidence estimated of 1/6000 to 1/10000 births and a carrier frequency of 1/35 to 1/50 [1]. Originally described by Guido Werdnig and Johann Hoffmann in the XIX century [2], after several decades in the XX century of clinical descriptions and eponymous classifications, the interest of SMA started to increase in 1995, when the causative *SMN1* gene was discovered by the group of Judith Melki [3] (Fig. 1). With the advent of animal models, preclinical studies contributed to test therapeutic alternatives (the translational research decade between 2000 and 2010). In 2011, clinical trials (CT) in humans where initiated.

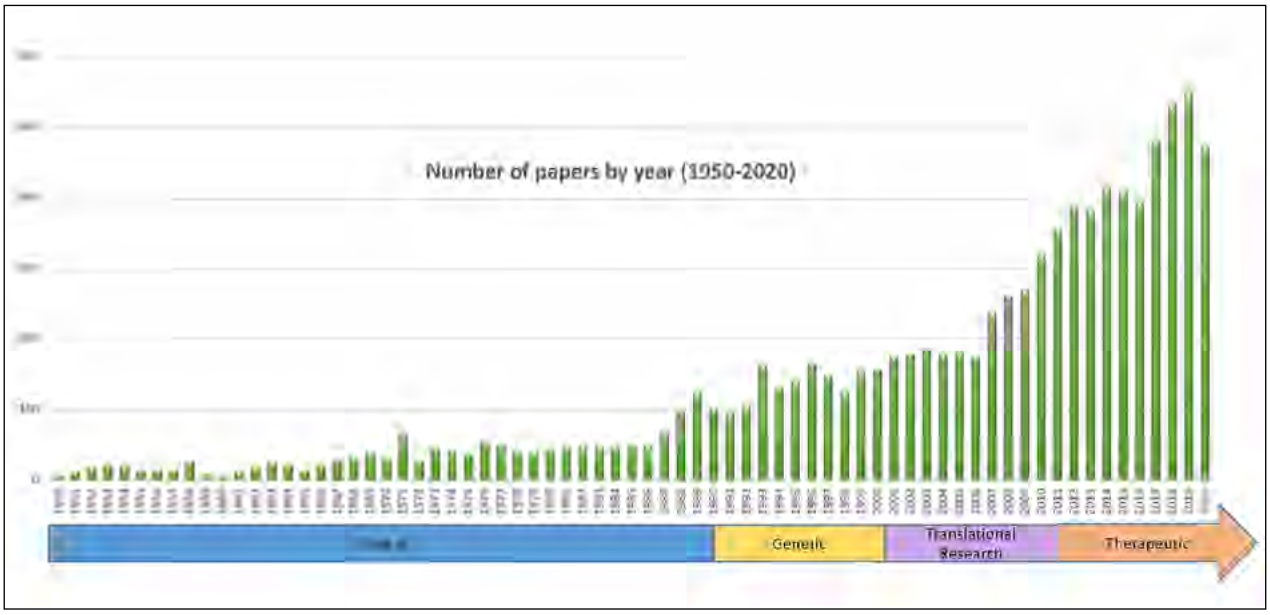

Figure 1. Stages of SMA progress in the last 70 years. Several decades considering 1950 onwards to the nineties were purely clinic. The genetic decade starts with the discovery of the SMN1 gene in 1995 doubling the number of publications. A translational research decade from 2000 to 2010 is following defined by the availability of animal models to test therapies and preclinical studies that also increased the number of publications. The last decade started in 2011, and includes the different clinical programs and the growing interest in SMA during the last years with more than 500 publications in 2019 and a higher number (n = 578) in the current unfinished year 2020 (Source: PubMED last entry December 2, 2020).

In less than ten years, three advanced therapies in SMA have been already approved by FDA. An antisense oligonucleotide (ASO) that affects splicing of the pre-mRNA (*nusinersen*, Spinraza®) in 2016 [4], a self-complementary adeno associated virus serotype 9 (AAV9) gene therapy (*Onasemnogene Abeparvovec*, ZolgenSMA®) in 2019 [5] and an oral compound that acts as splicing modifier (*risdiplam*, Evrysdi®) in 2020 [6].

SMA clinical picture is viewed as a continuous spectrum of manifestations ranging from serious congenital forms to minimal manifestations in adulthood. To better follow-up and categorize SMA patients, a classification into three main types based on age at onset and maximum milestones achieved have been reported in 1992[7] and several subtypes have been also defined [8].Type I, the most severe form, manifests early in the first weeks or months of life, with generalized hypotonia. Patients are so weak that never achieve the sitting position. Natural history studies indicate that more than 90% of these cases will have died by 2 years of age due to complications of respiratory problems [9,10]. Type I is known as the severe form given that the patients are so weak that never achieve the sitting position. Three subtypes can be identified: type Ia that starts very early after birth and also may overlap in some cases with the congenital (type 0) severely extreme form; type Ib corresponds to the typical form that start before the three months and usually patients never have head control; type Ic is detected after three months and patients may have some head control but never sit independently. In the type II form, patients manifest the disease after the 6 months of life and are able to sit but never walk independently and are permanently confined to a wheelchair (type IIa). Some stronger patients are able to stand up and even perform few steps with support (type IIb). In the type III form, patients can walk, but depending on the age of onset (less or more than 3 years), patients may lose the walking ability sooner in childhood (type IIIa) or later in adult life (type IIIb) respectively [8]. All these SMA types are the result of insufficient amounts of SMN protein which is encoded by two genes: Survival motor neuron 1 (*SMN1*) and Survival motor neuron 2 (*SMN2*) both located in a complex region of chromosome 5 (5q13) [3]. Although the SMN protein is ubiquitously expressed in all cells to guarantee living and survival, lower levels, as seen in SMA, are insufficient to protect motor neurons and the neuromuscular system [11,12].

SMA genetics and SMN protein function

The SMN genes are located in a region of chromosome 5q13 harbouring a segmental duplication [3]. *SMN1*

and *SMN2* share 99% homology, with few nucleotide changes in the coding region. Nonetheless, *SMN2* is an hypomorphic allele of *SMN1*, due to the alternative splicing of the 8th exon (exon 7), mediated by a C→T transition in position +6 [13-19]. This substitution disrupts interactions of the pre-mRNA with splicing enhancer and silencer proteins such that *SMN2* transcripts predominantly exclude exon 7 [13,19-24]. *SMN2* genes do not produce sufficient full length SMN protein to prevent the onset of the disease but, on the other hand, because each *SMN2* gene can still produce full-length SMN transcripts, no patient is devoid of SMN protein. Likely due to ancestral unequal crossing-over events, *SMN2* copy number is variable in patients and inversely related with the severity. Although *SMN2* is considered a good predictor of disease evolution, the correlation is not absolute and discordances may exist that need further investigations. (See Calucho et al. 2018, for a meta-analysis) [15,25].

Since the identification of the *SMN1* gene, a number of functions have been attributed to the SMN protein. So far, we know that SMN is ubiquitous, highly conserved across species, highly expressed during early development, and that SMN levels are higher in spinal cord and brain, but significantly down-regulated after birth [26-28]. SMN protein is member of a large, highly stable macromolecular complex that localizes in both the nuclear and cytoplasmic compartments of the cell [29]. While we know that SMN protein produced by the *SMN1* gene is fully functional, several lines of experimental evidence suggest that SMNΔ7 protein is rapidly degraded [30,31]. The SMN C-terminal domain is highly conserved and responsible for oligomerization, a process that is indispensable for its inclusion into the SMN complex. It has been hypothesized that the inability of SMNΔ7 protein to oligomerize, coupled with the resulting reduction in interactions with its own partners, might be responsible for the instability of this isoform [32].

The best-characterized function of the SMN complex is in the assembly of small nuclear ribonucleoproteins (snRNPs) which are involved in several aspects of RNA metabolism (see ref. 33 for a review). However, the link between SMN-snRNP biogenesis and SMA pathology remains unclear.

Several studies have evaluated the role of SMN protein in the two cell types which are more likely the specific targets of the disease: motor neurons and skeletal muscle. In motor neurons, SMN is localized in growth cones, along the axon and in the pre- and post-synaptic sides of the neuromuscular junctions (NMJ) [34-39]. SMN is subject to cytoskeletal-based, bidirectional transport between the soma and growth cones suggesting that SMN may have a cytoplasmic function related to neuronal transport of proteins and mRNA required at the distal tips of axons [38,40-42]. SMN protein deficiency could lead to the disruption of axonal transport and localization of several mRNAs, and/or of the assembly of specific snRNPs involved in transport and translation of a subset of axonal mRNAs: these defects would be responsible for the pathogenesis of SMA (see ref. 42 for a review). However, there is still debate why motor neurons are so sensitive to lower amounts of SMN in comparison with other neuronal cells.

Biomarkers in SMA

The landscape of SMA has been revolutionized over the last few years by the availability of effective treatments. The usual view of SMA type I-III needs to be updated for several reasons: firstly, the treatment of patients has revealed novel emerging phenotypes that do not fell in any of the classical forms [17]; secondly, the spreading of newborn screening programs is changing the diagnosis of SMA into that of subjects with a genetic defect who might or not develop early signs of the condition [43]. Additionally, the available outcome measures are not enough sensitive to detect tiny improvements that may still be clinically relevant, as in the case of the treatment of patients with a long story of disease. All these items have made mandatory the identification of prognostic, response and predictive biomarkers.

Even though some modifier genes have been reported (very recently reviewed by Kariyawasam et al., 2019) [44], so far the only genetic biomarker with clinical relevance is the determination of *SMN2* copy number, alongside with two alternative splicing-modulating variants (rs121909192 and rs1454173648, also known as NM_017411.3:c.859G > C and NM_017411.3:c.835-44A > G respectively [14,15]. Among *SMN2* gene products, full length transcript levels in peripheral blood correlate with the phenotype better than SMN protein levels [45,46]. For both, few longitudinal data are available [47]. Besides that, a number of efforts have been done to identify SMN-independent molecular markers, such as the SMA-MAP, neurofilament dosage, and few miRNAs [49,50]. Regarding the SMA-MAP, to our knowledge, beside the original cross-sectional study, no longitudinal data have been published so far. Among the other biomarkers, the most promising are thought to be the dosage in plasma of the phosphorylated neurofilament heavy chain (pNF-H) that allowed to differentiate SMA individuals from healthy controls. pNF-H levels were longitudinally dosed in patients treated with Nusinersen, showing a rapid decline and raising levels comparable to those of controls [50]. Albeit promising, the clinical impact of these data is limited by the insufficient number of healthy controls analysed; moreover we notice that the slope of pNF-H levels decay in patients is similar to that observed in controls with the highest levels of neurofilaments. Other biomark-

ers are also under study such as creatinine (Crn) in blood. A recent study showed that decreased Crn levels reflect progressive denervation and disease severity, suggesting that Crn is a candidate biomarker for SMA progression [51].

Beside molecular markers, some instrumental markers have also been evaluated: the majority of data available regards Compound Motor Action Potential (CMAP) and Motor Units Estimation Number (MUNE), the latter being the most reliable.

Newborn screening

The debate on the opportunity to perform newborn screening (NBS) for SMA has been issue of lively debate in the SMA community over the last years ahead of treatment availability [52,53]. At the time, the lack of effective therapies prevented the general consensus on this matter. The excellent results obtained with the pre-symptomatic treatment of SMA children in the NURTURE study [54], has changed the perspective and has made NBS a compelling need for both family associations and scientific community. Guidelines and operating workflows have been discussed and developed [43,55,56], pilot studies are ongoing or ready to start [43,57-61]. The results we are rapidly gaining are enlightening some crucial aspects and the pros and cons of the approach. Firstly, the advantage of the early treatment of expected severe patients is undoubtful, both in terms of health gain for children and of social, familial and economic burden [62]. Secondly, the scenario of SMA nosology is moving from the conventional classification based on the onset of clinical signs to the identification of oligo-asymptomatic subjects with an early molecular diagnosis. On the other side, some points remain open: 1) the different studies are providing quite variable incidence figures for SMA, ranging from inexplicably low levels (1/28137 in New York State) [57], to 1 in 11,545 in Australia [59], 1 in 7096 in Germany [58], 1 in 8398 in Belgium [60], 1 in 17,181 in Taiwan [63]. The preliminary data of our pilot study in two Italian Regions, indicate an incidence of 1 in 4861 (over the first 53477 neonates, updated at Dec 7th, unpublished data); 2) the stop-or-go for treatment starting remains *SMN2* copy number assessment, that still requires cross-validation and standardization across the different laboratories [15,16]; 3) the gold standard for treatment and follow-up of patients with 4 or more *SMN2* copies is still debated [15]; 4) the prevalence of asymptomatic subjects bearing *SMN1* homozygous deletion in the general population is unknown. The next few years will be of key relevance to discern these points and to get the widest spreading of NBS programs worldwide. The prevention programs of SMA are thus evolving from the treatment of symptomatic patients (tertiary prevention) to that of pre-symptomatic newborns (secondary prevention). Universal carrier screening programs (primary prevention) are also to be taken into account: these could constitute a complementary approach to allow couples to perform informed reproductive choices and eventually reduce the burden of the disease in general [43,64]. Once again, the availability of genomic biomarkers to predict the phenotypic severity is crucial.

The present therapeutic advances

After development of suitable animal models during the translational research decade (Fig. 1), the investigation of preclinical therapies has been successful to open the way to initiate clinical trials in patients [65-67]. A summary of the three approved SMN dependent therapies, including mechanisms of actions, administration and main trials involved is outlined in Table I. The earliest of the three programs was the nusinersen clinical program that started in 2011. *Nusinersen* (Spinraza®), an antisense oligonucleotide, can modulate *SMN2* splicing facilitating the inclusion of exon 7 to produce higher amounts of full-length SMN protein. Results of two pivotal clinical trials (ENDEAR and CHERISH) with loading doses and sustained intrathecal injection in type I SMA infants and late onset non-ambulant SMA patients led to wide label approval of this first tailored treatment in 2016 by FDA and in 2017 by EMA [4,68]. Expanded access programs as well as real world data confirmed safety and efficacy in more than 11.000 patients worldwide [69]. However, as mentioned above, the most impressive results have been obtained in pre-symptomatic patients with two and three *SMN2* copies detected because of previous family history of type I or type II disease (NURTURE clinical trial) [70]. These neonates started treatment up to 6 weeks of age and the majority of patients involved in this study were able to stand alone and walk independently.

A second clinical successful program started in 2013 with a single intravenous injection for a systemic-delivery of AAV9 with the coding part of *SMN1* as a gene transfer approach (AVXS-101) to replace *SMN1* in infants with SMA type I [5,72,73]. *Onasemnogene Abeparvovec*, (ZolgenSMA®) was approved in 2019 by FDA and in 2020 by the EMA becoming the most expensive drug in the market [73]. Ongoing studies and treatment access programs, targeting diverse population of patients, cover at present more than 400 infantile patients and also a number of pre-symptomatic cases. A third program refers to the oral compound RG7916 or Risdiplam, (Evrysdi®) which is a splicing modifier which also increase the inclusion of exon 7 and the amount of complete SMN protein. The results of their pivotal clinical trials in type I patients (FIREFISH) and type II-III patients (SUNFISH) led to the approval by FDA in 2020 [6].

The exclusive targeting of the central nervous system rather than the systemic approach is still an evolv-

Table I. Approved SMN dependent therapies for SMA (based and adapted from references 4,5,6,70,74,75,76 and www.clinicaltrials.gov).

	Nusinersen (Spinraza)	AVXS-101 (ZolgenSMA)	Risdiplam (Evrydi)
Type of therapy	18 mer antisense oligonucleotide specific to ISSN1	Self-complementary adeno associated virus 9 with human coding SMN1	Pyridazine derivative, binds to ESE2 on the 5'-ss site on exon 7
Mechanism of action	Increase amount of complete SMN protein from SMN2	Production of SMN protein from SMN1	Increase amount of complete SMN protein from SMN2
Administration route	Intrathecally (loading doses and sustained dose every 4 months)	Intravenously (one shot)	Oral (daily)
Pivotal Clinical trials	ENDEAR, CHERISH, NURTURE	AVXS 101, SPRINT, STRIVE	FIREFISH, SUNFISH, RAINBOWFISH
Number of patients treated (Clinical trials, Access programs and Real world data)	> 11,000	> 600	> 500
Approval	All SMA types (FDA 2016- EMA2017)	Age < 2 years: FDA (2019); type I up to 3 SMN2 copies: EMA, 2020	Age > 2 month: FDA 2020; EMA pending

ing issue [17]. Indeed, even though motor neurons appear the more sensitive cells to reduced levels of SMN, the protein is ubiquitously expressed and a number of extra neuromuscular findings has been reported, particularly in the most severe patients, including autonomic nervous system involvement, congenital heart defects, vascular defects, liver, pancreas, intestine and metabolic deficiencies [74].

Finally, although the three medications showed a therapeutic benefit when administered alone in most treated patients [4,5,75-77], they cannot be considered the cure of SMA, thus the investigation of combinatorial treatment is envisaged [17].

A number of other medications with a SMN-independent mode of action are under active investigation, and ergo might be transversally useful also in other neuromuscular disorders. These include for example neuroprotectors, neuromuscular junction stabilizers, muscle function activators or myostatin inhibitors. A summary can be found at www.clinicaltrials.gov and an updated pipeline in www.curesma.org. It is possible that in a near future, after their effectivity is demonstrated, these therapies may be incorporated into the protocols of SMA treatment. In this point, more preclinical studies and clinical investigation in patients should be performed to demonstrate their possible synergistic or additive effects.

What has changed during the last years in SMA and where are we going

We are witnessing an era of changes due to the live transforming therapies in SMA (Tab. II). There is an increasing interest in the disease that is reflected in the growing number of studies and publications (Fig. 1). More investigators and clinicians are discovering and becoming devoted to this fascinating disease and the possibility to apply advanced treatments [77]. This is also influencing other fields of rare genetic disorders in general and neuromuscular diseases in particular. SMA is an example of success that may encourage and give hope to patients, families, clinicians and researchers that an integrative collaboration could be successful to the main objective of stop the disease progression, rescue the phenotype or even an envisaged cure when therapy is applied as early as possible in some patients [78]. Research must go on: the awareness of the disease is now evaluating early manifestations for advancing the clinical detection, updates for wider genetic diagnosis programs (to give the patients the possibility to confirm disease and the option of treatment), and moving towards a better characterization of modifiers beyond the *SMN2* copies [15]. Other crucial issues are study and validation of biomarkers of disease evolution and response to treatments. Giving the rapid progression of severe SMA, a delay in treatment may impact the evolution with irreversible loss of function and reduced motor response. Therefore, the successful results in pre-symptomatic therapies support the inclusion of SMA in the newborn screening programs. A new SMA scenario of classification and progression trajectories is envisaged considering the increasing number of patients that will start the therapy during the neonatal period [43,64,77]. The impact of therapies in patients and families will modify the

Table II. What has changed in SMA over the last years and where are we going. More explanation in the text (Uppercase numbers show representative references of the text).

Increasing interest in the disease and record of scientific publications [77] (Fig. 1)
Defining of manifestations and awareness for early clinical detection [75-77]
Updating in genetic diagnosis, characterization of modifiers and validation of biomarkers [15,16,48-50]
Definition of new standards of care: from reactive to proactive [77,80]
Following up: the arrival of the multidisciplinary team [79,80]
Evolving of the SMA phenotypes and trajectories [17]
Changing perspectives in genetic counselling [64]
Managing expectations and sharing decision making for therapy [17,43,58,69,75,76]
Towards new SMA classifications [9,17,64,77]

burden of the disease and health policies.

We are also defining new standards of care moving from the traditional reactive approach to a more proactive and preventive approach that is also demonstrated by the expanding number of professionals and specialities that are involved in the follow-up of these patients [79,80]. These "new" patients under treatment present evolving phenotypes and trajectories that should be carefully defined in each case [17].

There is also a change in the genetic counselling of the disease [64]. A perception that SMA is no longer an untreatable disease is achieving consensus based on the promising results of therapies and the growing battery of available treatments. The perspective of families and reproductive decisions may evolve consequently. Although medications have demonstrated efficacy, patients with severe SMA are fragile and complications and death may happen to some patients even under therapy. For all these reasons, it is important to manage the expectations of the families with an adequate communication to establish a sharing decision making for therapy and psychological support. A further challenge that stands out is to accomplish the principle of wide access and equity for these expensive therapies to those in need. This requires the combined efforts of physicians, biomedical scientists, health-care economists, public-health experts, companies, funders and governments [78]. We all have to find a way to ensure that the costs in this Copernican revolution are not assumed by families that have already suffered SMA for too long.

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Myasthenia gravis: MuSK MG, late-onset MG and ocular MG

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Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction which affects all striated muscles, resulting in fluctuating weakness. Approaching MG as a disease with subgroups having different clinical, serological and genetic features is crucial in predicting the progression and planning treatment. Three relatively less frequently seen subtypes of MG are the subject of this review: MG with anti-MuSK antibodies (MuSK MG), late-onset MG (LOMG), and ocular MG (OMG). In addition to reviewing the literature, mainly from a clinical point of view, our experience in each of the subgroups, based on close to 600 patients seen over a 10 year period, is related. *MuSK MG* is a severe disease with predominant bulbar involvement. It is more common in women and in early-onset patients. With the use of high dose corticosteroids, azathioprine and more recently rituximab, outcome is favorable, though the patients usually require higher maintenance doses of immunosuppressives. *LOMG* with onset ≥ 50 years of age is more common in men and ocular onset is common. Frequency of anti-AChR and anti-titin antibodies are high. Although it can be severe in some patients, response to treatment is usually very good. *OMG* is reported to be more frequent in men in whom the disease has a later onset. Anti-AChR antibodies are present in about half of the patients. Generalization is less likely when symptoms remain confined to ocular muscles for 2 years. Low dose corticosteroids are usually sufficient. Thyroid disease is the most common autoimmune disease accompanying all three subgroups.

Key words: Myasthenia gravis, MuSK MG, Late-onset MG, Ocular MG

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Introduction

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction which affects all striated muscles, resulting in fluctuating weakness [1]. MG is mostly caused by antibodies against the acetylcholine receptor (AChR) and rarely by antibodies against muscle specific kinase (MuSK). In some of the patients without any detected antibodies (seronegative MG, SN MG), anti-AChR and anti-MuSK antibodies can be found with finer techniques [2]. The clinical significance of anti-LRP4 antibodies, many times coexisting with the classical antibodies in the same patient, is unknown.

Approaching MG as a disease with subgroups having different clinical, serological and genetic features [3-5] is crucial in predicting the progression and planning treatment. MG is associated with thymoma in 10-15% of patients. Among those without a thymoma, the largest subgroup consists of young women (age < 50 years) with anti-AChR antibodies. An increasingly important subgroup is MG with later onset (≥ 50 years). A very small subgroup is caused by antibodies against the MuSK antigen. MG usually tends to affect the oculobulbar and extremity muscles in all of these patients. In a small subgroup, it affects only the ocular muscles.

Three relatively less frequently seen subtypes of MG are the subject of this review: MG with anti-MuSK antibodies (MuSK MG), late-onset MG (LOMG), and ocular MG (OMG). Attempt was made to define the subgroups carefully with particular attention given to distinguishing generalized from ocular disease. In addition to reviewing the literature, mainly from a clinical point of view, our experience in each of the subgroups, based on close to 600 patients seen over a 10 year period, is related. To put our data on the subgroups into perspective, it is necessary to describe our cohort first.

Istanbul University MG cohort

Our cohort consisted of 576 patients, derived from our MG Database, who applied for the first time to the Neuromuscular Outpatient Clinic, Neurology Department, Istanbul Medical Faculty, Istanbul University (IU) during a 10 year period between 2001 and 2010. It is an unselected group with consecutive patients, subjected to the same criteria for inclusion in a set period of time.

Some patients with MuSK MG from this cohort were reported in two articles [6,7], they consisted of all MuSK MG patients seen until 2005 [6] and until 2009 [7]. Patients with LOMG in this cohort were also reported [8]. A manuscript on ocular MG, again with patients in this cohort, is in preparation.

Evaluating the frequency of the subgroups, thymoma-associated MG (TAMG) was found in 14%. Early-onset (< 50 years) generalized MG was the most common subgroup comprising 38% of the cohort. Late-onset (≥ 50 years) generalized MG and ocular MG (OMG) each made up about one fifth of the patients. MuSK MG comprised 7% of the cohort. Taking both onset age and antibody status into consideration, percentages were as follows. Anti-AChR generalized MG: early-onset (EO) 32%, late-onset (LO) 19%; MuSK MG: EO 6%, LO 1%; SN generalized MG: EO 6%, LO 2%; OMG: EO 10%, LO 10%; TAMG: EO 9%, LO 5%. In OMG, about half had anti-AChR antibodies. Almost all patients with thymoma had anti-AChR antibodies. It was striking to note that the subgroups of MuSK MG and SN MG were extremely rare in older ages. Also interesting was the fact that thymoma was more common in younger patients in this cohort.

Gender differences among the subgroups were noteworthy. Women predominated in early-onset generalized AChR MG (75%) and MuSK MG (82%). Women also predominated in seronegative generalized MG (72%), both early-onset and late-onset. Men predominated in late-onset generalized AChR MG (66%). The two genders were comparable in ocular MG and TAMG.

Our percentages in general reflected what is reported in the literature, except that thymoma was more common in younger patients in our cohort.

MuSK MG

MG with antibodies to the postsynaptically located MuSK protein is a relatively newly defined disease. It constitutes less than 10% of the MG patients, being more prevalent in the Mediterranean countries as compared to the northern ones in Europe, but more prevalent in the north as compared to the south in China [5]. The antibodies are mainly of the IgG4 class as opposed to IgG1 in MG with anti-AChR antibodies (AChR MG) [2]. There is a specific association with HLA-DRB1∗14, -DRB1∗16 and -DQB1∗05 HLA [9,10].

Onset age peaks in the late 30's and it is uncommon in prepubertal and elderly patients [6,11]. The disease is reported to be more common in women [11] although female preponderance was found to be comparable to AChR MG and SN MG in one report [6]. Clinically, it constitutes the most severe form of MG, paralleled by TAMG [5]. Bulbar, neck and respiratory muscles are involved with fast progression of the disease to life-threatening symptoms [11,12]. Dropped head syndrome with neck extensor muscle weakness can be the presenting symptom [13,14]. Ocular muscles are frequently mildly involved, and conjugate limitation of eye movements which is exceptional in MG can occur in MuSK MG [11]. Extremity muscles are usually spared or mildly affected. In those with ocular onset, generalization occurs in a short time. Rare patients generalize after a few years and pure ocular MG is extremely rare. Interestingly, we had one patient with onset at 3 years [15] who remained with purely ocular symptoms for a prolonged period before generalization, similar to another reported patient with MuSK MG [16], and in accordance with the frequent course of prepubertal-onset patients [17].

There are difficulties in diagnosis when the onset is indolent and fluctuations are not evident. At least half of the patients do not respond to anticholinesterases and a few can get worse, fasciculations and cramps are common [14]. Decrement is usually not present in the extremity muscles with repetitive nerve stimulation. The muscle with the best yield was found to be the orbicularis oculi [18]. Needle EMG may show a myopathic pattern.

Corticosteroids are the most effective drugs, but they are needed in high doses in many patients. Usually a second immunosuppressive is required at an early stage. Plasma exchange is considered to be more beneficial than IVIg in MuSK MG [19]. Although the disease is usually severe, many patients eventually do well with appropriate therapy [11,14]. We had previously compared all MuSK MG and generalized SN MG patients registered in our MG database with consecutive non-thymomatous 161 AChR MG patients [6]. Their outcome with conventional therapy was similar to AChR MG patients; however, they required higher dose of maintenance corticosteroid therapy.

Tongue atrophy has been noted in MuSK MG, but the role of refractoriness and the use of long term high dose corticosteroids is questioned [20].

There are MuSK MG patients who are clinically not distinguishable from non-MuSK MG [12]. Likewise, some MuSK MG patients can be easily treated. We had reported [7] that fast and good response within 3 months of starting corticosteroids predicted good outcome in MuSK MG patients. These patients, comprising one third of the study population, had pharmacological remission or minimal manifestations on a mean low maintenance dose of 6 mg/day (or as is generally done, 12 mg on alternate days) of prednisolone, usually with additional azathioprine.

Newer drugs such as rituximab have changed the scene in MuSK MG, particularly for refractory patients [21-23]. It is considered to be an early therapeutic option in the patients without a satisfactory initial response to conventional immunosuppressives [19]. Symptomatic treatment with 3,4-diamynopyridine (3,4-DAP) and albuterol is also being considered [11]. Over the years, a better outcome is noted in MuSK MG patients, attributed to early diagnosis and better treatment [11].

It has been difficult to evaluate the effect of thymectomy, because of the confounding effect of steroids, which have usually been started before the surgery. The pathology of the thymus showed involution in most patients [24], further suggesting that the thymus may not play a similar role in MuSK MG as compared to AChR MG. Thymectomy is now not considered to be indicated in MuSK MG. There are exceptional cases with small thymomas [25]. In parallel to the other subgroups of MG, thyroid disease was the most frequent autoimmune disease in MuSK MG [11].

One point needs to be emphasized. An important differential diagnosis of MuSK MG is with bulbar amyotrophic lateral sclerosis (ALS), particularly because of fasciculations [26] and myotonic discharges [27] in rare patients with MuSK MG. Absence of definite fluctuations, absence of response to anticholinesterases, and difficulty in finding decrement in classically-examined muscles in repetitive nerve stimulation in some patients with MuSK MG may suggest ALS in the face of bulbar symptoms/head drop without ocular symptoms. Even without fasciculations, the differential diagnosis of MuSK MG and bulbar ALS can be difficult. It is sometimes difficult to distinguish from ALS the speech in MuSK MG which may lack the nasal quality of typical MG. Furthermore, mild fluctuations, mild response to anticholinesterases and decrement can all be seen in ALS. Needle EMG of the extremities is usually normal when the symptoms are confined to bulbar muscles in ALS. Caution is necessary in the diagnosis of some cases with bulbar symptoms.

IU MG cohort: MuSK MG

In our cohort of 576 patients, 38 had MuSK MG. One patient, a man with onset at age 39, had a father with LOMG, who was negative for both anti-AChR and -MuSK antibodies. In the cohort, median age of onset was 34 years (range of 3-66 years). Below age 16, there was only one patient with onset at 3 years [15]. Onset age was ≥ 50 in 5 patients. Eighty-two percent were women. Onset with ocular symptoms was present in 15 patients (39%). None had ocular MG throughout the entire period of observation. Onset with bulbar symptoms was seen in 12 patients (32%). Three patients had head drop as an onset symptom. The rest had oculobulbar or extremity onset.

Most of our patients did not respond to anticholinesterases. However, some patients responded to lower doses so that anticholinesterases can still be an option to be used with caution in MuSK MG. Of note is the fact that the initial test dose was very good even in some of the non-responders. About half of the patients had hypersensitivity to anticholinesterases. As reported, we found that repetitive nerve stimulation of the orbicularis oculi muscles yielded the best results, both in the frequency and in the amount of decrement.

Maximum severity and outcome were evaluated in 36 patients excluding the two patients who had been seen only once. Median disease duration was 8 years (range: 1-20 years) and median follow-up was 6 years (range: 1-13 years). Using the Myasthenia Gravis Foundation of America (MGFA) Classification [28], 75% were MGFA Class 3 or above; 31% of these were 4b and 3 had been intubated (MGFA Class 5). Three patients had tongue atrophy; it was notable that two of these patients had been treated late and the response had taken a long time. Four patients had fasciculations, usually related to the beginning of MG or to exacerbations.

Treatment included corticosteroids and azathioprine. Prednisolone at 1 mg/kg/day or 60 mg/day was usually needed and it was continued for a longer period than usual in some patients. A look at the outcome revealed that two patients had died of MG. Twenty patients (56%) had reached MGFA post-intervention status (PIS) [28] of pharmacological remission or minimal manifestations with a mean maintenance dose of about 10 mg/day of prednisolone, but there was none with complete stable remission. Two were unchanged and the rest had improved to some degree.

At the time of this cohort, treatment of MuSK MG was a little different in that we did not use rituximab and had not yet discontinued performing thymectomies. In fact, 23 patients had been thymectomized. No patient in our cohort had a thymoma. In most of these patients, corticosteroids had been started before thymectomy and in a few within 3-4 months after thymectomy so that it was impossible to evaluate the effect of thymectomy properly.

Involution of the thymus was the main pathological result in these patients with a few showing mild hyperplasia. Overall, it is not possible to say that thymectomy has any effect in MuSK MG.

Late-onset MG

The prevalence of LOMG has increased in recent years, [29] worldwide [30]. It is not clear whether the increase reflects a biological phenomenon[31] or has been influenced by increased awareness, availability of the AChR antibody assay [29,32] and longer lifespan [29]. LOMG is different from early-onset MG (EOMG) with respect to demographic-clinical characteristics as well as serological properties. Specific HLA associations of LOMG have been discovered [33,34]. Comparison between studies on LOMG is somewhat difficult because of different cut-off ages used to distinguish LOMG from EOMG, and the inclusion/exclusion of thymoma. Despite the difficulties, there seems to be consensus on many characteristics of LOMG.

The percentage of men is higher in LOMG [8,17,35-37]. Anti-AChR antibodies were present in over 80% [8,17,35-38]. A rising frequency with higher decades of the percentage of anti-AChR antibodies [8,37] was reported, with 93% in very-late-onset MG (≥ 65 years) [37]. Anti-MuSK antibodies were found to be uncommon after 65-70 years of age [11,37]. The implication of these findings is that one has to be extremely cautious when diagnosing MG in double seronegative patients in the elderly and other causes have to be sought diligently.

Anti-titin antibodies were present in one third to over one half of the LOMG patients [8,38-40], being more frequent in LOMG than in EOMG. They increased in higher decades in LOMG [8,40]. Anti-titin antibodies were not found to be a poor prognostic factor in LOMG in two studies [8,40].

Ocular onset was more frequent in LOMG [8,17,36,37,41]. Myasthenic crisis occurred in 6-11% of the patients [8,17,36,37]. Although a higher percentage of patients presented with life-threatening events at onset, they responded better to medications; those over 65 were particularly better than early-onset patients in terms of drug requirements and drug refractoriness [37]. At the end of follow-up, over 80% were reported to be improved or better [8,17,35,42]. Beneficial effect of thymectomy in LOMG has been reported, but it is usually not advised beyond age 55. Thyroid disease was reported to be the most frequent autoimmune disease accompanying LOMG [8,36].

IU MG cohort: Late-onset MG

In an attempt to understand more about non-thymomatous generalized LOMG and its outcome, we analyzed separately 95 of the LOMG patients with generalized symptoms (ocular MG excluded) who had been followed for ≥ 3 years in the same cohort. Although reported [8], it might be useful to emphasize some of the findings. Men constituted 63% of the patients. Onset was ocular in 62%, bulbar in 23% and in the extremities in 15%. Anti-AChR antibodies were positive in 84% and anti-MuSK antibodies in only 5%. Anti-titin antibodies were present in 61%.

Half of the patients were MGFA Class 3 or above with myasthenic crisis in 6%. Outcome was good with 63% reaching MGFA PIS of complete stable remission, pharmacological remission or minimal manifestations with a mean maintenance dose of about 5 mg/day of prednisolone and a further 24% were improved. Patients in whom azathioprine was added to prednisolone did significantly better than those receiving only prednisolone. Another finding in the study was that many patients with mild disease who received a low maximum dose of prednisolone (≤ 30 mg/day), usually together with azathioprine, had a favorable outcome, implying that low-dose prednisolone with additional azathioprine may be sufficient in mild disease of older people. Thymectomy, done in 12 patients, was not found to be useful although it is not easy to evaluate thymectomy when corticosteroids are used.

Ocular MG

Ocular MG (OMG) refers to patients whose symptoms are confined to ocular muscles (levator palpebrae superioris and extraocular muscles), resulting in ptosis and diplopia; orbicularis oculi muscles are variably weak. Ocular muscles are involved in about 50% of patients at onset of MG. In about half of these patients, generalization occurs to non-ocular muscles, leaving about one fourth of patients with purely ocular symptoms for a prolonged/indefinite period [44-46]. Generalization usually occurs within one year, mostly within 2 years and it is uncommon after 2 years [44-46]. Generalization is reported to be more likely in anti-AChR positive [47,48] and TAMG [49]. Purely ocular MG is more common in Asians, particularly in juvenile-onset patients [50]. It is reported to be more frequent in men [51,52]. Spontaneous remissions with a mean of 4-5 years are reported to occur, mainly at earlier stages of the disease [46,51].

When a patient presents with ocular symptoms, there are several clinical clues which make the diagnosis of MG more likely. Complete or almost complete unilateral ptosis without pupillary changes, one sided ptosis alternating with ptosis on the other side, definite improvement in the mornings and presence of remissions are very suggestive of the diagnosis of MG. Ptosis can be mild. Eye movement limitation is usually asymmetrical, it can mimick all cranial nerve palsies as well as internuclear ophthalmoplegia.

Diagnosis is easy when anti-AChR antibodies, present in about half of them, are detected. Anti-MuSK an-

tibodies are extremely rare in MG with prolonged pure ocular symptoms. When antibodies are not detected, diagnosis can be difficult in some patients. Response to anticholinesterases may not be present in some patients; on the other hand, other entities such as intracranial mass lesions may show a positive response, usually requiring cranial magnetic resonance imaging for the differential diagnosis [47]. Repetitive nerve stimulation is not very useful with purely ocular symptoms. Single fiber EMG is very sensitive, but one has to be careful remembering that it is not specific to MG [53]. One other caveat about single fiber EMG is in order: Presence of abnormalities in limb muscles of a patient with OMG does not indicate generalization [54], the diagnosis of generalized MG is clinical. When all tests are negative in a patient with ocular symptoms, a trial of corticosteroids may be necessary.

In the differential diagnosis, mitochondrial myopathy (progressive external ophthalmoplegia, PEO) must be considered. Diplopia is not a feature of PEO; however, ptosis can be asymmetrical and fluctuations can be present, making the differential diagnosis with MG difficult. Oculopharyngeal muscular dystrophy is usually bilateral and without eye movement limitation, at least for a long time. Congenital myasthenic syndromes usually start in infancy and it is then easy to eliminate MG. The distinction with thyroid ophthalmopathy can be difficult. In thyroid ophthalmopathy, ptosis is rare; esotropia (inward deviation of the eye) and hypotropia (downward deviation of the eye) are usually present since muscles causing restriction are medial rectus and inferior rectus. Thus, ptosis and exotropia (outward deviation of the eye) are suggestive of MG in a patient with thyroid ophthalmopathy [55].

Anticholinesterases provide symptomatic treatment for some patients with ptosis, but they are usually not useful for diplopia since the required precise alignment cannot be achieved [50]. Corticosteroids have caused debates on whether they are beneficial or not [50]. Observational studies [47,56] and one clinical trial [57] with a small number of patients (11 patients) have found them to be effective. Generalization appeared to be less likely in the patients who received immunosuppressives. It is emphasized that low doses are sufficient: About 25 mg/day or twice the dose on alternate days usually results in pharmacological remission. A real concern is the appearance of relapses upon discontinuation of steroids in many patients, necessitating long term administration with small doses. High dose intravenous methylprednisone has also been advocated [58]. Azathioprine and mycophenolate mofetil have been found to be beneficial [50] and again lower than the standard doses may be sufficient in OMG. It takes a few months for them to take effect so that they are usually used as additive drugs. However, they can be given as the sole therapeutic agent in selected patients.

Intravenous immunoglobulins do not improve the symptoms [59]. Thymectomy, reported to be beneficial in some patients [60], is usually not considered to be indicated in OMG, although some centers might have revised their indications after the advent of videothoracoscopic thymectomy.

Eye patches for diplopia, prisms when eye movement limitation is mild and eyelid crutches for ptosis can be useful. There are rare patients who do not improve and have severe symptoms/signs despite all therapy. In these patients, if the signs are chronic and stable, blepharoplasty can be a good option, taking care not to cause the diplopia to be more disturbing once the ptosis is alleviated.

IU MG cohort: Ocular MG

In our cohort, 101 patients with a median disease duration of 8 years remained with purely ocular symptoms/signs throughout the entire period of observation. Two of the patients had thymomas. In the cohort, almost equal distribution was present regarding gender as well as onset age with very slight preponderance of men and early-onset. In patients with early-onset, women predominated while men predominated in late-onset. About half of the patients were anti-AChR positive.

Low dose prednisolone of 15-30 mg/day was sufficient in many patients. Addition of azathioprine, sometimes also at a low dose, appeared to be beneficial. Azathioprine was used as a single agent successfully in selected patients. We have also found that several courses of intravenous methylprednisone pulse, alone or in addition to oral steroids, was very helpful in difficult cases.

Myasthenia generalized after 2 years in another small group of patients. The majority of the patients were anti-AChR positive. Presence of thymoma was another risk factor for generalization. The two patients with anti-MuSK antibodies also generalized after several years.

Conclusions

In this review, the importance of analyzing the subgroups separately was emphasized because each has its own characteristics and different treatment approaches. Thymectomy, a very important treatment in MG, is not considered to be a widely-accepted therapeutic option in any of these subgroups. Corticosteroids are useful in all of the subgroups; however, while MuSK MG patients need high doses, low doses are usually effective in OMG, and LOMG patients can also respond well to low doses. Additional immunosuppressives are usually needed in order to taper corticosteroids to low maintenance doses and perhaps to be able to discontinue them. With the advent of new therapies, the differences between the subgroups are likely to play a more important role.

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Spinal cord injuries, human neuropathology and neurophysiology

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A correlative approach to human spinal cord injuries (SCI) through the combination of neuropathology and neurophysiology provides a much better understanding of the condition than with either alone. Among the benefits so derived is the wide range of interventions applicable to the restorative neurology (RN) of SCI so that the neurological status of the SCI patient is thereby much improved. The neurophysiological and neuropathological elements underlying these advances are described.

Key words: spinal cord injuries, restorative neurology, discomplete SCI

Introduction

Human spinal cord injuries (SCI) pose a massive human, public health and economic burden world-wide. In the USA alone it is estimated that over 250,000 persons are affected by SCI with the economic cost being in the order of a trillion dollars [1]. The enormity of the problem brings great pressure upon neuroscientists researching to find a cure for SCI despite the great difficulty imposed by the task. The major limiting factor in this respect is the inability of central axons to regenerate[2]. The setback due to lack of regenerative ability is further aggravated by Wallerian degeneration. In Wallerian degeneration the distal portion of the axon disintegrates progressively from the point of injury, caudally in the case of the descending efferent motor tracts and cranially for the afferent sensory pathways. Because of Wallerian degeneration experiments designed to rejoin the severed axons are totally flawed from the outset.

Therefore, an alternative approach to the problem posed by SCI is called for and this lies in the exploitation of limited but preserved neurological functions which may have escaped injury. In this regard it is a remarkable fact that in most SCI whether clinically complete or incomplete a small amount of residual white matter remains intact traversing the level of injury. This finding rests on both neurophysiological and neuropathological evidences and is the essence of this review. For instance, in a high proportion of otherwise clinically complete SCI patients conduction across the level of injury can be shown neurophysiologically. The term *discomplete* SCI has been introduced to describe this phenomenon. Various innovations have been introduced which enhance the function of these residual nerve fibers and as such improving the neurological status of the SCI patient. These methods form the armamentarium of the new discipline of restorative neurology (RN).

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Conflict of interest
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Neuropathology of spinal cord injuries

In a series of 220 SCI postmortems it is found that there is no standard lesion, each case of human SCI is distinct so that they are never exactly similar. Everyone is an individual. This means that when an effective treatment is discovered it will need to be customized to suit each affected person individually in which case the standardized lesions produced in experimental animals would not be generally applicable.

In diving or motor vehicle injuries the spinal column is hyper-flexed or hyperextended causing disruption of ligatures and bony fractures of the vertebral column. As a consequence, the spinal cord is contused, crushed and lacerated. Total transection of the spinal cord is a rare finding. More often there is some continuity across the site of injury. Extradural or subdural hematoma formation is not a feature of human SCI. Clinically SCI is classified as being "complete" when there is total loss of sensation and voluntary motor control below the level of injury. The SCI is clinically "incomplete" when some degree of sensation and/or retained but limited voluntary movement is present. There is a third clinical category of SCI for which the term "discomplete" has been introduced[3]. In discomplete SCI there is absence of all sensation and voluntary movement below the level of spinal cord injury but in whom transmission of signals across the lesion can be shown neurophysiologically. This partial retention of neurophysiological function is supported by post-mortem findings in SCI patients which show that there is most often a small quantity of white matter i.e. axonal sparing at the level of injury in which case the term "anatomically" discomplete SCI is applicable [4,5].

Briefly the pathological changes found at post-mortem consist of compression laceration and central hemorrhagic necrosis of the spinal cord with a variable amount of preserved white matter at the periphery. A few hours after injury polymorphs infiltrate the lesion followed by lymphocytes and after a few days by macrophages which engulf the necrotic debris. A small amount of traumatic demyelination can be found at the early stage, limited to the level of injury. Softening (myelomalacia) of the spinal cord is also in evidence within 24 hours. Macrophage activity leads to multilocular cavity formation with astrocytes forming its wall and glial trabeculae crisscrossing the cyst. Within the walls of the cavity a small number of myelinated axons may be found. Following the injury, the myelinated pathways efferent and afferent undergo Wallerian degeneration. The myelin sheath breaks up into globules and the axons degenerate progressively both proximally and distally [6].

Neurophysiology of spinal cord injuries

In the normal human spinal cord learned tasks are associated with changes in the microanatomy of the cord as new networks are established. Motor control of muscle activity and movement is modified by the learning process in which the propriospinal network participates in addition to the recognized corticospinal voluntary motor pathway [7]. Surface poly-electromyography (PEMG) allows one to distinguish total spinal cord disruption from partial damage even in cases in which the syndrome is clinically complete. Standardized PEMG recordings of motor tasks enable patterns between injured and non-injured subjects to be compared [8,9]. PEMG also provides insights on how complex spinal systems combine to produce functional outcomes [10]. Two human SCI models are recognized by PEMG. Firstly, the less common type of injury in which the spinal cord is clinically completely anatomically disconnected from the brain. Secondly there are SCI patients in whom there is a partial spinal cord injury so that the segments below the level of injury receive a reduced and altered cortical input [3].

In SCI cases where the spinal cord is completely separated anatomically and physiologically from the brain the cord generates its own ipsilateral unsustained phasic proprio- and exteroceptive reflexes. In incomplete and discomplete SCI where there is partially retained residual brain influences, tonic segmental reflexes are observed even in the absence of any volitional muscle control [11,12].

With increasing suprasegmental conduction there is better motor control ranging between large and poorly organized flexion and extension movements to highly organized volitional postural and gait activity in which there is complete integration of segmental and suprasegmental functions [13].

Recovery from spinal cord injury depends on the presence of conducting axons and the locations of their endings within the spinal central grey matter [13]. Long loop reflexes between the brain and the spinal cord are dependent upon the integrity of both systems, upper and lower motor neurons. Complete or partial separation from the brain results in motor activity arising from local structures and synapses. Functional hierarchy of the spinal cord anatomically integrated with the brain results in functions which are dependent or independent of brain influences as may be demonstrated physiologically. The differences between anatomically integrated or anatomically dissociated motor activities of the spinal cord may be clearly recognized by physiological assessments [14]. There are several pathways which descend upon the anterior horn cells and the interneurons of the central grey matter of the spinal cord. The best known is the cortico-

spinal tract for voluntary movement. There are also the corticobulbar, or extrapyramidal, tracts and the peripheral nerve primary, secondary and tertiary afferents connecting with the spinal interneuronal network which possess widespread connections over many spinal cord segments. These interneurons integrate the numerous functions involved in producing harmonious movements [14].

The discomplete spinal cord injury syndrome

The term 'discomplete' SCI is applied to ASIA-A clinically complete cases of SCI in whom cortical influences below the level of the lesion can be identified by surface PEMG[3]. In discomplete SCI although there is absence of voluntary motor control below the level of the lesion neurophysiological evidence of residual conscious volitionally induced influence on spinal reflex activity below the level of injury can be found. Motor control and the pattern of motor unit activity recorded by surface electrodes in discomplete SCI is elicited through stretch reflexes, tendon vibration, attempted volitional activation of clinically paralyzed muscle groups, voluntary suppression of plantar withdrawal reflexes below the lesion and by reinforcement techniques such as the Jendrassic maneuver above the SCI lesion.

Concerning the frequency of the discomplete syndrome, in our experience of 88 clinically complete SCI cases examined electromyographically by PEMG, 74 (84%) were found to be discomplete and thus were the majority [15]. On the other hand "absolutely" complete SCI in which there is total absence of voluntary movement or sensation below the level of the lesion and in whom there is no neurophysiological evidence of supraspinal influence on the spinal reflexes below the lesion are in the minority.

There is currently an increasing number of reports of 'motor' discomplete[16] and 'sensory' discomplete cases appearing in the literature [17]. Nevertheless, there are still many attending SCI clinicians who believe that most clinically complete SCI cases are also in 'absolutely' complete group. These physicians seem to be incognizant of the existence of the discomplete syndrome which represents the majority of clinically complete cases. This lack of appreciation may be due to their belief that prominent spasticity arises only when there is 'spinal cord transection' arising from a segmental generator of hyperactivity below the level of the lesion. This misunderstanding needs to be corrected as follows. The interneuronal network presents a structurally dynamic entity which integrates many functions to produce harmonious voluntary movement and is a unified entity rather than being separated into two groups one spinal and the other supraspinal [18]. There exists a premotor neuronal pool which receives signals from above and from peripheral afferents [19]. Lesions of the descending voluntary and extrapyramidal pathways release the interneurons from brain control which then becomes an independent "generator of spasticity". Thus, released from cortical inputs, the interneurons sprout and network with peripheral afferents, creating new connections and so develops into a "new anatomy" generating spasticity [20].

Anatomical aspects

The neurophysiological evidence of transmission of signals across the level of injury in discomplete cases of SCI is supported by anatomical data as follows. In a post mortem study of 220 SCI cases 53 were found to be anatomically discomplete i.e. during life they had no voluntary motor control or sensation below the level of injury but who had anatomically a variable number of preserved axons traversing the lesion. This residual white matter was quantified in 3 of these discomplete cases being 1.12, 3.89, and 1.09 square mm. respectively. It is this residual white matter consisting of myelinated axons which conducts the neurophysiological signals observed to cross the lesion in discomplete cases of SCI [5]. In the incomplete and discomplete SCI patients the preserved white matter is responsible for the newly established profile of the residual brain descending system connections and their integration with the spinal network. Moreover, in studies of motor control in chronic SCI patients with multichannel surface PEMG it was shown that brain motor control diminished by SCI results in the establishment of a new distinct pattern of residual brain motor control. This is also the case for the quality of gait performance, which depends upon the extent of residual suprasegmental brain influence and brain control [21].

Restorative neurology

Restorative neurology is the branch of neurological science which applies active procedures to improve functions of the impaired nervous system through selective structural and functional modification of altered neurocontrol. It was first defined in 1982 at a Symposium on the Upper Motor Neuron. In restorative neurology under-unrecognized or altered neural functions are modulated by techniques which act on afferents and surviving neural circuits thus improving neurological status of the patient [22,23].

The human CNS has the ability to conduct neural impulses as spikes carrying information from inside and outside the body to, from and between hierarchically placed nuclei of the brain and spinal cord which then process this information to produce appropriate sensory perception

and motor output. The conducting pathways are made up of multi-parallel axons of different diameters, conduction velocities and lengths reaching neural processors that are of different sizes and shapes, located within the gray matter and constructed from populations of interneurons with short axons. These two basic functional features of the CNS, to conduct and to process, provide sensory perception, cognition, and a wide variety of movements, from locomotion to learned skills and speech.

The systematic use of a comprehensive protocol for multichannel surface PEMG recording, which was developed as a method for brain motor control assessment (BMCA) is a very useful protocol in assessing SCI neurophysiology. BMCA is able to quantitatively describe the characteristics of motor control recovery in persons with SCI. Key information is contained in the overall spatiotemporal pattern of motor unit activity, observed in the PEMG envelope. In incomplete SCI patients, in addition to the methods that are applied in patients with complete motor lesion (proprioceptive and exteroceptive reflexes, motor unit and microneurographic studies, etc.) neurophysiological studies during volitional motor activity should also be included. Three functions of the upper motor neuron are studied in this context:

A. preservation or deterioration of volitional activity;
B. the effects of remote muscle contraction on paretic, or paralyzed muscles;
C. features of stretch and withdrawal reflexes.

In Restorative neurology, rather than focusing on the deficits and lost function caused by upper motor neuron lesions or disorders it is more advantageous to elucidate, in each individual, the specific neural functions that remain intact and from there, to build upon the preserved functions by designing a treatment protocols to optimize their effectiveness and thus improve recovery.

Theoretically there are two means by which neural processing and restoration of upper motor neuron functions may be modified, firstly anatomically, through reconstructive neurosurgery and secondly by fostering functional restoration through restorative neurology. Both approaches are applied in the modern practice of restorative neurology such as by physical therapy and applied neurophysiology as well as by pharmacological, functional neurosurgical and neurobiological means. Reconstructive neurosurgery consists of tendon or peripheral nerve transplantations. Neurobiological methods explore the potential for neurotrophins and stem cells to differentiate into becoming nerve cells, which may then theoretically be able to search for and recognize appropriate target neural circuits and thus to restore function – but to the present such results remain to be demonstrated.

In our experience SCI neural damage is almost never total as determined neurophysiologically or morphologically by autopsy (see above). In human SCI there are almost always some surviving and functioning neural tissues that, depending on their newly establish relationships; will generate clinical and sub-clinical residual movements. Neurophysiological methods designed for the assessment of processing and conducting neural systems are capable of elucidating and measuring the characteristics of such residual functions.

The spinal cord, being the output organ of the central motor system, has a fundamental role in the restoration of motor function. The spinal cord consists of neural networks located within its central gray matter with long white matter intersegmental connections as the pro-propriospinal system. Descending efferent tracts are the corticospinal, reticulospinal, vestibulospinal, rubro and tectospinal pathways. This spinal neural circuitry integrates the nervous system as a common final network within which is executed the motor control required for reflex activities, volitional, postural and gait. Their descending influence is exerted over the control of reflexes at the interneuron level. These descending voluntary and extrapyramidal motor systems converge upon primary afferents connecting to the interneuron pool and from there projecting to the motor neuron. The capacity of the spinal cord to integrate converging signals is retained even in the absence of brain input as shown by sustained electrical stimulation of posterior nerve roots. Different strengths, site and frequency of posterior root stimulation can elicit functional and non-functional motor output patterns [24]. This finding is applied in the clinical practice of restorative neurology through the augmentation and modification of residual motor control [25].In the clinical practice of restorative neurology the modification of the spinal cord network configuration is accomplished by electrical stimulation of afferents of peripheral nerves, posterior roots and posterior columns which elicits a central state of the excitability. Such excitability is required to be maintained at an operational level to generate a functional motor output. It is also essential that this central state should be in dynamic equilibrium between excitatory and inhibitory mechanisms within the processing networks. In addition, pharmacological intervention to support and maintain this equilibrium can be of additional value [26].

Discussion

The spinal cord has a regular segmental structure with long white matter ascending and descending pathways placed peripherally and with the gray-matter neural networks centrally located. The human lumbar spinal cord is not simply a relay system between brain motor pathways and spinal motor neurons [27]. Its role within an integrated nervous system is that of a common processing

network for the execution of voluntary motor movement as well as for local reflexes, posture, and gait. The lumbar spinal cord gray matter contains a large population of interneurons which form neural circuits, the functional organization of which is flexible and subject to plasticity giving it a multifunctional character providing modulatory actions, reconfiguration, and flexible operation. In this way the lumbar spinal cord may be considered to be a "spinal brain" [26,28].

Electrical and magnetic spinal cord stimulation provides a reliable way in which to characterize neurophysiological and pathological aspects of spinal cord functions. Moreover, transcutaneous direct current stimulation and repetitive magnetic stimulation holds therapeutic promise for patients with spinal cord injuries. To the present the number of findings from epidural or transcutaneous spinal cord stimulation in paralyzed chronic spinal cord injury is limited to observational and neurophysiological studies in a relatively small numbers of subjects. Well-designed scientific studies are needed to consolidate our theoretical and practical knowledge of Human Motor Control and the neurophysiology of upper motor neuron dysfunction. Briefly we are beginning to learn how to perform external modifications of upper motor neuron function by external control of afferents to the spinal cord. External electrical stimulation of posterior root afferents has the potential to facilitate neuroplasticity. Neuroplasticity is defined as the ability of neurons and neuron networks to adjust their activity and their morphology from alterations in their environment or patterns of use [29]. External control of afferents facilitates the development of neuroplasticity in SCI [30].

Conclusions

Clinical research designed to improve the quality of spinal cord patients' recovery has given promising results. For instance, we have discovered that there are many diverse methods which can restore movement after injury in complete, discomplete and incomplete SCI as shown above. This beneficial outcome is best explained in two parts: firstly by gaining extensive knowledge and data from healthy spinal cord systems and secondly by a new approach of managing spinal cord injury as a structured, multi-modal program with on point analysis and measurements. A remarkable fact is that SCI has little or no finality and what we may see as damaged spinal cord neural systems actually are useful in ways which we continue to discover. It will be seen that well administered care of the spinal cord injury patient which incorporates all possible approaches and new discoveries leads to remarkable spinal cord injury recovery rates.

Author contributions

MRD: neurophysiology; BAK: neuropathology

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NEWS FROM AROUND THE WORLD

AIM

From December 9th to 12th 2020 the first virtual congress was held as the joint initiative of two Italian scientific societies, the Italian Association of Myology (AIM) and the Italian Association for the study of the Peripheral Nervous System (ASNP), on the occasion of two important anniversaries in the current year: the 20th AIM National Congress and the 10th Annual ASNP Congress.

Due to the restrictions related to the SARS-CoV-2 pandemic conditions, the congress was online with the participation of over 700 members working in the field of neuromuscular diseases.

The congress, in its joint form, showed high scientific value and proved to be an opportunity for comparison and exchange between two Scientific Societies that share many subjects matters. It dealt with the current challenges on neuromuscular diseases and covering several topics such as new gene therapies prospective, therapeutic approaches of dysimmune and paraneoplastic neuromuscular disorders, clinical and rehabilitative aspects of genetic and acquired myopathies and peripheral neuropathies, prevention and neonatal screening, innovations in diagnostics and possible biomarkers, pharmaco-economics and artificial intelligence in neuromuscular diseases, and imaging methods in diseases of the skeletal muscle and peripheral nerve. As part of the congress, a round table was held with clinicians and representatives of the Italian Patients Associations (Unione Italiana Lotta alla Distrofia Muscolare UILDM, Famiglie SMA, Parent Project, Altro Domani Onlus, ACMT Rete), as an important moment to share common initiatives and to network in improving the management and quality of life of patients and their families.

During the congress 174 abstracts have been collected and presented as oral communications or posters, often-including results of multicentric projects and collaborations at both national and international level. At the end of the congress, ten young participants received an award for their contributions: one of these was in memory of Prof. Giovanni Nigro, while the other prizes were funded by Patients Associations.

The proceedings of the congress are available on Neurological Sciences.

by Giulia Ricci and Gabriele Siciliano, Pisa University, on behalf of the Scientific Committee of the joint Congress AIM/ASNP

MSM

The 14th Meeting of the Mediterranean Society of Myology (MSM) is moved to 2021. Proposals to organize and host the event are welcome.

WMS

The 25th WMS congress was performed virtually between 28 September-2 October. Under normal circumstances this congress would have taken place in Halifax, in Canada, however evident pandemic conditions forbid a live meeting. And this decision had to be taken only recently after monitoring the situation closely. Despite shortcomings, end results were very pleasing. There were 2967 registrants for the main congress and 1761 for the classical pre-congress muscle course (which normally can accomodate 40 students). Seventy-six countries were represented, and this was another record breaking occasion. Every year, WMS meetings are based on three selected topics. At this congress 'new developments in congenital muscle disease', 'gene modifiers and gene delivery in neuromusucular disorders', and as par tradition 'advances in the treatment of neuromuscular disorders' were the main sections. The format and style of main presentations were in a pleasant hybrid format. There were live events, pre-recorded sessions, and the last day late breaking news again live. Social gatherings were also possible to enrich collboration as well as seeing old friends. All activites have been recorded. You can still view and follow this congress until 4 January 2021 from the congress site to include social interaction. Then the files will be transferred onto the WMS website.

by Haluk Topaloglu on behalf of WMS Executive Board

FORTHCOMING MEETINGS

2020

November 30-December 2

Mitochondrial Medicine (Virtual Conference). Information: website: *https://coursesandconferences.wellcomegenomecampus.org/our-events/mitochondrial-medicine-2020*

December 8-10

ERN Neuro-NMD 4th Annual Meeting. Virtual Edition. Information: website: *https://ern-euro-nmd.eu*

December 9-12

20th National Congress of Italian Association of Myology and 10th Annual Congress of the Italian Association for the Study of the Peripheral Nerve. Virtual edition. Information: website: *https://www.miologia.org*. For the program, see page 361-371.

December 11-12

EACVI - Best of Imaging 2020. Virtual Edition. Information: website: *https://www.escardio.org/Congresses-&-Events*

2021

January 13-15

IRDiRC Conference and RE(ACT) Congress. Virtual Edition. Information: website: *https://www.react-congress.org*

February 28

Rare Disease Day. Information: website: *https://ern-euro-nmd.eu*

March 5-11

ICHG 2021 – International Conference of Human Genetics. Cape Town, South Africa. Information: website: *www.ichg2021.com*

March 22-24

ACSC | Genomics of Rare Diseases. Virtual Conference. Wellcome Genome Campus, Hinxton, UK. Information: website: *https://coursesandconferences.wellcomegenomecampus.org*

April 15-17

ESC Preventive Cardiology 2021. Virtual Edition. Information: website: *https://www.escardio.org/Congresses-&-Events*

May 15-18

Heart failure 2021. Virtual edition. Information: website: *https://www.escardio.org/Congresses-&-Events*

May 21-24

European MTM-CNM Family Conference, Bad Nauheim, Germany. Information: *website: https://ern-euro-nmd.eu/event/european-mtm-cnm-family-conference*

May 28-June

16th International Congress on Neuromuscular Diseases, Valencia, Spain. Information: website *www.icnmd.org*

June 12-15

The European Human Genetics Conference. Glasgow, United Kingdom. Information. *website: https://eshg.org*

June 19-22

7th Congress of the European Academy of Neurology (EAN), Vienna, Austria. Information: *website: www.ean.org*

July 20-22

12th Annual Congress of Cardiology-2021 (ICC-2021), Lisbon, Portugal. Information: website: *www.bitcongress.com*

September 20-24

International Course and Conference on Neuromuscular Imaging 2021, Rotterdam, The Netherlands. Information: website: *https://iccnmi2021.com*

September 21-25

26th Congress of World Muscle Society. Prague, Czech Republic. Information: website: *https://worldmusclesociety.org*

October 3-7

XXV World Congress of Neurology (WCN 2021), Rome, Italy. Information: website: *https://wfneurology.org/world-congress-of-neurology-2021*

October 19-23

ASHG Annual Meeting. Montreal, Canada. Information: website: *www.ashg.org*

2022

February 13-17

International Conference on Human Genetics. Cape Town, South Africa. Information: website: www.ichg2022.com

April 28-May 2

14th European Paediatric Neurology Society Congress, Glasgow, UK. Information: website: *www.epns.org*

October 10-15

27th Congress of World Muscle Society. Halifax, Canada. 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 600 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.

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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.

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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;343:230-8. doi.org/10.14639/0392-100X-1583

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

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