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(Myopathies, Cardiomyopathies and Neuromyopathies)

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

Three-monthly

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Estimating the impact of COVID-19 pandemic on services provided by Italian Neuromuscular Centers: an Italian Association of Myology survey of the acute phase

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Conflict of interest

The Authors declare no conflict of interest

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Introduction. Since February 2020, the outbreak of COVID-19 in Italy has forced the health care system to undergo profound rearrangements in its services and facilities, especially in the worst-hit areas in Northern Italy. In this setting, inpatient and outpatient services had to rethink and reorganize their activities to meet the needs of patients during the “lockdown”. The Italian Association of Myology developed a survey to estimate the impact of these changes on patients affected by neuromuscular disorders and on specialized neuromuscular centers during the acute phase of COVID-19 pandemic.

Methods. We developed an electronic survey that was sent to neuromuscular centers affiliated with the Italian Association of Myology, assessing changes in pharmacological therapies provision, outpatient clinical and instrumental services, support services (physiotherapy, nursing care, psychological support) and clinical trials.

Results. 40% of surveyed neuromuscular centers reported a reduction in outpatient visit and examinations (44.5% of centers in Northern regions; 25% of centers in Central regions; 50% of centers in Southern regions). Twenty-two% of centers postponed in-hospital administration of therapies for neuromuscular diseases (23.4% in Northern regions; 13.0% in Central regions; 20% in Southern regions). Diagnostic and support services (physiotherapy, nursing care, psychological support) were suspended in 57% of centers (66/43/44% in Northern, Central and Southern centers respectively) Overall, the most affected services were rehabilitative services and on-site outpatient visits, which were suspended in 93% of centers. Strategies adopted by neuromuscular centers to overcome these changes included maintaining urgent on-site visits, addressing patients to available services and promoting remote contact and telemedicine.

Conclusions. Overall, COVID-19 pandemic resulted in a significant disruption of clinical and support services for patients with neuromuscular diseases. Despite the efforts to provide telemedicine consults to patients, this option could be promoted and improved further. A close collaboration between the different neuromuscular centers and service providers as well as further implementation of telehealth platforms are necessary to ensure quality care to NMD patients in the near future and in case of recurrent pandemic waves.

Key words: SARS-CoV-2, COVID-19, neuromuscular diseases, myopathies, neuropathies, myasthenia gravis, neuromuscular services

List of abbreviations

AIM: Italian Association of Myology
 ALS: amyotrophic lateral sclerosis
 COVID-19: Corona Virus Disease 19
 DH: Day Hospital
 DMD: Duchenne Muscular dystrophy
 ERT: enzyme replacement therapy
 IVIG: intravenous immunoglobulins
 NMDs: neuromuscular diseases
 PLEX: plasma exchange
 SARS: severe acute respiratory syndrome
 SMA: spinal muscular atrophy

Introduction

Italy has been one of the first countries, after China, facing cases of local interhuman transmissions of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2 infection) worldwide ^{1,2}. The first confirmed case of coronavirus disease-2019 (COVID-19) was reported in Italy on the 21st of February 2020. Since then, more than 233.000 individuals across Italy became infected ¹. To assist COVID-19 patients, territorial health care services were reorganized and some hospitals were converted into “COVID hubs” (appointed by authorities), particularly in the most affected areas. Moreover, general population was imposed strict preventative measures to limit unnecessary gatherings and movements. Despite national laws and guidelines, regional differences in the management of the crisis exist ³⁻⁷. In this emergency setting, providing services to patients with chronic diseases and disability represented a major issue for the health care system ⁸. Due to preventive home isolation, fragile patients and their caregivers had to face many problems in terms of diagnosis, therapy, rehabilitation and support.

Neuromuscular diseases (NMD) may represent a risk factor causing a more severe course and outcome of SARS-CoV-2 infection. Indeed, several factors may contribute, namely respiratory and cardiac involvement, which are common NMD complications, muscle weakness, disability and, in some cases, dependency from caregivers ⁹⁻¹¹. The discontinuation of care and treatments can worsen the underlying condition, exacerbate symptoms, and increase anxiety, leading to a vicious circle and increased management concerns ¹²⁻¹⁶. Health services tried to address these needs by promoting remote patient contacts, referring patients to available nearby services and offering on-site visits only for urgent cases. Across the country, specialized NMD centers devoted their efforts and shared their knowledge in order to ensure quality care for NMD patients during these dramatic and unusual months, under the guidance of the Italian Association of Myology (AIM).

AIM associates developed a survey to quantify the extent of changes experienced by NMD centers and patients during these months, and to outline the most effective solutions undertaken, with the ultimate goal of improving NMD patients’ care.

Methods*Study design*

We designed a cross-sectional study based on a multicentric survey (Appendix 1).

The period considered in the survey runs from February 23rd to April 30th 2020, i.e. the most acute phase of the pandemic in Italy. Thirty-seven referral Centers for Neuromuscular Diseases were enrolled. The questionnaire was sent via e-mail to members of the AIM responsible for the enrolled centers. Geographical areas were divided into three macro-areas: Northern Italy, comprising the regions Valle d’Aosta, Piedmont, Liguria, Lombardy, Veneto, Trentino, Friuli Venezia Giulia, Emilia Romagna; Central Italy, including Tuscany, Lazio, Marche, Umbria; Southern Italy accounting for Abruzzi, Molise, Campania, Apulia, Calabria, Basilicata, Sicily and Sardinia.

Setting and participants

The survey included questions addressing how the activity of the center and clinical practice had changed and which services reported the worst difficulties from February 23rd, 2020 to April 30th, 2020. The questionnaire was divided into five sections:

1. Center and neurologist identification information;
2. Access of patients to standard home pharmacological therapy;
3. Access of patients to hospital-administered pharmacological treatments, including Nusinersen, intravenous immunoglobulins (IVIG), intravenous corticosteroids, rituximab, enzyme replacement therapy (ERT) for Pompe disease, plasma exchange (PLEX), edaravone and others;
4. Management of clinical trials;
5. Management of care-related services such as physiotherapy, home nurse service, psychological support for the patient and the caregiver, prenatal diagnosis.

Results

Thirty Italian Neuromuscular Centers completed the survey: 18/20 centers from Northern Italy, (response rate – r.r. 90%); 8/10 centers from Central Italy (r.r. 80%); 4/7 centers from South Italy, (r.r. 57%). Four centers were exclusively dedicated to the pediatric population (2 in

Northern Italy and 2 in Central Italy), while 26 assisted both pediatric and adult patients. Regional differences in the impact of COVID-19 epidemic emerged, partly as a consequence of the different geographical viral spread. The impact on activities and services provided by Neuromuscular Center on the national territory is summarized in Table I.

Outpatient visit and exams

From February 20th, 2020, a marked reduction in both outpatient visits and exams was observed in all Italian Neuromuscular Centers. Only 16.7% of centers all over the country continued to provide in-hospital visits and 43.3% of them limited the visits to medical emergencies and urgencies (Fig. 1). However, neuromuscular specialists were generally available on-call for neurological emergencies and a substantial part of the planned visits were replaced by remote telephone contact. In Northern Italy, the worst-hit area, 44.5% of centers were not able to provide outpatient visits and instrumental procedures, while in Central and Southern Italy outpatient visits were not performed in 25 and 50% of centers respectively. Cancelled visits were delayed or replaced with remote contact in the same proportion. The 75% of pediatric-dedicated centers guaranteed outpatients visits and exams.

Therapy administration and availability

Overall therapeutic pharmacological provisions (chronic pharmacological treatments, in-hospital administered therapies, experimental therapies) were guaranteed in 64% of interviewed centers (Fig. 1).

Chronic outpatient treatments remained available for the majority of patients (93.3% of Italian centers and the totality of the exclusively-pediatric centers).

Neuromuscular patients in regular clinical practice often refer to specialized centers for day-hospital (DH) treatments, particularly for intravenous drug administration. In this regard, 38% of centers provided in-hospital therapeutic administration without changes and 50% of centers with minor changes in Northern Italy. In Central and Southern Italy this service was mostly provided without major interruptions, but some difficulties were reported by 57 and 75% of centers in Central and Southern regions respectively.

We analyzed effectively performed DH treatments. Pulse high dose intravenous corticosteroids were administered in 94% of scheduled patients, while Intravenous Immunoglobulins (IVIG) administrations were provided in 74% of planned patients. Conversely, the monoclonal antibody Rituximab was administered only in 53% of expected cases. Referral centers for Nusinersen succeeded

Table I. Impact on activities and services provided by Neuromuscular Center on the national territory (yes: performed; no: not performed; DH: Day Hospital drug administration; FKT: physiokinesitherapy).

		Outpatient visit and exams	Therapy				Services				
			Total	DH	Chronic home therapy	Clinical trials	Total	FKT	Psychological support	Home nursing service	Pre-natal diagnosis
Italy	Yes	16.7%	64%	39%	93.3%	63%	43%	7%	66.7%	33%	87.5%
	No	40.0%	22%	7%	3.4%	37%	57%	93%	33.3%	67%	12.5%
	Partial (urgencies only)	43.3%	14%	54% (some)	3.3% (some)						
North	Yes	11%	63.6%	41%	89%	63%	34%	0%	84%	20%	77.5%
	No	44.5%	23.4%	12%	5.5%	37%	66%	100%	16%	80%	22.5%
	Partial (urgencies only)	44.5%	13%	47% (some)	5.5% (some)						
Center	Yes	0%	73%	43%	100%	76%	57%	12.5%	50%	87.5%	100%
	No	25%	13%	0%	0%	24%	43%	87.5%	50%	12.5%	0%
	Partial (urgencies only)	75%	13%	57% (some)							
South	Yes	0%	60%	25%	100%	62%	56%	20%	50%	75%	100%
	No	50%	20%	0%	0%	38%	44%	80%	50%	25%	0%
	Partial (urgencies only)	50%	20%	75% (some)							

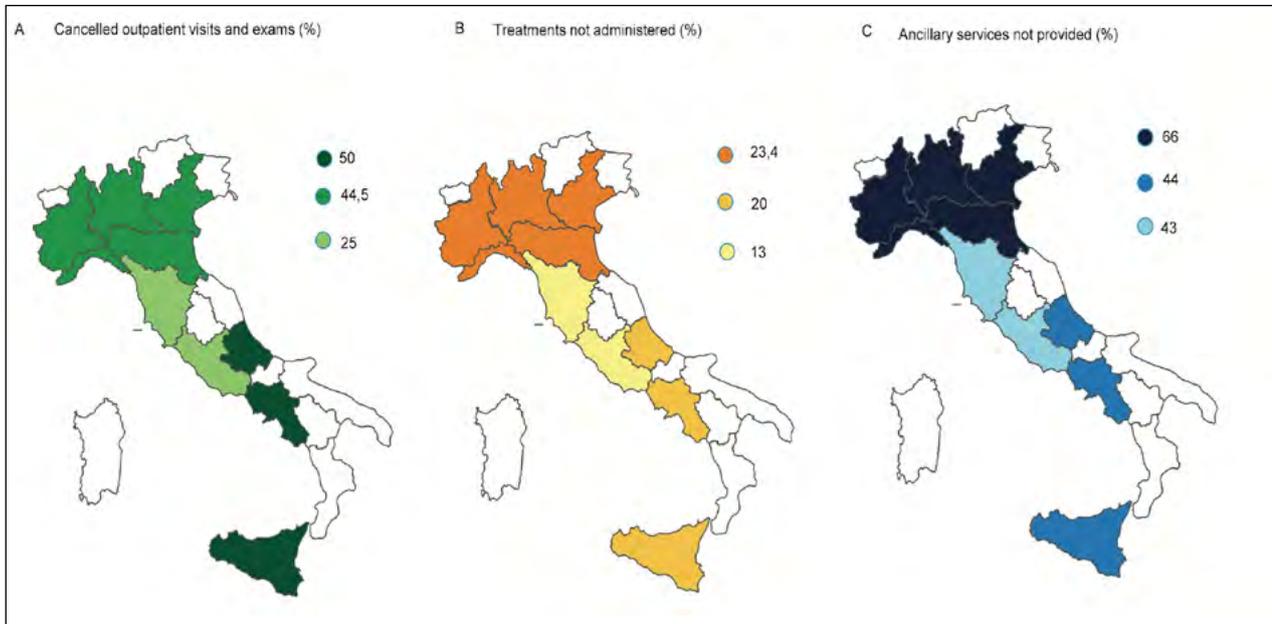


Figure 1. Geographical distribution of the impact of COVID-19 on the activity of NMD centers in Italy. The figure displays the percentage of outpatient visits and exams, treatments and ancillary services that have not been performed in three different part of Italy. Panel A shows that percentages of visits and exams that have been cancelled within the considered time interval in Northern, Central and Southern Italy. Panel B and C highlight the bigger impact of COVID-19 pandemic on treatment administration and ancillary services in Northern Italy than in the other parts of the country.

in 77% of scheduled administrations. In centers providing these treatments, Edaravone was administered in 98% of programmed patients, while ERT was maintained in 87% of patients with Pompe disease. Conversely, plasma exchange was performed in a higher number of patients than expected (Fig. 2).

The most relevant changes in treatment administration in this period were those for myasthenia gravis, followed by those affected with neuropathies, spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), inflammatory and metabolic myopathies. Two centers referred their patients (with a diagnosis of myasthenia gravis, ALS, SMA and Pompe disease) to other institutions for the prosecution of treatment.

The reasons why patients did not receive in-hospital therapy administration were mainly related to personal decisions, e.g. fear of the contagion. In most cases, pharmacological in-hospital treatments were postponed or rearranged, while, in few cases, drug administration was temporarily interrupted due to the high risk for the patient to develop serious COVID-19 complications in case of infection.

Clinical trials

Clinical trials continued with scheduled visits and exams in 63% of centers, with a minor percentage of inter-

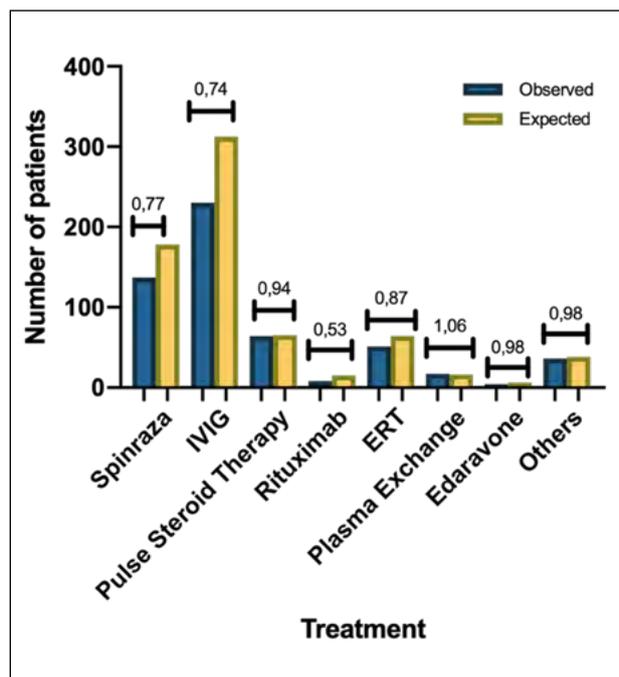


Figure 2. In-hospital administered therapy versus expected. Percentage of patients who received in-hospital administered therapies in the worse-hit period compared to the scheduled administrations for Nusinersen, IVIG, pulse high dose intravenous corticosteroids, Rituximab, ERT for Pompe disease, Edaravone and PLEX.

ruptions in the regions of Central Italy. Reasons for interruption were logistic problems, safety issues and patients' fear of contagion.

Experimental trials involving ALS and Pompe disease faced greater difficulties. As regards ALS patients, 60% of trial-related visits were delayed or cancelled for precautionary reasons. Patients with Pompe disease did not undergo visits and exams in 50% of cases.

Conversely, more than 75% of the centers participating in clinical trials for other neuromuscular diseases, including myasthenia gravis, inflammatory myopathies and neuropathies, DMD and SMA, performed the scheduled visits and exams with only minor lags. Particularly, visits and exams scheduled for clinical trials were conducted in the 87.5% of pediatric-dedicated centers.

Services

Services provided by neuromuscular centers, such as rehabilitation, home care nursing, psychological support and prenatal diagnosis, faced a reduction in 57% of centers, showing higher reduction rates in geographical areas with higher numbers of SARS-CoV-2 cases (aggregate services reduction in 66% of Northern NMD centers, 43% of Central centers and 44% of Southern centers) (Fig. 1). The most affected service was rehabilitation, since physical therapy sessions were suspended in 93% of centers, and postponed or delayed in a minor number of centers due to patient-related travel problems. Indeed, only 7% of surveyed centers reported that physical therapy sessions continued without variations (all Northern centers faced the interruption of sessions, while 12.5% of Central centers and 20% of Southern centers maintained the service).

Despite some difficulties, psychological support to families was provided in 66.7% of centers upon request.

Home nursing care service was not provided in 67% of centers, with a higher reduction rate in Northern Italy regions, where only 20% of expected patients obtained regular assistance. Also, session duration was reduced. The suspension was mainly determined by a choice of the families to protect their relatives from external contacts.

Services associated to prenatal diagnosis remained accessible in 87.5% of centers providing this service, in some cases with minor delays.

Fifty% of pediatric-dedicated centers reduced physiotherapy and home care nursing, while no center reported any defection in psychological support and prenatal diagnosis services.

Discussion

During the first wave of COVID-19 pandemic, Italian health care system had to rapidly and heavily reorganize inpatient and outpatient services, particularly in the worst-

hit areas^{3,6,17,18}. In this setting, specialized NMD centers were no exception. Under the guidance of AIM, they tried to address the needs of fragile NMD patients and to maintain essential services. However, despite these common intents, many NMD patients suffered from home isolation and temporary interruption of a part of outpatient visits and exams, thus struggling to receive standard therapies and rehabilitative sessions and facing the risk of worsening of their disease or exacerbation of symptoms^{7,19}.

With this survey, AIM aimed to assess the impact of SARS-CoV-2 pandemic on NMD centers and NMD patients' care in Italy. We found that changes related to the pandemic partly followed the regional burden of infections across Italy. Centers in Northern Italy experienced a heavier reduction of inpatient therapy administration and ancillary services if compared to NMD centers in Central and Southern Italy. On the other hand, outpatient neurological visits and exams suffered from a marked reduction in Southern regions. Concerning these results, however, we acknowledge the possibility of a sampling bias, since centers from Southern regions were less represented.

From the survey, it emerged that most centers ensured in-hospital visits for urgent cases and tried to limit the worsening of NMD diseases through remote contact advice. Provision of chronic pharmacological therapy was unaffected, whereas many centers faced difficulties in the administration of in-hospital therapies. Particularly, rituximab and IVIG were the most challenging to deliver appropriately, and patients who suffered the greatest change in in-hospital treatment delivery were myasthenia gravis patients. Indeed, patients with auto-immune and inflammatory NMD receiving immunosuppressive treatment generally present a higher risk of complications from infectious diseases^{14,20}. Thus, treatments might have been cancelled in order to let these patients avoid unnecessary travels.

Furthermore, the pandemic partially affected the regular course of clinical trials as well. Scheduled visits and exams were performed without major problems and with the necessary precautions only in 63% of centers.

Ancillary services such as rehabilitation, home care nursing and psychological support were markedly affected nation-wide. Due to the additional risk of infection from close patient-therapist contact²¹, rehabilitation services were generally suspended. Remote physiotherapy was not provided, possibly because of the difficulties experienced by disabled patients and their caregivers in conducting physical sessions without support. Home nursing care was provided in a higher number of cases as compared to physical therapy, despite the risks deriving from close contact with operators being similar. Psychological support for patients and caregiver was generally available, since it could be provided through remote contact. Finally, services related to prenatal diagnosis were

accessible in most centers. Exclusively-pediatric centers deserve a further comment, since their activity resulted less affected and services were generally maintained throughout the pandemic; however, rehabilitative activities requiring personal contact were reduced and extra-regional travels were avoided. This is partly due to an effort of the Health Care System to guarantee assistance to little fragile patients. Moreover, we hypothesize that buildings and spaces dedicated to pediatric population was less involved in the conversion to assist COVID-19 patients.

This cross-sectional, multicentric study was the first to specifically address the issues experienced by NMD patients in our country and to provide a picture of the pandemic burden for NMD specialists in different regions. Limitations of the study include the fact that we depicted the pandemic burden during a reduced time span, in a rapidly developing context in terms of preventative measures, organizational settings and law framework. A further limitation consists in the different restrictive measures applied regionally that may have led to a heterogeneous impact on the access to care for NMD patients.

In addition to the abovementioned sampling bias, methodological limitations associated to the survey itself could be the adhesion bias, as centers volunteering for the study might not represent a uniform sample, and the referral bias, as centers which encountered problems might have been more prone to answer promptly. On the other hand, centers heavily involved in the emergency might have experienced difficulties in answering the survey due to lack of time. Nonetheless, surveys are an effective tool to rapidly gather information without direct in-person contacts. In addition, our survey reached a consistent part of NMD centers across Italy.

Overall, COVID-19 pandemic caused a public health crisis with a potentially severe impact on the most fragile part of our society – including many NMD patients. In this context, neuromuscular centers played a pivotal role in ensuring an adequate support and care to these patients, and to reach this aim they had to rapidly reorganize their services. Some of the strategies and innovations that were experimented, such as telemedicine, could prove useful in the nearby future, after the acute phase of the pandemic²², and deserve diffusion and standardization for their use in clinical practice. Surveys, at-distance meetings and virtual platforms will likely be valuable tools to help addressing the future concerns and challenges of NMD centers, aiming at maintaining the best standards of care for NMD patients in these difficult times.

Ethic statement

The survey has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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Appendix 1
Italian Association of Myology
Survey on the impact of the COVID-19 pandemic on the management of
neuromuscular patients from 24/2/2020 - 30/4/2020

Clinical Center: _____ Number of patients followed by the center in the period considered: _____ Date of compilation: _____		
1. Did the patients accessed regularly to pharmacologic home treatments during the COVID-19 pandemic? <input type="checkbox"/> Yes <input type="checkbox"/> No, why: _____		
2. How many patients could regularly access to the following in-hospital administered therapies (day hospital/hospitalization)?		
Therapy	Treated	Expected
Spinraza		
Intravenous immunoglobulins		
Pulse high dose endovenous Corticosteroids		
Rituximab		
Enzyme replacement therapy		
Plasma exchange		
Other		
For patients who could not regularly access to in-hospital therapies, which were issues reported? (one or more options): <input type="checkbox"/> Calendar administrations' variations <input type="checkbox"/> Administration denied due to center-related organization problems <input type="checkbox"/> Patient's personal choice, logistic problems or fear of the contagion		
3. Could patients enrolled into clinical trials participate to scheduled onsite visits and exams? (one or more options): <input type="checkbox"/> Yes, without variations (specify the pathology _____) <input type="checkbox"/> Yes, but with calendar variations (specify the pathology _____) <input type="checkbox"/> No, visits and exams were postponed (specify the pathology _____) <input type="checkbox"/> No, because of patient's personal choice, logistic problems or fear of the contagion (specify the pathology _____)		
4. Could patients access to the planned outpatient neurological visits and exams? (If available, please specify the number of patients) <input type="checkbox"/> Yes, without variations _____		

<p><input type="checkbox"/> Only for neurological urgent evaluations _____</p> <p><input type="checkbox"/> Yes, but with calendar variations _____</p> <p><input type="checkbox"/> No, visits and exams were postponed _____</p> <p><input type="checkbox"/> No, because of patient's personal choice, logistic problems or fear of the contagion _____</p> <p><input type="checkbox"/> No, visits were replaced by telephone contact _____</p>
<p>5. Have patients who required physiotherapy the possibility to access it?</p> <p><input type="checkbox"/> Yes, without variations _____</p> <p><input type="checkbox"/> Yes, but with calendar variations _____</p> <p><input type="checkbox"/> Yes, but only privately arranged _____</p> <p><input type="checkbox"/> No, sessions were abolished _____</p> <p><input type="checkbox"/> No, because of patient's personal choice for logistic problems or fear of the contagion</p>
<p>6. Have patients who received home care nursing service the possibility to obtain it regularly? (one or more options):</p> <p><input type="checkbox"/> Yes, without problems or time changes</p> <p><input type="checkbox"/> Yes, but with time reduction</p> <p><input type="checkbox"/> Yes, but only privately arranged</p> <p><input type="checkbox"/> No, because of professionals' refuse</p> <p><input type="checkbox"/> No, because of patient's personal choice or fear of the contagion</p>
<p>7. Could patients and caregivers who necessitate psychological support obtained it?</p> <p><input type="checkbox"/> No, it was not possible</p> <p><input type="checkbox"/> Yes, but with some denials</p> <p><input type="checkbox"/> Yes, without problems</p>
<p>8. Could patients and their family access to prenatal diagnosis services associated to neuromuscular diseases? (one or more options):</p> <p><input type="checkbox"/> Yes, without problems</p> <p><input type="checkbox"/> Yes, but with calendar variations</p> <p><input type="checkbox"/> No, investigations were not available or have been postponed</p> <p><input type="checkbox"/> No, because of patient's personal choice or fear of the contagion</p>

Limb girdle muscular dystrophy due to *LAMA2* gene mutations: new mutations expand the clinical spectrum of a still challenging diagnosis

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Conflict of interest

The Authors declare no conflict of interest

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Mutations in *LAMA2* gene, encoding merosin, are generally responsible of a severe congenital-onset muscular dystrophy (CMD type 1A) characterized by severe weakness, merosin absence at muscle analysis and white matter alterations at brain Magnetic Resonance Imaging (MRI). Recently, *LAMA2* mutations have been acknowledged as responsible of LGMD R23, despite only few cases with slowly progressive adult-onset and partial merosin deficiency have been reported. We describe 5 independent Italian subjects presenting with progressive limb girdle muscular weakness, brain white matter abnormalities, merosin deficiency and *LAMA2* gene mutations. We detected 7 different mutations, 6 of which are new. All patients showed normal psicomotor development and slowly progressive weakness with onset spanning from childhood to forties. Creatin-kinase levels were moderately elevated. One patient showed dilated cardiomyopathy. Muscle MRI allowed to evaluate the degree and pattern of muscular involvement in all patients. Brain MRI was fundamental in order to address and/or support the molecular diagnosis, showing typical widespread white matter hyperintensity in T2-weighted sequences. Interestingly these alterations were associated with central nervous system involvement in 3 patients who presented epilepsy and migraine. Muscle biopsy commonly but not necessarily revealed dystrophic features. Western-blot was usually more accurate than immunohistochemical analysis in detecting merosin deficiency. The description of these cases further enlarges the clinical spectrum of *LAMA2*-related disorders. Moreover, it supports the inclusion of LGMD R23 in the new classification of LGMD. The central nervous system involvement was fundamental to address the diagnosis and should be always included in the diagnostic work-up of undiagnosed LGMD.

Key words: limb girdle muscular dystrophy, merosin, *LAMA2* gene, brain MRI, muscle MRI, leukoencephalopathy

Introduction

Laminin-2 (also called merosin), a heterotrimeric protein composed of three different subunits ($\alpha 2\beta 1\gamma 1$), is a major component of the basement membrane of skeletal muscle fibres, Schwann cells in peripheral nerves, brain capillaries and submandibular glands ¹. It belongs to a large family of glycoproteins involved in extracellular matrix architecture, cell adhesion and differentiation and mediates the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components ^{1,2}. Mutations in *LAMA2* gene (chromosome 6q22.33), which encodes alpha 2 chain, have been so far identified as the cause of two different clinical phenotypes: an early-onset congenital muscular dystrophy (CMD), also known as CMD1A (congenital muscular dystrophy 1A), and a milder late-onset limb-girdle type muscular dystrophy (LGMD) phenotype.

CMD1A is one of the most frequent forms of classic CMD in Western countries. This form, commonly associated with complete absence of laminin- $\alpha 2$, is characterized by severe muscle weakness and hypotonia at birth or in the first six months of life, delayed motor development, inability to walk unsupported, joint contractures, high creatine-kinase (CK) levels and widespread white matter abnormalities on brain magnetic resonance imaging (MRI) ²⁻⁴.

In 1998 Bushby et al. described for the first time a case with adulthood onset and mutations in *LAMA2* gene ⁵. These forms are generally characterized by slowly progressive proximal muscular weakness, white matter lesions on MRI and partial protein loss at muscle analysis ⁶⁻⁸.

Recently the 229th ENMC International Workshop revised LGMD nomenclature and classification, providing a precise definition of LGMD and including *LAMA2* gene as causative of LGMD R23 ⁹.

A potential correlation between the degree of merosin expression, the genotype and the clinical features has been described ¹⁰. In particular, mutations that lead to partial laminin- $\alpha 2$ deficiency are generally associated to milder phenotypes, which range from mild forms of CMD to adult-onset presentations. Mild CMD forms present a benign evolution and slow course, but are always characterized by delay in motor milestones acquisition ¹¹⁻¹⁴, while the adult-onset LGMD forms generally do not show symptoms at birth. The adult-onset cases are generally due to missense or in-frame splice-site mutations while CMD cases are more frequently associated to nonsense or out-of-frame mutations.

While the prevalence of MDC1A is estimated to be 0.6/100,000 in Northern England (around 10-30% of all

the MDC) ¹⁴⁻¹⁵, the prevalence of LGMD due to *LAMA2* mutations is unknown. However it is believed to be very rare and since now only few cases with adult LGMD presentation have been reported ^{6-8,17-22}.

Here we describe a small group of Italian subjects carrying homozygous or compound heterozygous mutations in *LAMA2* gene, who developed mild and slowly progressive muscular weakness with limb girdle muscular dystrophy phenotype.

The description of these cases, which include patients with late onset seizures, very late disease onset as far as marginal muscle involvement, contributes to enlarge the spectrum of *LAMA2* gene disorders. In many of these cases the incidental finding of brain white matter abnormalities was fundamental in order to direct the diagnosis.

Materials and methods

Clinical evaluation

Patients were selected from a cohort of adult LGMD patients evaluated at IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico of Milan and at Department of Neurosciences of Padua. All patients underwent a systematic clinical characterization, including comprehensive neurological, cardiac (electrocardiogram and echocardiogram) and respiratory (spirometry) assessments. Data about familiar and personal history, in particular about age of onset and progression of symptoms, were collected. Muscle strength was evaluated using the Medical Research Council (MRC) Scale. Electromyography (EMG) was performed at the beginning of the disease. The patients were also studied with both Brain and Muscle Magnetic Resonance Imaging (MRI). In selected cases further studies such as electroencephalography were performed.

Written informed consent was obtained (and preserved in the original form) from all subjects or their caregivers when primary diagnostic procedures were performed, with explicit consent to future uses for research purpose, according to the Declaration of Helsinki.

Brain and muscle imaging

Brain imaging data were obtained in all patients by nuclear Magnetic Resonance Imaging (MRI). A 1.5T scanner was used and spin echo (SE), turbo spin echo (TSE) and fluid attenuated inversion recovery (FLAIR) sequences with T1 and T2 weighted images in the three orthogonal planes were acquired.

Muscle MRI was performed using a 1.5T scanner, axial TSE T1 weighted- images and axial short tau inversion (STIR) T2-weighted images were acquired. Both proximal upper and lower limbs' muscles were studied.

Muscle tissue analysis

All probands underwent muscle biopsy after giving written informed consent, according to Institutional guidelines. Morphological examination was performed according to standard procedures²³.

The proteins involved in LGMDs were studied using immunohistochemical (IHC) analyses with monoclonal antibodies directed against dystrophin (Novocastra, Newcastle upon Tyne, UK), sarcoglycans (Novocastra, Newcastle upon Tyne, UK), alpha-dystroglycan (a-DG) (Upstate Biotechnology, Lake Placid, NY), and caveolin-3 (Transduction Laboratories, Lexington, KY) as previously described. Calpain-3 and dysferlin expression were analyzed through Western blot (WB) analysis.

Immunostaining of laminin $\alpha 2$ was performed using two commercially available antibodies directed against the 80-kDa carboxyl-terminus (MAb1922; Chemicon, Temecula, California) and against the amino-terminus (NCL-MER3, Novocastra, Newcastle Upon Tyne, UK). Laminin- $\alpha 2$ immunoblot was performed using the MAb1922 antibody (Chemicon)²⁴.

Molecular analysis

Genomic DNA was extracted from peripheral blood samples according to standard procedures (Flexi Gene DNA Handbook, Qiagen). Molecular analysis of *LAMA2* gene was performed through the amplification by Polymerase Chain reaction (PCR) and direct sequencing (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA) of all the exons and the adjacent intron regions. Primer sequences and PCR conditions are available upon request. Mutations were named according to the Leiden Muscular Dystrophy database (www.dmd.nl). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

The pathogenic nature of the mutations was confirmed by screening of 160 control healthy subjects and the parental origin of each mutation was assessed through analysis of parental genomic DNA when available. Furthermore, the amino acid conservation was confirmed by

comparison with the sequence in different species. In few patients, mRNA was isolated from muscle tissue with Eurozol. Then cDNA was produced through reverse transcription polymerase chain reaction (RT-PCR) using the Ready-To-Go RT-PCR kit (Amersham Pharmacia) and analysed by mRNA amplification and sequencing.

In one patient the diagnosis was achieved with Next Generation Sequencing (NGS) techniques and confirmed by Sanger sequencing. DNA samples underwent Motor HaloPlex Target Enrichment protocol, analyzing the coding regions of 89 genes; libraries were run on a HiSeq instrument and analyzed through a custom pipeline of bioinformatics analyses²⁵.

Results

We selected a cohort of 5 patients clinically diagnosed as LGMD and carrying mutations in *LAMA2* gene. We identified 7 different mutation, 6 of which are novel. The molecular, clinical and biophysical features of the patients are summarised in Table I, Table II and Table III.

Patient I

Patient I was an Italian 30-year-old man without family history for neuromuscular disorders. He presented to medical attention with a LGMD phenotype characterized by progressive limb girdle weakness, predominantly involving lower limbs, and moderate CK levels increase (2-5x; range 454-1000 UI/L). He was the third son of non-consanguineous parents; his parents and his daughter did not show any muscular involvement and had normal CK levels. The patient was born after a normal pregnancy and had a normal psycho-motor development. He presented the first symptoms at 28 years of age when he started to complain difficulty in climbing stairs. The disease had a slowly progressive course with worsening of lower limbs weakness and asymmetrical involvement of proximal upper limb muscles starting from the age of 31 years. Electromyography showed neurogenic alterations without myopathic signs. Clinical evaluation at 59 years of age showed severe prox-

Table I. Molecular data showing the two pathogenic mutations and their proteic effect for each patient. Most of the mutations were not previously described in literature.

Pt	Nucleotide 1	Protein 1	Exon 1	Ref.	Nucleotide 2	Protein 2	Exon 2	Ref.
I	c.6742delC	p.Leu2248TrpfsX23	48	New	c.8544C > G	p.His2848Gln	60	New
II.1	c.4005T > C	p.Lys1469Arg	30	New	c.4005T > C	p.Lys1469Arg	30	New
III	c.2750 + 2 insT	p.Glu892Ala del 893_917	IVS19	New	c.2750 + 2 insT	p.Glu892Ala del 893_917	IVS19	New
IV	c.752T > C	p.Leu25Pro	5	New	c.7586_7589dup	p.Phe2531X	55	New
V	c.752T > C	p.Leu25Pro	5	New	c.7147C > T	p.Arg2383X	50	[35]

Table II. Clinical data about the patients described.

Pt	I	II.1	III	IV	V
Sex	M	F	M	M	F
Age (yrs)	62	51	75	41	52
Onset (age)	28 y	Childhood	40s	Childhood	Childhood
Symptoms at onset	Proximal weakness	Proximal weakness	Fatigability and distal weakness	Proximal weakness	Proximal weakness
Phenotype	LGMD	LGMD	LGMD	LGMD	LGMD
CK (UI/L)	454-1000	300-400	500-800	677-1640	180-680
Loss of ambulation	No (59 y)	No (51 y)	No (75y)	No (41y)	no (52y)
Joint retractions	No	Mild TT	No	TT and elbows	TT
Sural hypertrophy	Yes	Yes	Yes	No	No
Cardio-pulmonary involvement	BBD	PSVT	Dilated cardiomyopathy, atrial flutter	None	Mild MIP and MEP reduction
Muscle MRI	Severe asymmetric fibro-fatty replacement biceps brachii, triceps brachii, thigh. Selectively sparing of deltoid, sartorius, gracilis, rectus femoris and short head of the biceps femoris	Fatty substitution in trapezius, supraspinatus, subscapularis, infraspinatus and pectoral thigh and leg muscles with relative sparing of the sartorius, gracilis, short head of the biceps femoral and tibialis posterior muscles	Diffuse fibro-fatty substitution of the gluteal muscles, of the posterior thigh, adductor magnus and of the quadriceps, sparing only the rectus femoris. The shoulder muscles were mild affected	NA	Moderate fatty substitution of scapular girdle muscles, severe substitution of pelvic girdle and thigh muscles, with sparing of sartorius, tensor fasciae latae, obturator and iliopsoas, gracilis, biceps femoris and adductor longus on the right side (52 years)
Brain MRI	WMA (U fibres sparing)	WMA	WMA	WMA	WMA
CNS	None	None	Migraine, MCI, polyneuropathy	Epilepsy	Epilepsy
EMG	Neurogenic	NA	Mixed signs	Myogenic	Myogenic
Other features	Asymmetric weakness	Myalgia	Distal legs involvement	Generalized muscle atrophy	Hyperlordosis, tiptoe gait

WMA: white matter abnormalities; MCI: mild cognitive impairment

Table III. Bioptical data of the patient described.

Patient	I	II.1	III	IV	V			
Age at muscle biopsy (yrs)	40	45	ND	67	75	ND	11	30
Biopsy pattern	Dystrophic	Myopathic	Dystrophic	Dystrophic	Dystrophic	ND	Myopathic	ND
Merosin WB (residual %)	ND	ND	30%	60%	60.00%	ND	ND	20%
Merosin IHC 300 kDa	Partial reduction	Partial reduction	ND	Normal/mild reduction	ND	ND	ND	Mild reduction
Merosin IHC 80 kDa	Partial reduction	Partial reduction	ND	Normal/mild reduction	Partial reduction	ND	ND	Mild reduction

imal atrophy (more pronounced at thigh and brachial biceps) and proximal lower limb weakness associated with bilateral calf hypertrophy. Weakness was asymmetric and involved predominantly the quadriceps (MRC 4+/5) and ileopsoas (MRC 4/5). At upper limb, brachial biceps was affected as well (MRC 3.5/5). Tendon reflexes were reduced at lower limbs. There was no distal, facial and bulbar involvement. Scoliosis, tendon retractions, cramps and myalgia were absent. The patient had a waddling hyperlordotic gate, possible on tiptoes but not on heels, and was able to walk for 400 meters at 6 minute-walking test. He raised from a chair pushing himself with upper limbs and was able to climb stairs with bilateral support. The patient lost the ability to raise from floor without external support at 55 years of age. Electrocardiogram and echocardiogram showed a normal cardiac function (ejection fraction 55%) and a right bundle branch block. Respiratory evaluation (spi-

rometry and nocturnal saturation) did not reveal pulmonary involvement. The patient did not show cognitive impairment.

The patient underwent two muscle biopsies respectively at the age of 40 and 45 years. The first biopsy, performed on quadriceps muscle, showed a dystrophic pattern with connective tissue substitution. The second biopsy, obtained from deltoid muscle, showed mild fiber size variability, nuclear centralization and fiber splittings, without necrosis or inflammation. Furthermore the connective tissue was normally represented (Fig. 1). At IHC analysis, dystrophin, sarcoglycans, alpha-dystroglycan, caveolin-3 and emerin showed a normal signal. WB study revealed a normal signal of dysferlin and alpha-dystroglycan and a mild reduction of calpain-3 without mutations in the corresponding gene.

At the age of 57 years, after two transient episodes of tinnitus and blurred vision, the patient underwent

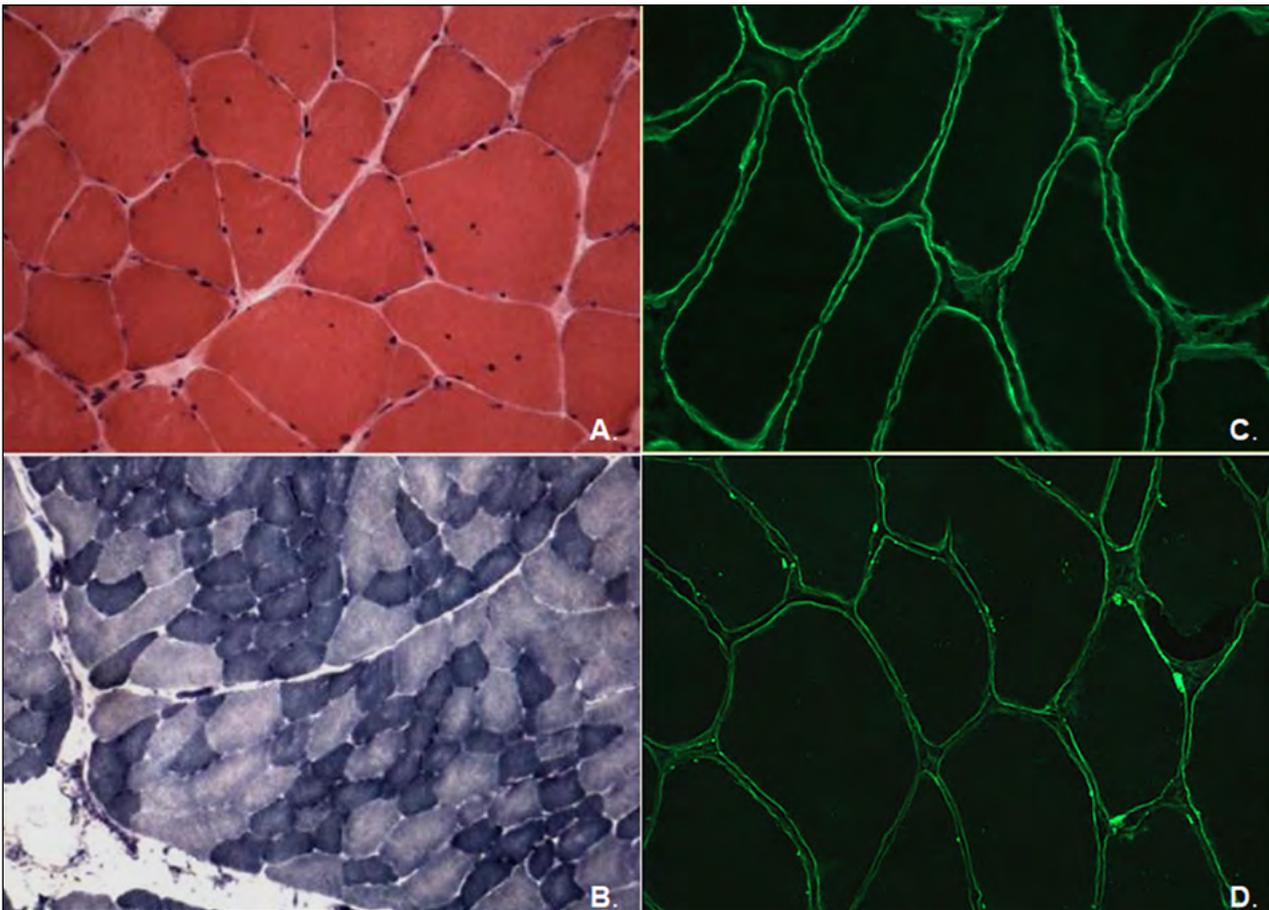


Figure 1. Muscle biopsy Patient I. (A) Haematoxylin and eosin (H&E), 40x: few fibres with internal nuclei and fibre splittings; (B) Reduced nicotinamide adenine dinucleotide dehydrogenase- tetrazolium reductase (NADH-TR), 25x, showing mild variation in fibre size. Immunolabelling of laminin alfa2 to 80 kDa fragment in a control muscle (C) and in our case (D), showing a partial protein expression in some areas of the sarcolemma (arrows). (Immunolabelling with amino-terminus antibody is not shown).

brain MRI which showed radiological signs of leukoencephalopathy with bilateral, symmetric and diffuse T2 hyperintensity of peri- and sovra-ventricular white matter, extending to the subcortical regions and sparing U fibres. Posterior limb of internal capsule was only mildly involved. No signs of atrophy was found and the corpus callosum and posterior fossa structures were regular (Fig. 2). Eye examination was also normal.

According to the brain MRI finding, IHC and WB of merosin were performed. At IHC merosin labelling showed a partial reduction with both antibodies in few muscle fibers (Fig. 1), while the WB analysis showed a severe reduction of the protein expression (Fig. 3).

Muscle MRI, performed at 59 years of age, revealed a significant muscular impairment at both arms and thighs. In particular the most involved muscle of the upper limbs was the biceps brachii, followed by the triceps brachii, while deltoid was selectively spared. The muscles of the thigh showed atrophy and an high signal in T1-weighted images, which suggested fibro-fatty replacement. Some muscles were selectively spared, in particular the sarto-

rius, the rectus femoris and the short head of the biceps femoris. The right thigh was more impaired than the controlateral one, where also the semitendinosus muscle was partially spared. Atrophy and substitution were detected also in the medial portion of gastrocnemius. No inflammatory signs were found on STIR T2 images (Fig. 4).

Molecular analysis showed the presence of two compound heterozygous mutation in *LAMA2* gene: the frame-shift c.6742delC (p.Leu2248TrpfsX23) in exon 48 and the missense mutation c.8544C > G (p.His2848Gln) in exon 60. Both mutations are novel. Both parents as far as the daughter of the patient carried the mutation in a heterozygous state.

Patient II

Subject II.1 is a 51-years-old woman presenting for progressive muscular weakness involving predominantly limb girdle muscles. She started to complain muscular weakness during childhood with difficulties in running. The disease showed a progressive course during the following years. The patient lost the ability of rising from

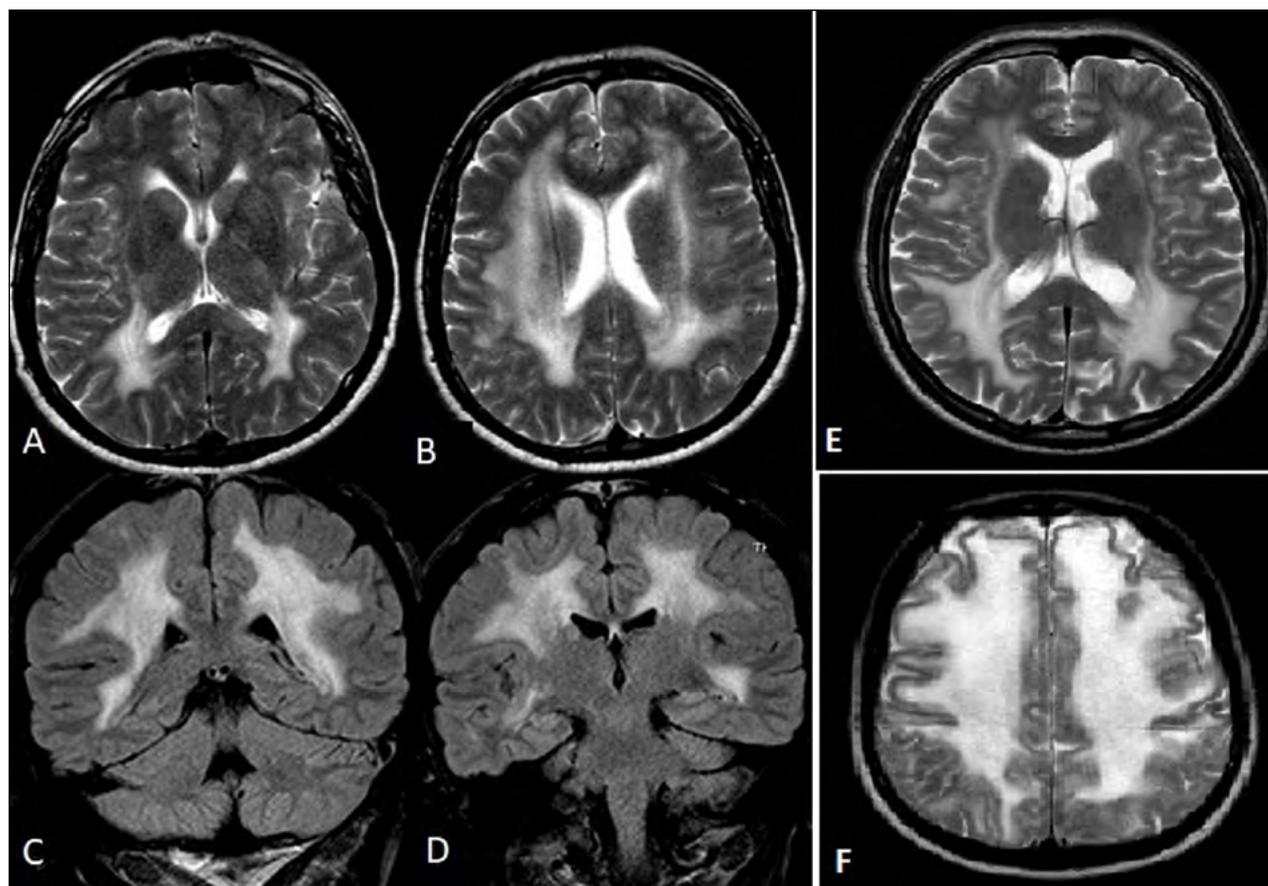


Figure 2. Brain MRI showing diffuse abnormal high signal intensity involving peri and sovra-ventricular white matter in axial T2 images (patient I: A-B; patient III: E; patient II: F) and coronal T2 FLAIR images (patient I: C-D).

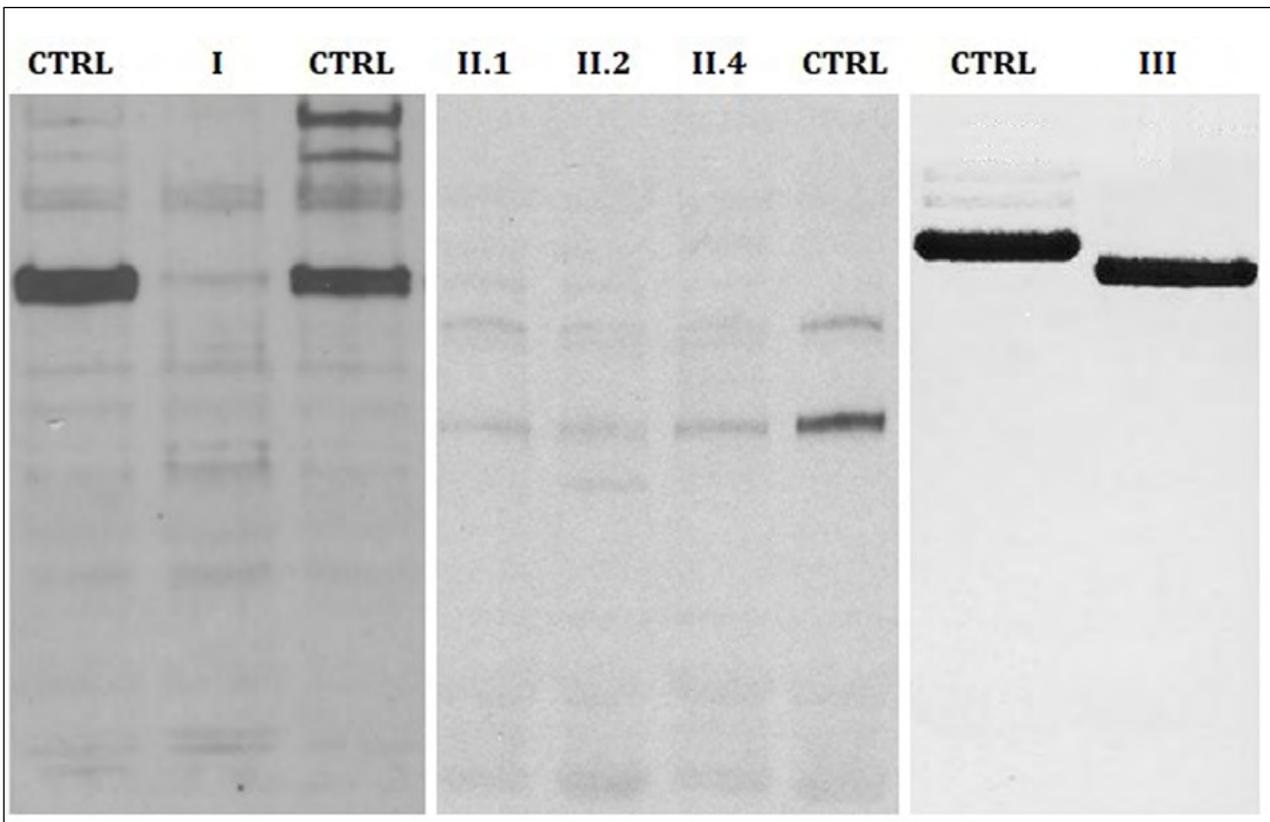


Figure 3. Western-blot analysis. Merosin Western-blot analysis showing a severe deficiency with monoclonal MAb 1922 antibody.

supine position at 10 years of age and of climbing stairs at 30 years of age. Myalgias were reported from adolescence. The neurological examination at 50 years of age showed symmetrical limb girdle weakness, sural pseudo-hypertrophy, bilateral mild Achilles tendon tightness, lumbar hyperlordosis and waddling gait. Weakness mainly involved lower limbs muscles, in particular gluteus (MRC 2/5), ileopsoas (MRC 4/5), quadriceps (MRC 4/5) and tibialis anterior (MRC 4/5). Deltoid (MRC 4/5) was the only muscle involved at upper limbs. The patient was able to raise from a chair only with external support. Cranial nerves and cognitive functions were normal. No episodes of loss of consciousness were reported. CK levels were mildly elevated (300-400 U/l). Pulmonary function was normal. The patient was affected by paroxysmal supraventricular tachycardia since the age of 45 years old and was treated with beta-blocker therapy.

Her parents were not apparently consanguineous but carried the same last name and were born in the same town. The patient had two unaffected children, but five relatives on her mother's side presented muscular involvement (Suppl. Fig. 1), suggesting a pseudo-dominant pattern of inheritance. The patient's mother (Subject II.2)

showed proximal muscular weakness and lost independent ambulation at 55 years of age. Three of her brothers and one sisters also showed neuromuscular involvement. In particular, one aunt (Subject II.5) lost ambulation at 60 years of age. Another uncle (Subject II.3) showed sural pseudohypertrophy since adolescence and lower limbs weakness since adulthood. In the following years he developed atrophy in gluteus and thigh muscles, waddling hyperlordotic gait and upper limb involvement with deltoid pseudo-hypertrophy and right fingers common extensor atrophy. His electromyography displayed myopathic signs. He died at 47 years of age in a road accident. Another aunt (Subject II.4) presented slowly progressive weakness in pelvic girdle muscles since childhood, associated with axial muscle involvement, sural pseudo-hypertrophy, lower limb areflexia, myopathic signs at EMG and CK increase (400 UI/L). At 71 years of age she required support for walking. Moreover, the maternal grandfather (Subject II.6) was also affected by an unspecified neuromuscular disease.

Muscle biopsy was performed in our proband and in subjects II.2 and II.4. The proband showed a dystrophic pattern, subject II.2 neurogenic alterations and subject

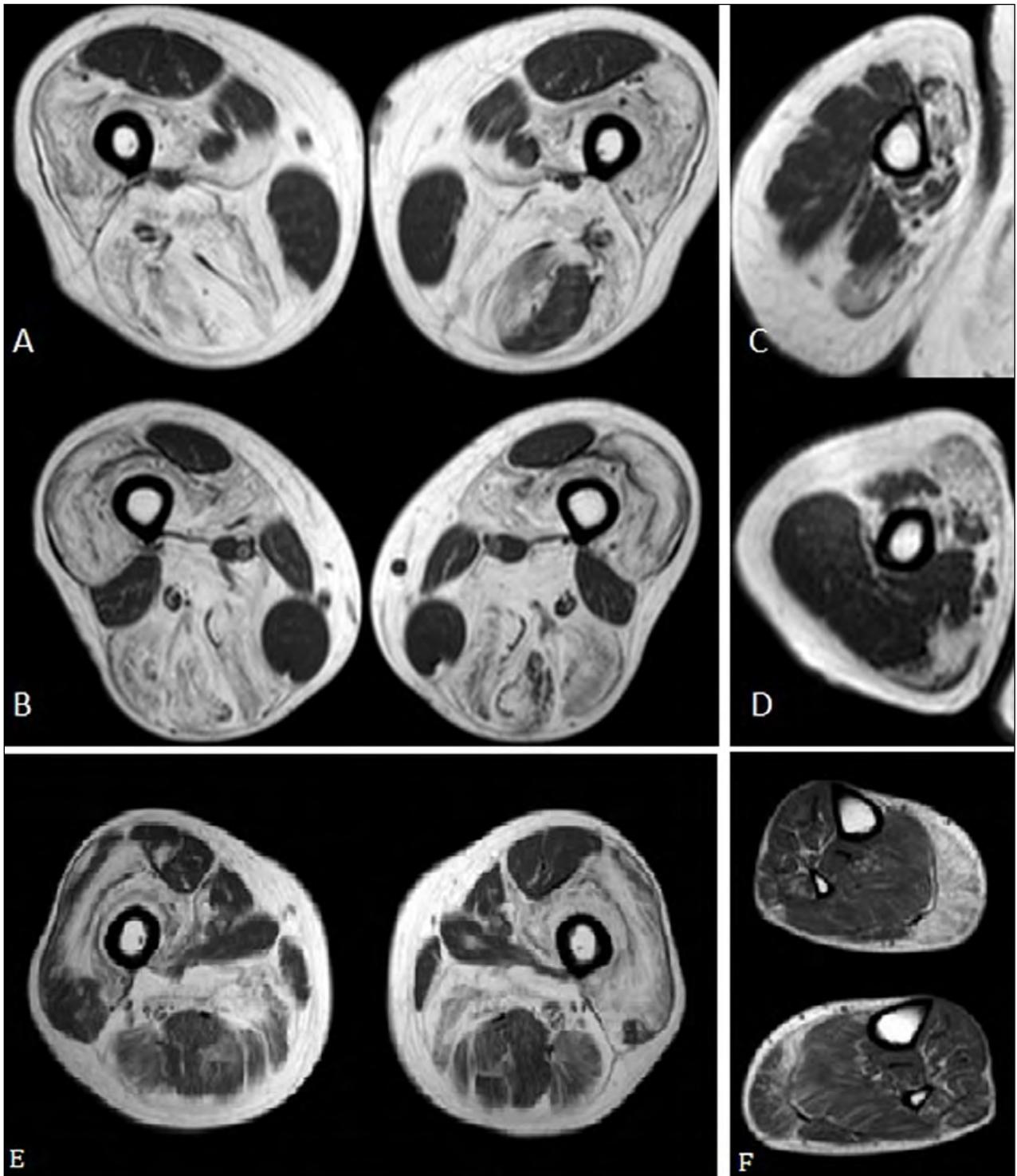
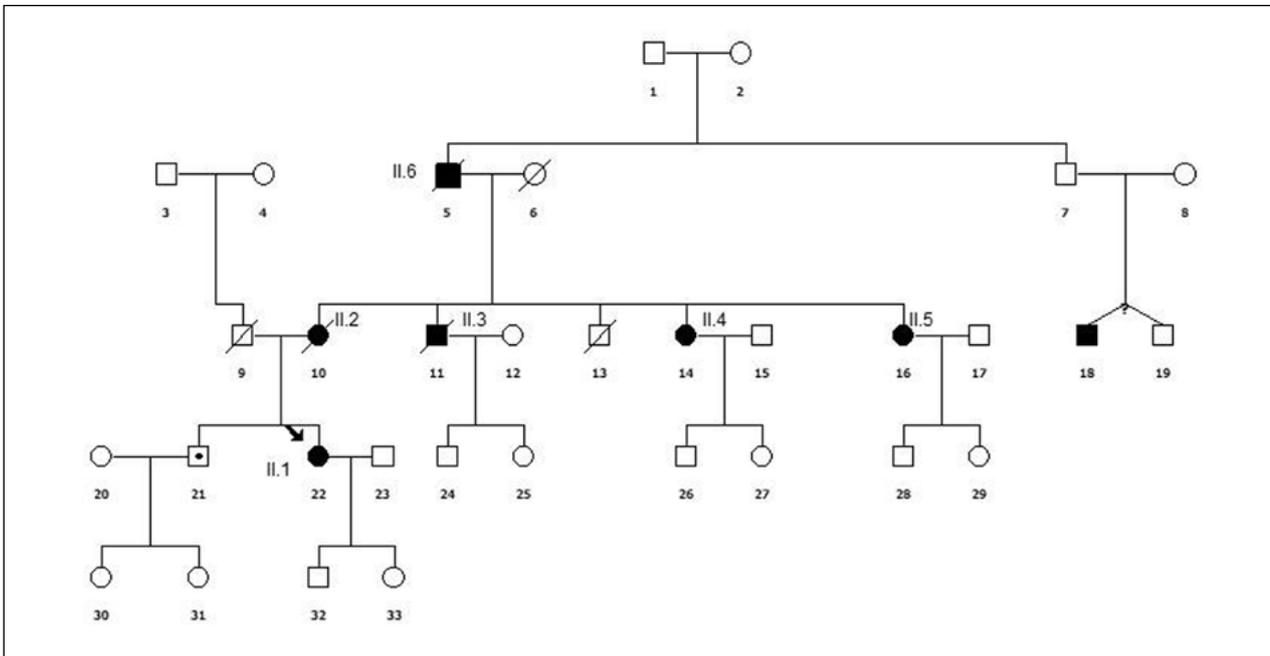


Figure 4. Muscle MRI. Axial TSE T1 images of the thighs (A-B) and of the right arm (C-D) of patient I, showing a diffuse atrophy and hyperintensity signal, as for fatty degeneration; some muscles were selectively spared, in particular the deltoid, the sartorius, the gracilis, the rectus femoris and the short head of the biceps femoris; the left thigh semitendinosus muscle was partially spared too. Axial T1 images of the thighs (E) and legs (F) of patient III, showing diffuse fibro-fatty substitution of the gluteal muscles, of the posterior thigh, adductor magnus and of the quadriceps; milder connective substitution in the medial gastrocnemius bilaterally.



Suppl. Figure 1. Pedigree of patient II suggesting a pseudo-dominant inheritance.

II.4 a myopathic pattern. In the proband immunohistochemical analysis for caveolin-3, alpha-sarcoglycan, gamma-sarcoglycan, alpha-dystroglycan, dysferlin and dystrophin were normal, while Calpain-3 (94 kD) was mildly reduced.

NGS analysis revealed an homozygous missense mutation (c.4405T > C; p.Lys1469Arg) in exon 30 of *LAMA2* gene. This mutation was previously described in compound heterozygosis in a patient with adulthood onset of mild proximal myopathy, polyneuropathy, dilated cardiomyopathy with conduction defects, histological features similar to Inclusion Body Myositis and partial laminin alpha-2 reduction¹⁷. This homozygous mutation was confirmed by Sanger sequencing both in the patient and in subject II.4. Unfortunately it was not possible to confirm the segregation in the other members of the family; however Western blot analysis was performed in subject II.1 II.2 and II.4 demonstrating severe merosin deficiency (30% of residual protein) in all of them (Fig. 3).

Brain MRI was performed in subject II.1 and revealed widespread periventricular and subcortical white matter hyperintensity in T2-weighted sequences and a reduction of ventricular system dimensions and cortical sulci, suggestive for white matter edema (Fig. 2). Signal abnormalities were found also in the corticospinal tract and to a lesser extent in the splenium of corpus callosum, posterior portion of the thalamus and dentate nucleus (basal ganglia were spared)²⁵⁻²⁸.

Muscle MRI, performed at 50 years of age, detected almost complete fatty substitution in thigh and leg muscles with relative sparing of the sartorius, gracilis, short head of the biceps femoral and tibialis posterior muscles. In the upper limbs the trapezius, supraspinatus, subscapularis, infraspinatus and pectoral muscles were mainly involved; milder signs of fibro-fatty infiltration were present also in deltoid muscles.

Patient III

Patient III is a 75-year-old male, born from consanguineous parents (first cousins). He suffered from migraine since adolescence, almost unresponsive to common headache drugs. Family history was positive for dementia (the mother and two maternal aunts affected by). None of the other family members had neuromuscular disease history. The patient started complaining of mild exercise intolerance in his forties. Although he referred a worsening in fatigability, the muscular involvement remained almost stable over the years. At last evaluation he presents just a mild muscular weakness in distal legs associated with a mild calf hypertrophy. He always had high serum CK levels (500-800 U/l). The EMG showed a mixed neurogenic and mild myogenic pattern. EMG also documented a chronic polyneuropathy even if the patient has never complained neuropathic symptoms. Over the years the patient developed a dilated cardiomyopathy, recently worsened by atrial flutter episodes due to an abnormal ventricular pre-excitation, treated with catheter ablation when he was 73.

In his sixties a brain MRI, performed during the migraine follow-up, showed diffuse white matter lesions, spreading over the years to almost the whole brain white matter (Fig. 2), without clinical significant correlation. MMSE was of 26/30 and psychometric tests were globally normal. Because of headache, family history of dementia and MRI findings, a gene analysis for CADASIL was performed but turned out normal.

The patient underwent to muscle biopsies respectively at the age of 65 and 73 years (Fig. 5). The first biopsy showed some scattered COX negative fibres leading to a mitochondrial disease suspect. The immunofluorescence study for dystrophin, caveolin-3 resulted normal. Based on the suspicion of mitochondrial pathology, mtDNA long time PCR on muscle tissue was performed showing multiple deletions. Several nuclear genes involved in multiple deletions were screened

(POLG1, ANT1, POLG2, TYMP and PEO1) but turned out negative.

Eight years later, a second muscle biopsy and a muscle MRI were performed. The MRI showed a diffuse fibro-fatty substitution of the glutei, the posterior thigh, adductor magnus and quadriceps, sparing only the rectus femoris. A diffuse but milder connective substitution was also present in lower legs muscles, particularly in the medial gastrocnemius twins. The shoulder muscles were mild affected. No inflammation was detected (Fig. 4). The biopsy showed the presence of diffuse fibre atrophy and several necrotic fibres with a mild increase in muscular connective tissue. A few COX negative fibres were still present. At immunofluorescence, a mild, diffuse reduction of merosin alpha 2 came out (Fig. 5). Western Blot analysis of merosin on muscles shown a reduction of 40% in both the muscle biopsies (Fig. 3).

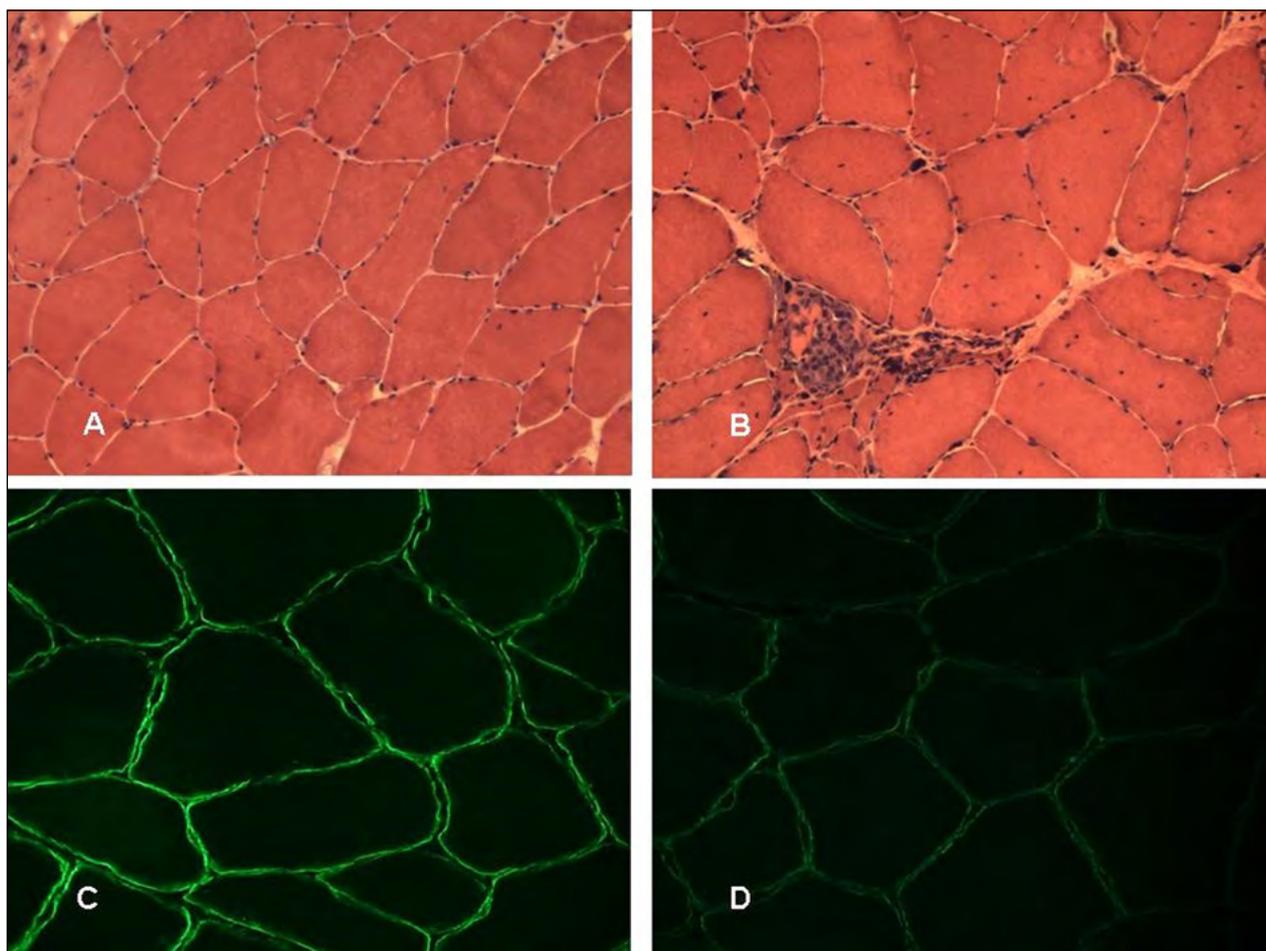


Figure 5. Muscle biopsy Patient III. Hematoxylin and Eosin stain on the the first muscle biopsy (A) and on the second (B). Scattered hypotrophic fibers in (A). Dystrophic pattern showing necrosis, nuclei centralizations and increase in connective tissue (B). Immunofluorescence staining (Merosin antibody) on the first muscle biopsy (C) and on the second (D). Original Magnification 40X (Merosin Laminin Alpha2). Normal Merosin binding (C) and scattered mild Merosin reduction of binding (D).

According to the MRI and bioptical results *LAMA2* gene was analyzed showing the presence of the following homozygous new mutation: c.2750+2 insT (IVS19), bringing to an in frame deletion of 75 nucleotides (c.2675-2749 del 75nt) as demonstrated by cDNA analysis. cDNA electrophoresis showed a band shorter than normal control, which corresponds with the production of a shorter protein product compared to the usual laminin size (p.Glu892Ala del893_917). The healthy sister of the patient resulted heterozygotic carrier of the mutation.

Patient IV

Patient IV is a 42-year-old man with progressive muscular weakness starting during childhood. The parents described him as clumsy. He showed difficulties in running since 18 years of age and in climbing stairs since 30 years of age. He underwent surgery for tibio-tarsal retraction. He did not show cognitive involvement but presented since 35 years of age partial seizures. Cardiac and respiratory involvement were absent. Last neurological examination showed waddling gait, proximal weakness (2-4/5 at MRC evaluation) and elbow tendon retractions. CK levels were mildly increased (1640-677 U/L) and muscle biopsy showed mild connective tissue increase and fibre I atrophy. Merosin was not tested. Brain MRI showed widespread white matter alteration.

Molecular analysis of *LAMA2* gene revealed the presence of the following heterozygous mutations c.752T > C (p.Leu251Pro) in exon 5 and c.7586_7589dup (p.Phe2531X) in exon 55, confirming the diagnosis.

Patient V

Patient V is a 53-year-old patient with negative family history. She came to medical attention at 30 years of age for progressive muscular weakness, with difficulties in climbing stairs and in rising from floor since 45 years of age. Retrospectively she presented motor delay with achievement of independent ambulation at 2 years of age and difficulties in running. At last evaluation neurological examination showed waddling gait, proximal weakness at four limbs (4/5 at MRC scale), tibio-tarsal retractions. CK were mildly elevated (180-680 U/L). Muscle biopsies showed myopathic signs. The patient did not show cognitive impairment. At 26 years of age she presented generalized seizures (both absences and tonic-clonic episodes). Brain MRI showed widespread alterations of brain white matter.

Molecular analysis showed the following compound heterozygous mutations in *LAMA2* gene: c.752T > C (p.Leu25Pro) in exon 5 and c.7147C > T (p.Arg2383X) in exon 50; both mutations were not previously described.

Review of the literature

We performed a wide PubMed search of the literature concerning LGMD-like presentation associated to mutation in *LAMA2* gene. We used the most commonly used entries and selected only papers written in English and reporting a complete molecular characterization.

We selected 14 papers^{6-8,17-22,30-34}. One paper³⁰ was excluded because it described three siblings with adulthood onset but predominant distal phenotype.

The clinical and molecular characteristics of the patients described in the remaining 13 papers are summarized in Table IV.

Overall 21 cases with LGMD presentation have been described since now. Age of onset spanned from 14 months to 59 years of age. However the patient with onset at 14 months complained waddling gait and frequent falls, but achieved independent ambulation at 12 months of age and did not show progression of weakness until 17 years of age. Only two patients showed mild delay in motor milestones, while all the other subjects achieved independent ambulation and had normal milestones. CK levels were moderately increased (average 1032 +/-926 UI/L, range 280-4100 UI/L). All patients were still ambulant at last evaluation (age range 11-65 years).

Contractures were reported in 6 patients, associated to rigid spine in two cases. One patient showed mild respiratory involvement while cardiac involvement was reported in two cases. Brain white matter abnormalities were present in all patients and were generally severe confluent white matter lesions, with exception of one case who showed only subtle bilateral signal abnormalities on the deep parietal lobe²⁰. Cerebellar abnormalities were present in two subjects. Central nervous system involvement was variable and included epilepsy (3 patients), sporadic episodes of loss of consciousness (2 cases), mild mental retardation (2 subjects) and mild deficit of executive functions (2 patients). The remaining subjects had normal cognitive performances. Three patients showed peripheral neuropathy. Interestingly merosin staining generally showed partial reduction with exception of two cases which showed respectively absence at IHC study and severe reduction at WB analysis. In some cases merosin immunostaining revealed only a subtle decrease, in particular when antibody that recognizes the 80 kDa fragment was used, which could be erroneously interpreted as normal (Tab. III). Muscle biopsy showed a dystrophic pattern, rimmed vacuoles were present in three cases. As far as mutational analysis is concerned the majority of patients carried at least one missense mutation with the exception of the patient described by Di Blasi et al. which presented a nonsense mutation which however was associated with the skip-

Table IV. Review of the literature. LGMD patients with LAMA2 gene mutations described in literature: clinical and molecular characteristics.**Clinical aspects**

Pt	Sex	Onset (age)	Ambulation (age)	Brain MRI	CNS/PNS	CK	Other features	Ref
1	M	12 yrs	16 m	WMC	None	2417	Three sister affected	[16]
2	M	< 29 yrs	18 m	WMC	IQ 85	250-1236	Rimmed vacuoles; dilated cardiomyopathy, arrhythmias	[17,29]
3.1	M	15 yrs	Normal	WMC, cerebellar hypotrophy	Demyelinating neuropathy	4x	Severe contractures; mild respiratory involvement	[18,33]
3.2	F	Childhood	Hip dislocation	WMC	None	2x	Contractures	[18,33]
4	M	4 yrs	18 m	WMC	None	NA	Contractures	[32]
5	M	Childhood	18 m	WMC	Epilepsy, sensory-motor neuropathy	1429	Contractures	[31]
6	M	14 mo*	12 m	WMC	Low normal IQ, epilepsy	655	-	[6]
7.1	M	23 yrs	Normal	WMC	Epilepsy ?	309	-	[6]
7.2	F	40 yrs	2 yrs	WMC	Mild executive function deficit, epilepsy ?	405	-	[6]
8	M	10 yrs	Normal	WMC	Epilepsy	1053	-	[6]
9	M	59 yrs	Normal	WMC	Mild executive function deficit, trigeminal neuralgia	859	-	[6]
10.1	F	30 yrs	Normal	Subcortical and deep WMC	Epilepsy	280	Occasional rimmed vacuoles	[7]
10.2	M	NA	Normal	WMC	None	NA	Rimmed vacuoles	[7]
11	F	5 yrs	12 m	Deep parietal WMC	Sensorimotor neuropathy	653	-	[19]
12	M	56 yrs	Normal	WMC	None	1171	-	[8]
13	F	1 yr	Delayed	WMC	None	2148	Contractures	[8]
14	M	10 yrs	Normal	WMC	Epilepsy	1053	-	[8]
15.1	M	8 yrs	16 m	WMC	None	398-2103	Hyperreflexia	[21]
15.2	F	< 3 yrs	14 m	WMC	None	4100	-	[21]
16.1	M	Childhood	Delayed	WMC, globi pallidi involvement	None	400	Contractures, rigid spine, dilated cardiomyopathy, atrial fibrillation	[20]
16.2	M	7 yrs	Normal	ND	Sensorimotor neuropathy	400	Contracture, rigid spine	[20]

* no progression until 17 yrs; WMC: white matter changes

Table IV. Review of the literature. LGMD patients with LAMA2 gene mutations described in literature: clinical and molecular characteristics.**Bioptical and molecular aspects**

Pt	Merosin ICH 300/80 kDa	Merosin WB	Molecular analysis	Ref
1	Severe / mild reduction	NA	Linkage analysis	[16]
2	Partial / partial reduction	Severe reduction	c.4405T > C (p.Cys1469Arg); c.4645C > T (p.Arg1549X)	[17,29]
3.1	Moderate / moderate reduction	Partial reduction	c.2230C > T (p.Arg744X + skipping of exon 15) in homozygosis	[18,33]
3.2	Slight / slight reduction	Partial reduction	c.2230C > T (p.Arg744X + skipping of exon 15) in homozygosis	[18,33]
4	Absent / absent	Absent	NA	[32]
5	Moderate / moderate reduction	NA	c.1847G > A (p.Gly600Arg) in homozygosis	[31]
6	Severe / mild reduction	50% reduction	c.850G > A homozygous (p.Gly284Arg)	[6]
7.1	Severe / mild reduction	65% reduction	c.728T > C (p.Leu243Pro); c.4860+2T > G_4860+3insGCC (p.Phe1573_Lys1620delinsSerfsX49)	[6]
7.2	ND	ND	c.728T > C (p.Leu243Pro); c.4860+2T > G_4860+3insGCC (p.Phe1573_Lys1620delinsSerfsX49)	[6]
8	ND / mild reduction	50% reduction	c.397-1_397-15del (p.Val133_Gln135delinsArgX5); c.7431A > T (p.Arg2477Ser)	[6]
9	NA	Absent	c.454T > G homozygous (p.Trp152Gly)	[6]
10.1	Mild reduction / normal	NA	c.2749+1G > A (Spl?); c.1177T > G (p.Cys393Gly)	[7]
10.2	NA	NA	c.2749+1G > A (Spl?); c.1177T > G (p.Cys393Gly)	[7]
11	Subtle / subtle reduction	Normal	c.391C > T (p.Gln131X); c.4487C > T (p.Ala496Val); (associated c.595T > A p.Cys199Ser exon 4)	[19]
12	Residual / NA	1/3 residual	NA	[8]
13	Residual /NA	Absent	c.3758T > GT (?) (p.Leu1253Arg); c.35T > G (p.Leu12Arg)	[8]
14	Residual / NA	1/3 residual	NA	[8]
15.1	NA / normal	NA	c.1358G > C (p.Cys453Ser); deletion exon 36-65	[21]
15.2	NA	NA	c.1358G > C (p.Cys453Ser); deletion exon 36-65	[21]
16.1	NA	NA	c.4533delT (p.Gly1512fsX); c.611C > T (p.Ser204Phe)	[20]
16.2	NA	NA	c.4533delT (p.Gly1512fsX); c.611C > T (p.Ser204Phe)	[20]

ping of the exon containing the mutation, thereby resulting in a restored open reading frame ¹⁹.

Discussion

Since now only 21 cases of patients with *LAMA2* gene mutations associated with adult-onset phenotype have been reported worldwide (Tab. IV). These cases are generally associated with partial reduction of merosin, the presence of almost one in-frame mutation, moderate increase of CK levels.

In this paper we describe 5 new subjects with *LAMA2* gene mutations associated with late onset phenotype characterized by mild, slowly evolving, proximal muscular involvement.

Mean age of onset was 23.2 ± 11.3 years; all patients showed normal milestones and achievement of independent ambulation; only one patient was described as clumsy during childhood, all patients were ambulant at last evaluation. In particular, patients I and III showed a very mild clinical phenotype, with the latest age of onset (respectively 62 and 75 years of age) described since now.

Moreover patient III also showed a very mild muscular involvement. The other patients showed predominantly proximal muscle weakness, with distal involvement described only in one subject. CK were moderately elevated (640 ± 267.8 UI/L). Cardiac involvement was present in two patients who developed respectively paroxysmal supraventricular tachycardia and dilated cardiomyopathy associated to atrial flutter. Respiratory involvement was absent in all patients. Joint retraction and sural hypertrophy can be present. Electromyography showed a myopathic pattern occasionally associated to neurogenic signs (2 patients). Neurogenic signs were also detected in the biopsy of one patient (II.2). Peripheral neuropathy has been described in LAMA2-related diseases, mainly as a sensorimotor demyelinating polyneuropathy^{14,32}, with possible axonal involvement²⁰ due to abnormal myelination linked to the deficient expression of merosin in the peripheral nerve Schwann cells. Two patients showed CNS involvement with epilepsy starting in adulthood, respectively at 26 and 35 years of age; seizures can be common in CMD but are rare in LGMD cases and generally start at younger age (Tab. IV). Migraine was present in patient III and never described before in association with this form of LGMD.

Brain MRI, which was abnormal in the majority of the patients, is fundamental to detect some specific aspects that can address the diagnosis. It should be performed even if central nervous system involvement is entirely subclinical. For example in patient I the molecular diagnosis was reached only after the accidental finding of white matter abnormalities at brain MRI, which addressed protein analysis and molecular investigation.

Muscle damage detected at muscle MRI is hard to compare with what is described in other patients because literature lacks MRI data in patients with partial merosin deficiency and LGMD phenotype. Only in a single report, a younger patient showed in her adolescence similar muscle MRI findings, although more severe than those found in the present series²⁰.

Our patients showed diffuse atrophy and fatty degeneration of thighs with selective sparing of the sartorius, the gracilis, the rectus femoris and the short head of the biceps femoris also at later stages. A diffuse but milder connective substitution was also present at lower legs, particularly in the medial gastrocnemius twins while upper limbs were less affected. No inflammatory signs were found.

Molecular analysis showed seven different mutations in *LAMA2* gene, six of which are new. Merosin deficient patients with a LGMD phenotype carry at least one missense or in-frame mutation. Patient III carries the novel homozygous mutation IVS19+2 insT which affects splicing bringing to a 75 bp in-frame deletion in cDNA

transcript (c.2675-2749 del 75nt). We did not notice any correlation between mutations and disease severity.

Muscle biopsy analysis showed a myopathic pattern. In patient III the availability of two different muscle samples, collected at 13 years of distance, allowed to study disease progression from a histological point of view. In this case, at disease onset, disease epiphenomena like fibres atrophy and subtle mitochondrial abnormalities may lead to misdiagnosis.

All patients showed partial reduction of merosin at protein analysis. Western-blot data are available only in three patients, among them the patient with milder reduction (60% of residual protein) showed a later onset than the two patients with more severe deficiency (30% of residual protein), but this data should be confirmed in larger samples.

Furthermore we noticed a considerable variability in merosin IHC and WB staining pattern depending on the antibody used. This variability has been previously described. Jones et al. analysed with two different antibodies 58 muscle biopsy samples demonstrating that 40% of them showed a differential staining³³. In particular the Chemicon antibody MAB1922, which was used in our patients, showed a milder degree of deficiency when compared to that observed with the Alexis MAB4H8-2. This significant difference between IHC and WB analysis results also by Di Blasi et al.¹⁸. Interestingly also Bushby et al.⁵ described a group of patients with a late onset myopathy and the unusual finding of a reduction of merosin on immunoblotting but not on IHC analysis. The presence of severe merosin reduction at WB but not at IHC in this group of patients and in our probands could be explained by different reactivity of the antibody, by the extensive processing of homogenization which is necessary for WB analysis or by a patient's specific predisposition to merosin fragility.

Furthermore in some cases protein analysis could be misleading because, especially when only amino-terminus antibodies are used, it can underestimate merosin deficiency. WB analysis with antibodies directed against both the amino- and carboxyl-terminus of the protein must therefore be used when a LGMD associated to merosin deficiency is suspected.

Conclusions

Since now only few cases of LGMD with *LAMA2* gene mutations have been described. LGMD R23 is a rare cause of LGMD, although this entity might be under-recognized. This could obviously be due to its rarity, but also to the fact that some contributory examinations, such as brain MRI, are not routinely performed in LGMD patients.

Overall the description of our cases further enlarges the spectrum of clinical phenotypes associated to mutations in *LAMA2* gene. In particular our case sample includes patients with very late age of onset and very mild muscular involvement. Furthermore, the description of muscle MRI in our sample expands the knowledge about muscle imaging in this form of LGMD. WB analysis and brain MRI should be useful in order to suspect this form of LGMD also in pauci-symptomatic patients.

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Maximum bite force in patients with spinal muscular atrophy during the first year of nusinersen therapy – A pilot study

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Conflict of interest

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Objectives. Spinal muscular atrophy is a monogenic disease characterized by progressive spinal and bulbar muscle weakness and atrophy. It is caused by the degeneration of alpha-motoneurons. The recent approval of the antisense oligonucleotide nusinersen highlights the need for reliable clinical tools to evaluate motor function in patients with neuromuscular disorders. Measurement of the bulbar neuromuscular function (e.g., bite force) could be an extension to existing motor scales, sensitive to more nuanced changes, especially in symptomatic patients with severely reduced functional abilities.

Materials and methods. Maximum bite force measurement was used to quantify changes of the masticatory function in adult monozygotic female twins with SMA type II. Using piezoelectric transducers, 550 observations were recorded for each patient during the first year of nusinersen therapy.

Results. During the application of four loading doses of nusinersen, bite force levels steadily increased and reached a statistically significantly higher level compared to the initial state in both patients. Subsequent maintenance doses coincided with smaller or no statistically significant changes in maximum bite force.

Conclusions. This pilot study indicates that the measurement of maximum bite force may be a useful tool to detect changes of the bulbar function in SMA patients. As such, it may supplement existing scales to identify treatment-related changes in motor function.

Key words: spinal muscular atrophy, motor neurons, masticatory force, antisense oligonucleotide

Introduction

Spinal muscular atrophy (SMA) is an orphan disease with an incidence of approximately 1:11,000 in Caucasians ¹. Characterized by progressive neuromuscular degeneration, this autosomal-recessive disorder is caused by biallelic deletions and/or point mutations of the survival of motor neu-

ron (*SMN1*) gene, leading to SMN protein deficiency ^{2,3}. Depending on age of onset, achieved motor milestones and life expectancy, SMA is subcategorized into different types ⁴: infants with SMA type I show symptoms within the first 6 months of life, never achieve the ability to sit independently and have previously often died within the first two years of life. SMA type II patients who usually become symptomatic after 6 but before 18 months of life achieve independent sitting, but never gain the ability to walk. Life expectancy is reduced in these patients, but most of them live into adulthood. Patients with SMA type III learn to stand and walk but may lose these abilities during the course of the disease. Usually these patients have a normal life expectancy ^{5,6}.

On 1 June 2017, the European Medicines Agency approved the antisense oligonucleotide drug nusinersen (Spinraza) for SMA therapy. Nusinersen alters the splicing of *SMN2* pre-mRNA and thus increases the production of the full-length SMN protein. The drug has to be administered intrathecally. The initial treatment phase comprises four loading doses within two months. Maintenance doses are administered once every four months thereafter ⁷. Two multicenter, randomized, double-blind, sham-procedure controlled studies with infantile-onset SMA patients and later-onset SMA patients previously showed significant improvements in motor milestones under nusinersen medication ^{8,9}.

With upcoming treatment options for SMA and other neuromuscular diseases ¹⁰, there is an urgent need for sensitive clinical tools to observe motor functions and thus drug efficacy, especially in symptomatic patients with a chronic course of disease and already limited motor abilities. The heterogeneity across different types and stages of SMA complicates the development of motor scales and reproducible outcome measures ¹¹. Here, we developed a method to quantify changes of masticatory function, which is representative of patients' bulbar neuromuscular function.

SMA usually first affects the spinal muscles and, later, to a lesser degree, the craniofacial and bulbar musculature ¹². As such, patients in the advanced stages of SMA show reduced maximum masticatory muscle strength ¹³, often resulting in problems with mastication and swallowing ^{12,14}. Common descriptive scales evaluating the motor function of SMA patients – such as the Hammett Functional Motor Scale Expanded (HF MSE) ^{15,16} or the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) ^{17,18} – do not account for limitations in the bulbar function and are inadequate to assess severely reduced motor function.

Assuming that nusinersen might also lead to an improvement in the bulbar function of patients with chronic SMA, we set out to test the usefulness and reliability

of maximum bite force measurements in patients with chronic minimal neuromuscular function where well-established outcome scales, such as the HF MSE or CHOP INTEND, cannot pick up changes. Quantifying maximum bite force in the course of treatment might generate initial hypotheses about the impact of nusinersen in such patients.

The purpose of this pilot study was to assess the variation of maximum bite force over time, using piezoelectric transducers. Focusing on intraindividual, instead of interindividual variation, the study assessed maximum bite force in two subjects with SMA type II over the entire first year of nusinersen therapy. Eliminating as many confounding influences as possible, the study quantified the extent of relative change in maximum bite force, providing a first indication of the method's feasibility in SMA patients.

Material and methods

Sample

Two adult monozygotic female twins with infantile onset SMA, later classified as SMA type II (patient 1 and 2) in view of their long-term survival, participated in this pilot study. Both have genetically confirmed SMA with a homozygous deletion of *SMN1* and three copies of *SMN2*. At the time of the first bite force measurement, they were 49 years old. Their motor abilities were limited to minimal movements of single fingers and to a reduced facial mimic and bulbar function affecting chewing and swallowing. HF MSE scores remained at 0 (out of 66 possible points; a score of 66 points corresponds to the best motor function) in both patients before and after one year of nusinersen therapy. CHOP INTEND remained at 12 points in patient 1 and increased from 15 to 16 points in patient 2 (out of 64 possible points; a score of 64 points corresponds to the best motor function). Both patients had several teeth missing, a severe class II malocclusion and, in addition to limb contractures, bilateral temporomandibular contractures with limitation of mouth opening. Active maximum mouth opening, measured both at the outset and after one year, remained stable at 24.5 mm in patient 1 and 21 mm in patient 2, being about 50 % less than normal ¹⁹. Prior to their participation, the patients gave informed consent. The study was conducted in accordance with the Declaration of Helsinki. Measurements were taken during the first year of nusinersen therapy, starting 21 days before the first application.

Recording methods

Bite force was measured using calibrated piezo-

electric transducers (Flexiforce A201-100, Tekscan Inc., South Boston, USA) in the form of thin plastic foil sheets (0.203 mm thickness, \varnothing 9.53 mm), with a maximum capacity of 445 N. Changes in the resistance of the sensor were detected by the multi-sensor measurement box CE-BO-MSA64 (CESYS, Herzogenaurach, Germany) and digitally recorded.

The measurements were conducted unilaterally on the posterior teeth in a region of good intercuspitation. To improve contact to the sensor, the teeth were partially covered by intraorally fabricated occlusal bite blocks (Pattern resin LS, GC America Inc., Alsip, Illinois, USA). Self-adhesive, convex bumpers (Stabilit, Bahag AG, Mannheim, Germany, \varnothing 8.00 mm) were placed on the sensor, guiding it into a predefined concavity on the surface of the occlusal bite blocks. The intermaxillary distance was kept at a minimum.

Bite force was repeatedly measured over the course of one year at two different times of the day (9.00 am and 5.00 pm). The patients were asked to bite 10 times with maximum effort for a period of one second, each followed by one second of recovery. One measurement lasted 20 seconds. The parameter of interest, maximum bite force, was defined as the maximum value of each bite (in N), yielding 10 values per measurement point. All measurements took place at the home of the patients, keeping their head positions constant in a familiar lying position in order to minimize the influence of neck muscle strength. All measurements were conducted by a single trained examiner (TK).

Statistics

The outlined procedure resulted in two patient-specific datasets consisting of 550 observations clustered in 55 measurement points each. The data were descriptively analyzed using locally weighted scatterplot smoothing (LOESS)²⁰; the means of the 10 values of each measurement point are illustrated (Fig. 3). Inferential statistics relied on patient-specific models using ordinary least squares regression, regressing patients' maximum bite force on the time points at which they were measured. Given that maximum bite force tended to decrease with each repetition, the position of each of the ten bites in a given measurement point was included as an additional control variable to account for systematic fatigue (values ranging between 1 and 10). Predictions of the quantities of interest are illustrated (Figs. 1-2). To account for the clustered data structure (i.e., observations nested in measurement points), all models relied on the clustered sandwich estimator²¹. Differences in bite force over time were generally considered statistically significant at $p < 0.05$. Conducting multiple comparisons over time, the statistical tests were Bonferroni corrected²². All statistical

analyses were conducted using the statistical software R (version 3.5.1).

Results

Over the period of one year, the two patients received six doses of nusinersen each. It was analysed how their application coincided with changes in maximum bite force, separately for the initial loading phase and for the subsequent maintenance phase.

Loading phase

During the application of four loading doses, maximum bite force steadily increases from baseline levels of 60 N / 37.7 N to 83.9 N / 47.3 N in patients 1 and 2 (Fig. 1). For patient 1, the increase is statistically significant after the second loading dose. The increase is weaker for patient 2, showing a statistically significant difference only after the fourth loading dose.

Maintenance phase

Following the loading doses, maintenance doses were applied every four months. After the application of the first maintenance dose, bite force levels are at 79.8 N / 42.7 N in patients 1 and 2, which is an increase relative to baseline levels (though weaker in size than after the fourth loading dose and statistically significant only for patient 1; see Figure 2). The application of the second maintenance dose is not accompanied by an increase in patient 1 over the subsequent two weeks (68.5 N). In the case of patient 2, the level of maximum bite force is even lower than before the first application of nusinersen, both before and after the second maintenance dose (31.7 N).

Changes in bite force over one year

Figure 3 descriptively summarizes all measurements over the complete period of observation. Maximum bite force decreases during the two observed four-month periods between drug administrations; temporary increases coincide with the application of the maintenance doses.

Inter- and intraindividual variation in bite force

Interindividually, patient 2 shows lower absolute values in maximum bite force than patient 1; intraindividually, values vary between measurement points. This variation between measurement points makes up ~50 % (patient 1) / ~90 % (patient 2) of the overall variation observed (as compared to variation across the 10 bites within measurement points). Intraindividually, variation between measurement points is also present before the application of nusinersen.

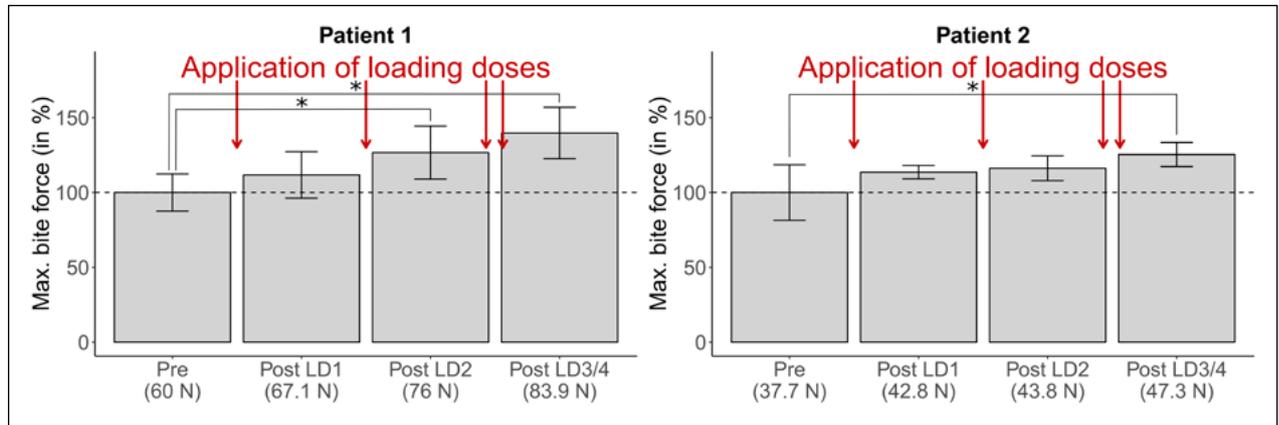


Figure 1. Changes in maximum bite force during the application of four loading doses of nusinersen (LD1/4, predicted values, in %). Pre entails seven measurement points before first application (=70 observations); each Post LD entails two measurement points within two weeks after application (=20 observations).

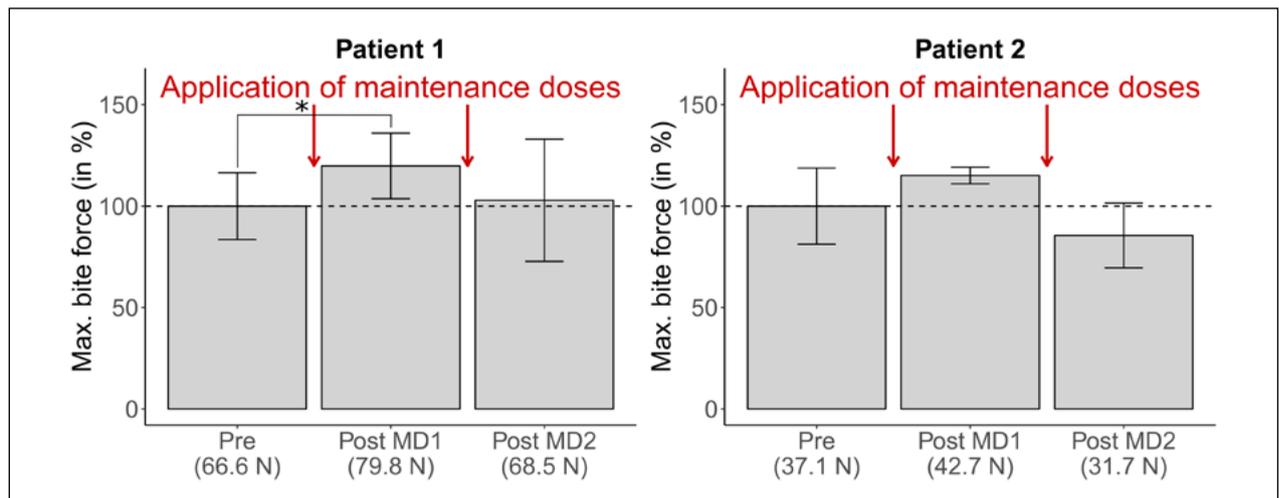


Figure 2. Changes in maximum bite force during the application of two maintenance doses of nusinersen (MD1/2, predicted values, in %). Pre entails seven measurement points before first application (=70 observations); Post MD1/2 entails two/four measurement points within two weeks after application (=20/40 observations).

Discussion

The results of this pilot study, based on data from two SMA patients, generate two hypotheses that need confirmation in further patients: first, that changes in bite force over time coincide with the application of nusinersen; second, that they can be detected by isometric bite force measurements.

At initially 35.7 N and 63.4 N, the maximum bite forces of the two surveyed adult patients with SMA type II were substantially lower than normal (supplemental measurements in a healthy female adult yielded values at ~300 N). Despite the use of thin piezoelectric foil sheets – facilitating bite force measurements in SMA patients with a limited range of mandibular movement – jaw

separation may have affected the absolute size of these values²³. Uninformative in absolute size, the respective values for the initial maximum bite forces underline the respective clinically observed limitations in the bulbar function of the two SMA patients and align with previous findings¹³. Interindividual differences of pre-treatment levels of bite force are irrelevant for our serial measurements, given that our analyses relied on intraindividual changes over time.

Time-invariant measurement error may thus be a minor concern. Instead, greater attention should be paid to time-variant confounding. A number of potential time-variant confounders could be accounted for – either during measuring itself (e.g., patients’ head positions or

time of the day) or ex-post statistically (e.g., fatigue due to repeated biting). However, unobservable factors, such as short-term fluctuations in physical fitness or individual motivation, remain more difficult to control. They may be the reason behind the unexplained variation between measurement points. In a similar vein, initial increases in bite force, as seen in patient 2, may be due to a learning effect²⁴. Similar patterns were also found in a healthy, untreated person (data not shown). A central task for future studies is thus to come up with ways to account for such potential influences of learning.

Notwithstanding these different sources of variation, the results indicate a systematic and statistically significant increase of maximum bite force that coincided with the application of nusinersen. Most notably, maximum bite force steadily increased with every additional application of the four loading doses in the first two months of treatment. The first maintenance dose coincided with a statistically significant increase in maximum bite force compared to baseline levels (though weaker in size than after the fourth loading dose). After the second maintenance dose, maximum bite force did not increase (in one patient, values were even below baseline level).

Overall, it could be speculated that maximum bite force changes with the application of nusinersen in adult SMA patients, especially at the onset of treatment, potentially indicating an altered bulbar function. Established scales did not capture any changes, potentially due to three reasons. First, neither HFMSE nor CHOP INTEND would capture changes that occur only in patients' bulbar function. Second, CHOP INTEND has not been validated for SMA type II and HFMSE evaluates gross motor skills only. Third, in line with current practice, both scores have been assessed at much greater intervals.

This pilot study is the first to record bite force of SMA patients over a long time period during a causative treatment of SMA. It relies on a unique and so far, rare population: patients at an advanced state of SMA being treated with nusinersen (the patients here are among the first, hence the small sample size). The study builds on previous work that assessed bite force in patients with other neuromuscular disorders (e.g., Duchenne or Becker muscular dystrophy), either in terms of one-time measurements²⁵⁻²⁷ or in terms of changes over time²⁸ – underlining the diagnostic potential of the approach.

The pilot study led to a number of insights that may be useful for further improvement of the method. First, measurement intervals should be chosen deliberately. This study covered the first year of nusinersen medication with 55 continuous measurement points. Being explorative in nature, the measurements took place at short, irregular intervals. Generally, future investigations may simplify the approach without risk by opting for fewer

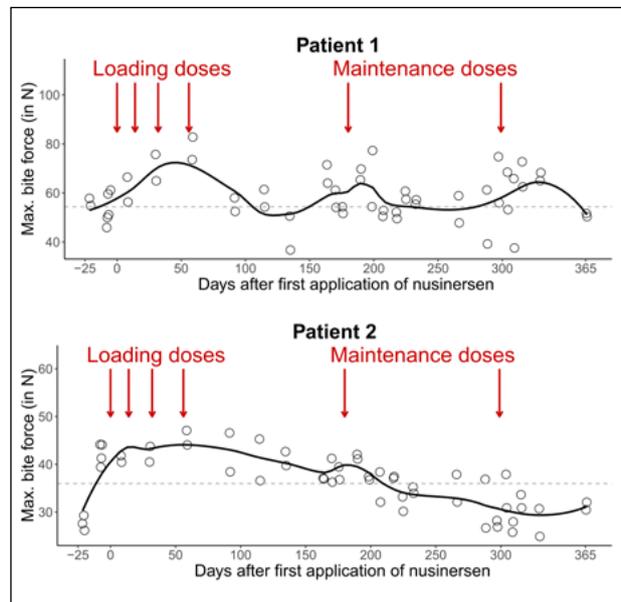


Figure 3. Maximum bite force in patient 1 and patient 2 over the first year of nusinersen therapy. Each circle indicates the mean value of 10 observations of maximum bite force per measurement point. Solid lines indicate the LOESS curve over time. Dashed lines indicate the mean maximum bite force before the first application of nusinersen.

measurement points that focus on time periods close to the application of nusinersen. Also, to avoid any bias from learning effects, the number of measurement points before the first application of nusinersen should be sufficiently large. Second, a central challenge is the reduction of time-variant confounding between measurement points. The analyses showed that the largest share of unexplained variation existed *between* measurement time points – even so before the first application of nusinersen. In comparison, 10 repetitions per measurement seemed sufficient to capture existing variation *within* a measurement point. This finding suggests that the use of piezoelectric transducers provides reliable and reproducible results, aligning with previous studies²⁹⁻³². The continuous and ongoing development of piezoelectric sensors and their software may further simplify the process, especially so for measurements in larger populations³³. Concerning unexplained variation between measurement points, more knowledge is needed, including for healthy subjects. Finally, beside maximum bite force, future investigations may also look at changes in neuromuscular endurance. The present study focused on serial maximal levels of bite forces. Decreases in bite force over repetitions were treated merely as confounders and controlled for in the multiple regression analyses. Given that there is evidence for faster fatigue among SMA patients¹³, it

may be worthwhile focusing on muscle endurance as a potential outcome measure.

Conclusions

The quantitative measurement of maximum bite force could constitute a promising tool to evaluate the masticatory function as a readout for the bulbar function among patients with degenerative, neuromuscular diseases. It could supplement biomarkers such as electrophysiology or other recently suggested approaches³⁴ as a cost-efficient measurement to identify functional changes due to treatment. The above suggested improvement for its future application may help to realize the diagnostic potential of bite force quantification in patients with neuromuscular disorders.

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Authors' contributions

TK collected, analyzed, and interpreted the data. She wrote the first draft of the manuscript. RH initiated the study, contributed to patient recruitment, gave advice about the interpretation of the results, and revised the manuscript. BW gave advice about the interpretation of the results and revised the manuscript. JG provided support in the development of the method. CDW and ACL conducted the treatment of the patients with nusinersen and revised the manuscript. BB gave advice about the study design and revised the manuscript.

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Efficacy and safety of ropivacaine HCl in peribulbar anaesthesia for cataract surgery in patients with myotonic dystrophy type 1

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Myotonic dystrophy (DM1) is the most common muscle disease in adults, affecting approximately 1:8000 individuals, characterized by myotonia and muscular wasting and a multisystemic involvement that includes heart, brain, respiratory and endocrine system, and eye. Conduction system is selectively involved, often causing cardiac sudden death. Early onset posterior subcapsular cataract is a characteristic feature of myotonic dystrophy, requiring surgical treatment. However, DM1 is associated with many anesthetic hazards; sensitivity to anesthetic drugs, especially muscle relaxants and opioids, may complicate postoperative care. Local anesthesia also requires attention. We investigated the heart response to local anesthesia Ropivacaine HCl administration in 16 DM1 patients (12M:4F) consecutively undergoing cataract surgery, by analyzing heart rate, ventricular and supraventricular ectopic beats, runs of tachycardia and pauses ≥ 2.5 sec., through a 24h-Holter monitoring, registered before and within 24 hours after surgery. The average age of patients was 47.4 years (range 30.2-55.9). At baseline, one patient had a pacemaker and 3 a defibrillator. Two patients presented a first-degree atrio-ventricular-block; three showed ectopic ventricular beats, on anti-arrhythmic drug treatment. No significant differences in heart rate values (73 ± 15 b/m versus 76 ± 13 b/m) were observed after cataract surgery, nor in the onset of ectopic beats. Only patients who presented ventricular ectopic beats at baseline, showed an increase in their number after surgery, likely related to an arbitrary interruption of the specific treatment. These data confirm the safety and efficacy of ropivacaine HCl used as a local anesthetic in patients with myotonic dystrophy.

Key words: peribulbar anaesthesia, ropivacaine HCl, myotonic dystrophy, cataract surgery

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Conflict of interest

The Authors declare no conflict of interest

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Introduction

Myotonic dystrophy type 1 (DM1) or Steinert's ¹ disease is the most frequent muscle disease in adults caused by the expansion of the trinucleotide CTG into the 3' UTR of the *DMPK* gene (19q13). Age of onset and severity of the disease are closely related to the number of repeats: 5-37 repeats are present in normal individuals, 50-150 in individuals mildly affected, from 100 to more than 1,000 repeats in patients with the classic DM1 phenotype. Repeats greater than 2000 are found in subjects with congenital onset pathology ².

DM1 is a multisystemic disorder involving muscle, heart, brain, respiratory and endocrine systems, and eye³ that requires a multidisciplinary approach and management⁴. Cardiac involvement, due to selective damage of the conduction system consequent to fibro-adipose accumulation in the sinus node and His bundle, is characterized by atrio-ventricular (a-v) blocks, bundle branch blocks, tachy/brady arrhythmias, which predispose to sudden death^{5,6}.

A variety of ocular signs is described in DM1 patients, like cataract, retinal degeneration, low intraocular pressure (IOP), eyelid ptosis, epiphora, corneal lesions, extraocular myotonia, extraocular muscle weakness, abnormal central control of eye movement⁷⁻⁹. Early onset (< 50 years of age) posterior subcapsular cataract is a characteristic feature of myotonic dystrophy type 1 and 2 and, at least for DM1, is known to be a key feature for timely diagnosis. So, sometimes, the ophthalmologist is the first doctor a patient would visit, as cataract often precedes all other symptoms¹⁰⁻¹³. In these cases, and especially in the absence of other secondary causes of cataract, awareness by the treating physician that early onset cataract can be a presenting manifestation of a multisystemic disease, is essential for appropriate referral of patients, avoidance of unnecessary examinations and timely diagnosis. Nevertheless, to our experience, many patients with DM1, despite being diagnosed and operated at a young age for posterior subcapsular cataract, remain undiagnosed for years and are referred for evaluation at a neuromuscular unit only after appearance of muscle weakness or other symptoms of the disease¹⁴.

Myotonic dystrophy is associated with numerous anesthesiological risks^{15,16}. Myotonia can be triggered by hypothermia, chills, electrical and/or mechanical stimulations. Moreover, sensitivity to anesthetic drugs, especially muscle relaxants and opioids, can complicate the post-operative course. The presumed hypersensitivity to neostigmine and a double response to the caffeine test impose a cautious evaluation in the choice of anesthetics, while the predisposition to arrhythmic events poses problems in the use of local anesthetics, with the result of a veiled resistance by anesthesiologists to subject these patients to anesthesiological procedures¹⁶.

Ropivacaine HCl, a long-acting member of the amino amide class of local anesthetics, indicated for surgery and acute pain management, has been recently used for ocular surgery. Compared to lidocaine and bupivacaine, it presents the advantage to have a less deep and shorter motor block, and minor cardiac toxicity¹⁷.

Aim of this work was to investigate through 24h-Holter monitoring, performed before and within 24 hours post-surgery, the heart response to the local retrobulbar administration of ropivacaine HCl (naropin), in a group of patients with DM1 undergoing cataract surgery.

Patients and methods

Sixteen patients (12M:4F), mean age 47.4 year (range 30.2-55.9) affected by myotonic dystrophy type 1, confirmed by molecular analysis and followed at the Cardiomyology and Medical Genetics of the University of Campania "Luigi Vanvitelli", underwent cataract surgery, through phaco-emulsification. Four patients had a cardiac device (1PM and 3ICD) due to atrio-ventricular (A-V) blocks or tachyarrhythmias, at the time of surgery. Three patients were on anti-arrhythmic drugs (amiodarone or bisoprolol) for tachyarrhythmias.

Before cataract surgery, all patients had a full cardiological assessment including clinical investigation, standard and 24h-Holter ecg monitoring, echocardiography and respiratory assessment. The following cardiological parameters were evaluated at baseline and after the surgery: heart rate (HR), supraventricular and ventricular ectopic beats (SVB, VEB), runs of tachycardia, pauses ≥ 2.5 sec.

Anesthesia was induced through peribulbar block, effective both on pain sensitivity and ocular motility, obtained by multiple infusions of 0.8 ml of 10% ropivacaine HCl, a member of the amino amide class of local anesthetics. This drug was chosen for its prolonged sensitive and motor block, and scarce cardiac and systemic toxicity. The mydriatic effect, necessary for a good visualization of the operative field, was obtained by the insertion of tropicamide 0.28 mg/phenylephryne 5.4 mg (mydriastert) in the inferior conjunctival sac, two hours before the surgery. The crushing of the lens was obtained through ultra-high frequency ultrasounds, followed by the insertion of an elastic intraocular lens.

Statistical analysis

Student t test for paired data was applied to compare mean values; Statistical significance was set at p-value < 0.05 (nominal significance).

Results

Table I shows cardiological data obtained before and after cataract surgery in DM1 patients. None of patients had episodes of tachycardia or pauses > 2.5 sec, at 24h-Holter baseline. Two male patients presented ventricular ectopic beats > 500/die and were on amiodarone and bisoprolol drugs, respectively.

The analysis of the ecg tracings registered during and for 24 hours after the phacoemulsification procedure, showed that 13/14 patients with no ventricular ectopic beats at basal 24h-Holter, did not develop arrhythmias in 24 hours after surgery. One of the patients having an ICD, developed ventricular ectopic beats within 24 hours

Table I. 24h-Holter cardiological data before and after surgery in DM1 patients studied group.

	Mean HR (b/m)		VEB		SVB		Couples		Bigeminism		Trigeminism		P value
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	
Pt. 01	60	58	1	67	154	0	0	0	0	0	0	0	
Pt. 02	100	103	0	0	0	0	0	0	0	0	0	0	
Pt. 03	75	68	0	0	0	0	0	0	0	0	0	0	
Pt. 04	88	93	0	0	10	0	0	0	0	0	0	0	
Pt. 05	80	90	0	61	0	0	0	0	0	0	0	0	
Pt. 06	75	76	0	0	372	86	0	0	0	0	0	0	
Pt. 07	65	72	0	0	0	0	0	0	0	0	0	0	
Pt. 08	90	82	0	0	0	0	0	0	0	0	0	0	
Pt. 09	60	75	0	0	0	0	0	0	0	0	0	0	
Pt. 10	93	75	1355	3613	163	16	3	178	1	65	0	13	
Pt. 11	60	64	0	741	0	0	0	0	0	0	0	2	
Pt. 12	53	57	302	1372	26	78	0	0	1	2	0	6	
Pt. 13	70	86	0	0	0	0	0	0	0	0	0	0	
Pt. 14	55	66	0	0	8	16	0	0	0	0	0	0	
Pt. 15	82	79	0	0	0	5	0	0	0	0	0	0	
Pt. 16	79	70	0	0	2	5	0	0	0	0	0	0	
Mean	74,1	75,9											0.68
SD	14.4	12.7											

HR : Heart Rate; VEB: ventricular ectopic beats; SVB: supraventricular beats

after surgery, but not in close relation to it. The patients who had ventricular ectopic beats > 500/die at baseline, showed an increase in the number of extrasystoles within 24 hours after surgery. However, they have arbitrarily stopped medication the day before surgery.

Statistical analysis

The analysis of heart rate values – before and after surgery – did not show differences statistically significant.

Discussion

Myotonic dystrophy (DM1) is the most common muscle disease in adults characterized by myotonia and muscular wasting and a multisystemic involvement that affects heart, brain, respiratory and endocrine system, and eye¹⁻⁴. There is a high risk of cardiac sudden death due to conduction system anomalies^{5,6}. A variety of ocular signs is described in DM1 patients, the most frequent and characteristic of them is posterior subcapsular cataract requiring surgical treatment. DM1 poses serious anesthesiological problems due to the associated neuromuscular and multi-organ implications, in both general and local anesthesia⁷⁻¹³. Adrenaline and lidocaine, alone or in combination with other anesthetics, generally used to induce vasoconstriction and to prolong the anesthetic effect

and control pain, are contraindicated for the known side effects on the cardio-vascular system (tachy-brady ventricular and supraventricular arrhythmias, hypertensive or hypotensive crises).

The use of local anesthetics of the amide and ester group, is preferable – whenever possible – in these patients. Several eye surgery studies¹⁸⁻²² requiring peribulbar blockade, conducted on patients not affected by DM1, have shown that ropivacaine is preferable to other drug combinations, because it produces long lasting rapid and deep blockage – sensory and motor – of the eye, normal recovery of the ocular motor function, and minor topical and systemic side effects.

Gioia et al.²⁰, evaluating clinical properties of 0.75% ropivacaine and a 1:1 mixture of 2% lidocaine and 0.5% bupivacaine for peribulbar anesthesia, demonstrated that ropivacaine has an onset similar to that of the lidocaine-bupivacaine mixture and provides a better quality of postoperative analgesia.

Zhou et al.²¹, in a prospective, randomised, double-masked comparison of local anaesthetic agents for vitrectomy, compared the intraoperative and postoperative clinical properties of 1% ropivacaine, 0.75% bupivacaine, 2% lidocaine and a mixture of 0.75% bupivacaine and 2% lidocaine (bupi+lido) administered for peribulbar anesthesia in 140 patients. They reported that 1% ropivacaine alone provides an adequate intraoperative anaesthesia similar to that provided by the bupivacaine,

lidocaine and lido+bupi solutions, as well as a better quality of postoperative analgesia. Furthermore, they showed that the incidence of postoperative subconjunctival haemorrhage was decreased in the ropivacaine group compared with the other three groups.

Our data – though limited to a small group of patients with myotonic dystrophy type 1 – confirm these studies and provide anesthesiologists with further evidence of the efficacy and safety of ropivacaine for ocular surgery also in patients with neuromuscular disorders.

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Mild myopathic phenotype in a patient with homozygous c.416C > T mutation in *TK2* gene

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The mitochondrial DNA depletion syndrome (MDDS) is characterized by extensive phenotypic variability and is due to nuclear gene mutations resulting in reduced mtDNA copy number. Thymidine kinase 2 (TK2) mutations are well known to be associated with MDDS. Few severely affected cases carrying the c.416C > T mutation in *TK2* gene have been described so far. We describe the case of a 14months boy with the aforementioned *TK2* gene pathogenic mutation at a homozygous state, presenting with a mild clinical phenotype. In addition to severe mitochondrial pathology on muscle biopsy, there was also histochemical evidence of adenylate deaminase deficiency. Overall, this report serves to further expand the clinical spectrum of *TK2* mutations associated with MDDS.

Key words: mtDNA depletion syndrome, TK2

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Conflict of interest

The Authors declare no conflict of interest

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Introduction

Mitochondrial diseases are clinically and genetically heterogeneous disorders due to respiratory chain disturbance with subsequent failure of aerobic metabolism, leading to dysfunction of multiple organs, especially the highly energy dependent. The pathogenic mutations may be either in the mitochondrial DNA (mtDNA) or in the nuclear DNA (nDNA), which encode mitochondrial proteins¹. Mitochondria are essentially the source of high-energy intermediates and are considered as the lever of cellular function. To fulfill their role, they have to constantly fuse and divide, processes that are quite complex and dependent on mtDNA replisome enzymes, nucleotides supply and balanced mitochondrial dynamics. Defects in mtDNA replication may be due to any of the aforementioned stage, resulting in usually multisystemic disorders^{1,2}.

Mitochondrial thymidine kinase (TK2) is the catalytic enzyme for the phosphorylation reaction of pyrimidine deoxyribonucleosides, which is the first step in the conversion of deoxynucleosides into deoxyribonucleotide triphosphates (dNTPs) (2). Therefore, *TK2*-mutations are responsible for disorders of mtDNA nucleotides supply encompassing a broad phenotypic spectrum, with myopathy and progressive external ophthalmoplegia

(PEO) being some of the most prominent clinical characteristics². The clinical variability of mtDNA depletion syndrome due to *TK2*-mutations may be partly related to the different extent of enzymatic activity, which is determined by the mutant gene³.

Herein, we describe a now 4-year-old boy, diagnosed at 14 months of age, with a mitochondrial DNA depletion syndrome (MDDS) harboring a *TK2* gene pathogenic mutation at a homozygous state with a milder phenotype than previously reported.

Case report

The index patient, a boy aged 14-months on his first admission, was firstly investigated in the pediatric department for persistently high creatine phosphokinase (CK) serum levels and mild transaminasemia, which were initially randomly detected. CK values (normal range 0-180 U/L) fluctuated from 700 U/L up to a maximum of 17.870 U/L, without any evidence of myoglobinuria.

The patient is the second child, born at term, to a healthy, non-consanguineous couple. His birth weight was 3.008 kg and he had a normal perinatal period and psychomotor development. His 4-yr old brother had an unremarkable medical history with just milk allergy. None of the patient's relatives had increased CK and there was no family history for any neuromuscular disease. Clinical examination, including muscle strength, muscle tone, tendon reflexes, cranial nerves and coordination, was normal. Although, the infant could sit and stand unsupported, he had not yet at that time achieved independent walking. With the exception of muscle enzymes, other laboratory tests, including virological markers and immunological parameters, were normal. Heart evaluation with ECG and echocardiogram did not reveal any abnormalities. Brain MRI was also normal. Genetic testing for deletions and duplications in the dystrophin gene was also normal.

A vastus lateralis muscle biopsy revealed myopathic changes with abnormal variation of muscle fiber diameter and multiple cytochromoxidase-negative (COX-negative) muscle fibers with few ragged red (trichrome Gomori staining) and/or ragged blue (succinate dehydrogenase staining), confirming the diagnosis of mitochondrial myopathy (Fig. 1). Histochemistry showed also myoadenylate deaminase deficiency (Fig. 2). Biochemical analysis of the muscle biopsy revealed a combined deficiency involving complexes I, III and IV, pointing either to a mtDNA mutation or a defect in the mitochondrial protein synthesis due to a nuclear gene mutation.

Genomic DNA from muscle biopsy revealed an already known pathogenic mutation in the *TK2* gene in homozygosity (NM_004614.5: c.416C > T, p.Ala139Val)^{4,5}, which was further confirmed by Sangers analysis. Both

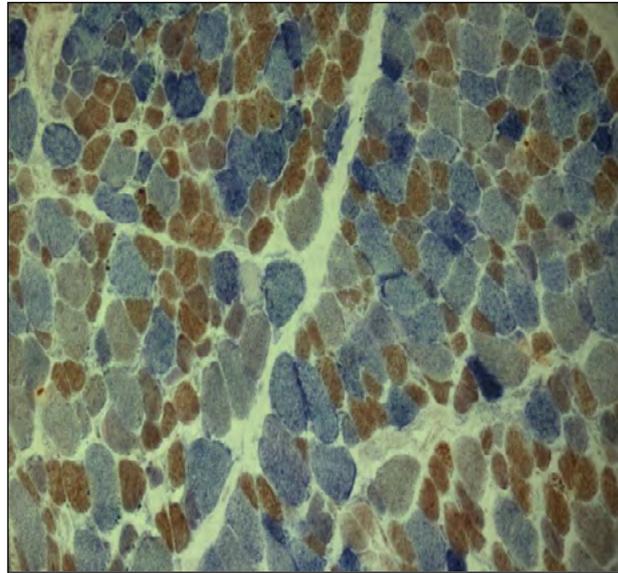


Figure 1. Transverse muscle biopsy section double stained with cytochromoxidase (COX) plus succinate dehydrogenase (SDH) – (COX-SDH x20) showing multiple COX-negative and SDH-positive fibers in blue.

parents were also tested and were found to be carriers of the same mutation.

Further clinical examination at a later stage showed delayed motor milestones with walking at 3 years of age. Today, at the age of 4 years, the index patient has difficulties in walking, climbing stairs and rising up from the floor. He has also a symmetrical muscle weakness of upper and lower legs (3-4 according to MRC scale in most muscle groups).

The patient's legal representative has signed written informed consent for the data to be published in a scientific journal. There was no need for ethical approval from an ethics committee, as any diagnostic step was within the routine management of such patients.

Discussion

This is a report of a toddler who presented with incidentally detected constantly high serum CK levels and was finally diagnosed with MDDS due to *TK2* mutations.

The mtDNA maintenance defects, involve, basically, either the most severe mtDNA depletion syndrome (MDDS) or the usually milder multiple mtDNA deletion syndrome. MDDS is an inherited autosomal recessive disease, caused by a reduction of mtDNA copy number and as such, it may be considered as a quantitative disturbance, with the phenotypic severity being related to the remaining normal mtDNA levels^{3,6}. The clinical spectrum of the syndrome is quite diverse with variable symptoms,

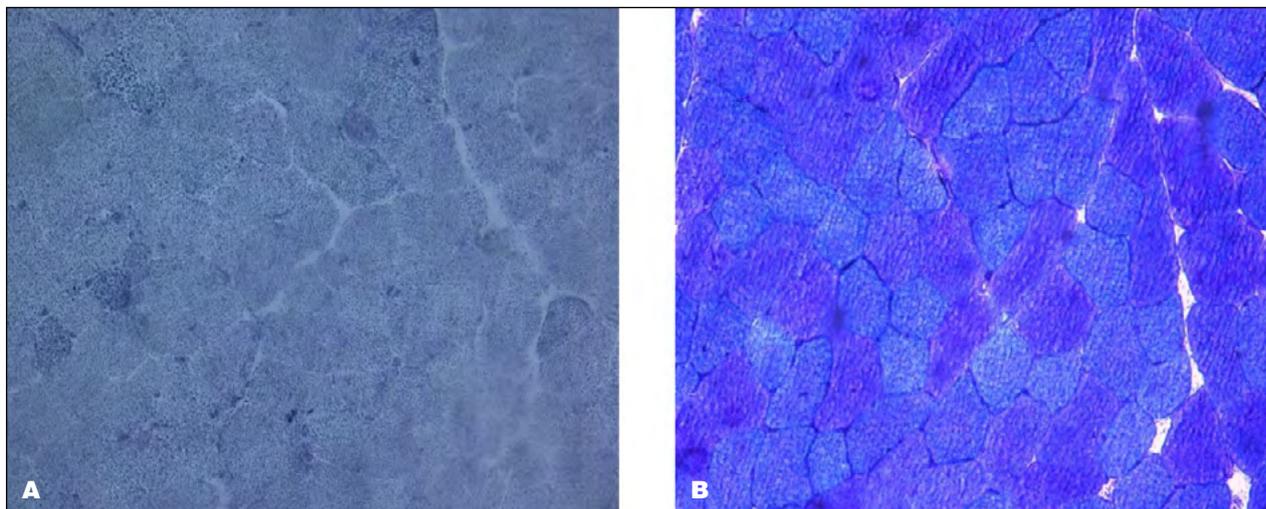


Figure 2. Myoadenylate deaminase (AMPDA x40) staining from the patient showing AMPDA deficiency (A) and from a healthy control, with type 1 muscle fibers more intensely stained than type 2 (B).

mainly related to mutations in certain genes. MDDS can be recognized by the following prevailing phenotypes: a myopathic, an encephalomyopathic, a hepatocerebral and a neurogastrointestinal ⁷.

A predominantly myopathic form, associated with high CK and onset in infancy or early childhood, has been related with *TK2* mutations ⁷. Typically, the children develop normally over the first months after birth, but they later lose some acquired motor milestones, usually before the second year of life. The most common presentations are hypotonia, exercise intolerance and especially proximal muscle weakness, while there may occasionally be some bulbar involvement with facial weakness, dysarthria and dysphagia ^{7,8}. Respiratory difficulties are also listed high among the most frequently symptoms of the disease ⁸. Noteworthy, the ongoing recognition of new manifestations has expanded the phenotypic spectrum of *TK2*-related MDDS. More specifically, there may be a broader age range of the first symptoms with even a milder adult onset presentation or a multi-organ involvement, including the brain with cognitive decline, epilepsy, cerebral atrophy, cerebellar degeneration and diffuse white matter changes, the heart with cardiomyopathy, the liver with hepatomegaly etc. ^{8,9}. Although, the condition has no approved treatment so far, there is robust evidence that deoxynucleoside monophosphates and deoxynucleoside administration may improve the clinical outcome in both early and late onset patients and potentially modify the natural course of the disease ¹⁰.

The patient of the present study is now a 4-yr old boy with a, so far, relatively mild pure myopathic phenotype. Among the initial diagnostic work-up tests, was the screening for deletions and duplications of the *dystro-*

phin gene by MLPA. After the negative results, it was not considered necessary, at that time, to perform sequencing of the gene, mainly due to a low clinical suspicion for a possible dystrophinopathy. The next diagnostic step was muscle biopsy, which revealed mitochondrial pathology with numerous COX-negative muscle fibers, few ragged red/blue fibers and myopathic changes mainly consisted of increased fiber size variation. The above-mentioned histological abnormalities, although not specific for any particular mitochondrial mutation, are amongst the most frequent features of *TK2*-related MDDS ¹¹. Genetic studies revealed the already described pathogenic mutation c.416C > T, p.Ala139Val mutation in homozygosity, in the *TK2* gene.

This mutation has been firstly described in homozygosity in two Greek siblings with normal early developmental milestones. The older brother run a progressively declining course after the very first years of life, rendered him, at age 5 years, wheelchair-bound with severe cognitive dysfunction, while her younger sister followed a rapid deterioration after the age of 2 years with hypotonia and inability to stand, six months later ⁵. The same mutation has been already reported in a compound heterozygosity in two affected brothers with a very severe clinical phenotype. Both probands were born without any sign or symptom of a possible underlying neuromuscular disorder, but after a few months of normal early development, they showed a rapid psychomotor regression and severe respiratory failure. The older brother suffered also from aggravating epileptic seizures and died at the age of 12 years after being tracheostomized for many years ⁴. In a more recent paper, there is a similar description of a young girl carrying this mutation in compound heterozygosity, who presented with

motor skill regression at the age of 13 months and died at the age of 22 months after a rapid deteriorating course⁹. Contrary to the poor outcome of the disease in all those affected children, the patient of the present study has run a more benign clinical course, so far. This discrepancy may reflect a lack of genotype-phenotype correlation, suggesting that a possible contribution of other epigenetic or environmental factors may determine the course of the disease. In any case, the existing literature based on few case reports does not permit to draw general conclusions on the underlying genetic mechanisms.

Moreover, an additional histological finding in the muscle biopsy of our patient was the absence of myoadenylate deaminase (AMPDA) activity. This is a quite common metabolic disturbance, estimated in approximately 2% of the population and can be coexist with other neuromuscular disorders. Exertional myalgia is the most frequently observed symptom, although most individuals are asymptomatic. The disorder was not genetically investigated in our patient, as it was considered coincidental, although it is difficult to determine whether AMPDA deficiency may partially contribute to the clinical picture, when combined with another metabolic disease. However, it has been shown that even a near total AMPDA deficiency does not influence exercise capacity and cellular energy charge, bringing into question a potential association to an add-on effect¹².

In conclusion, we present a paediatric patient with MDDS due to a homozygous pathogenic mutation in *TK2* gene, which has been already described in a homozygous or heterozygous state, in few severely affected patients. Interestingly, in addition to mitochondrial pathology, muscle biopsy also revealed AMPDA deficiency, which is a frequent histochemical abnormality and occasionally coexist with other metabolic disorders. We thereby expand the clinical and mutational spectrum of the *TK2*-related MDDS, although more cases are needed to investigate genotypic correlations and whether other modifiable factors may contribute to phenotypic variations and differing outcomes.

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Respiratory muscle involvement in *HNRNPDL* LGMD D3 muscular dystrophy: an extensive clinical description of the first Italian patient

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Limb girdle muscular dystrophy is a genetically inherited condition that primarily affects skeletal muscle leading to progressive, predominantly proximal muscle weakness at presentation. Autosomal dominant LGMD represent 10% of all LGMDs. *HNRNPDL*-related muscular dystrophy, LGMD1G/LGMD D3 (MIM#609115), is an extremely rare autosomal dominant adult onset myopathy described in a handful of families. Here we fully characterized the muscular and respiratory involvement of a 58 years old Italian woman presenting the previously reported pathogenic variant c.1132G > C p.(Asp378Asn) in the *HNRNPDL* gene.

Key words: Limb girdle muscular dystrophy, LGMD D3, *HNRNPDL*, respiratory muscle involvement

HNRNPDL-related muscular dystrophy (MIM#609115), Limb girdle muscular dystrophy D3 (LGMD D3), previously known as LGMD1G, is an extremely rare autosomal dominant adult onset myopathy thus far reported in fewer than 30 cases from one Chinese and four Latin-American families¹⁻⁵. All patients harbored mutations hitting the same residue located in the prion-like protein domain. They presented a limb girdle or combined proximo-distal muscular weakness without evident respiratory or cardiac involvement. Some had cataract.

We describe a 58-year-old Italian woman who presented in her forties difficulties in rising stairs followed by difficulties in arm elevation. She reported frequent and sudden falls on her knees. She also had frequent nocturnal awakenings and occasional headache in the morning when waking up. Her deceased mother, uncle, and grandfather presented a similar clinical history with proximal muscle weakness in their 40-50's.

Neurologic examination at age 58 showed a waddling gait, asymmetric scapular winging (Fig. 1a), upper limb muscle weakness and atrophy (Fig. 1b) (4/5 MRC), finger flexor and extensor and *interossei* muscle weakness, proximal lower limb weakness (2/3 MRC), and foot flexors and extensor weakness and atrophy (4/5 MRC) predominant in the left side (Fig. 1c). Serum CK levels were normal.

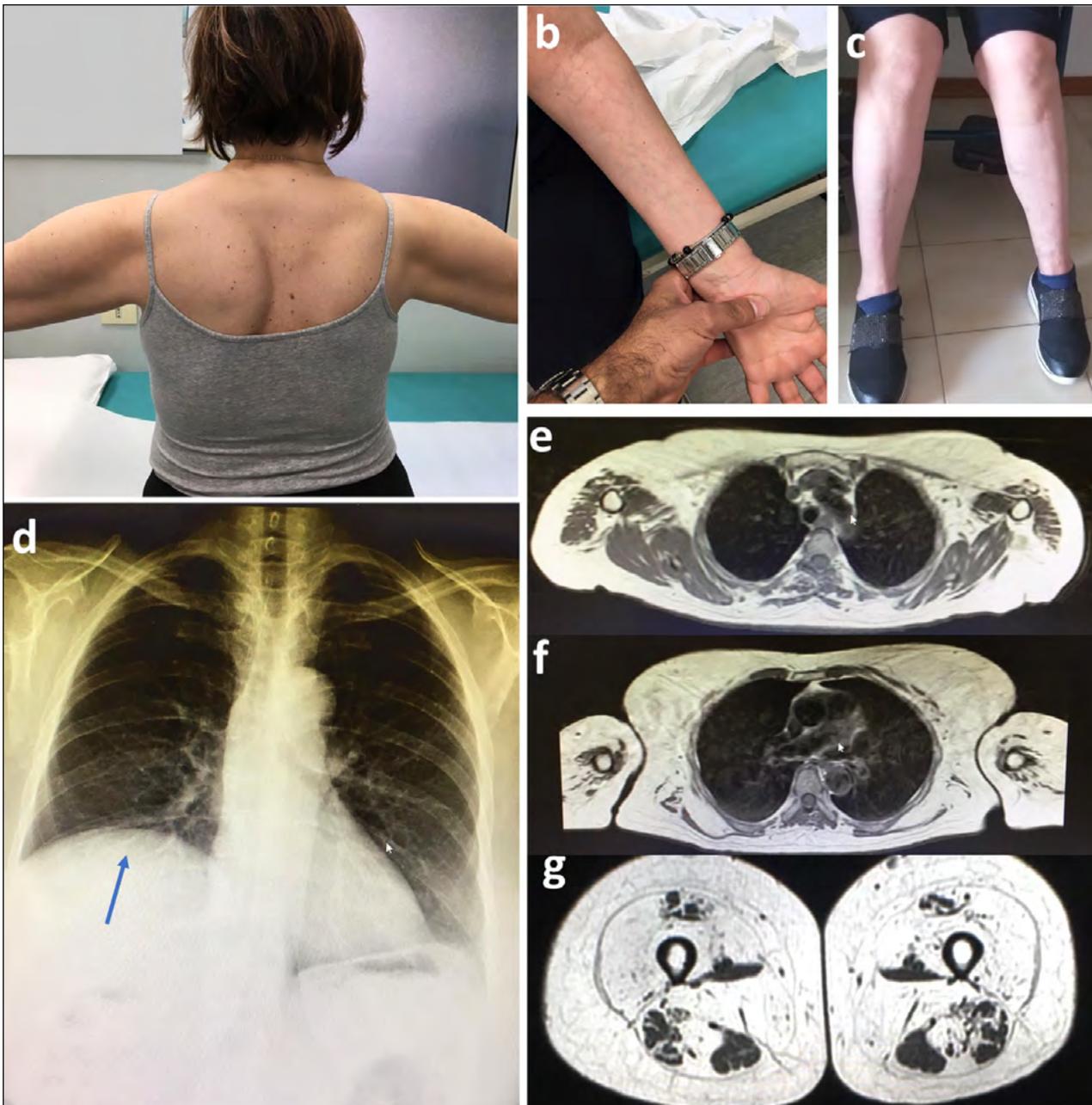


Figure 1. a. Asymmetric scapular winging; b. Distal arm atrophy; c. Asymmetric leg atrophy; d. Chest X-Ray showing elevation of the right hemidiaphragm muscle (indicated by an arrow); e-f. Upper limb T1 weighted MRI sections performed at 58 years. Severe fatty infiltration was noted on the deltoid muscles. At lower level there was a marked atrophy and fatty infiltration on pectoral, biceps, and triceps muscles; g. Thigh MRI, T1 weighted sections showing very relative muscle tissue sparing in *rectus femoris* and *adductor longus* muscles. *Vastus lateralis*, *adductor magnus*, and *sartorius* muscles are completely fatty infiltrated. In the posterior compartment only the semimembranosus, semitendinosus, and *biceps femoris* are relatively spared.

Pulmonary workup revealed a FVC at 51% of predicted values, dropping at 45% in supine position, suggestive of diaphragmatic weakness. Chest X-Ray denoted right hemidiaphragm elevation (Fig. 1d). Pulse oximetry

during sleeping hours showed an average 91% of Sa-tO₂, CO₂ = 53mmHg, and an EGA showed a PCO₂ = 49mmHg. Based on this, a non-invasive ventilation (NIV) during sleeping hours was set up. Cardiac workup includ-

ing EKG and ultrasound was normal. Of note the patient developed bilateral cataract in her 40s.

Shoulder MRI showed mild hypotrophy and fat replacement of the deltoid muscle bilaterally, and marked hypotrophy and fat replacement of the pectoral, and flexor-extensor muscles of the elbow (Figs. 1e-f). MRI of the lower limbs revealed a specific pattern with *adductor longus* and *rectus femoris* sparing in the thigh, and relative sparing of the semimembranous and long head of biceps in the posterior compartment (Fig. 1g).

A *vastus lateralis* muscle biopsy performed elsewhere was not contributive as constituted by fibro-fatty connective tissue.

Massive and parallel sequencing of a gene panel containing over 200 genes associated with inherited muscle disorders identified the previously reported c.1132G > A p.(Asp378Asn) in *HNRNPDL*³⁻⁴. The mutation was numbered according to the GenBank NM_031372.3 with +1 corresponding to the A of the ATG translation initiation codon and is not currently listed in gnomAD.

Common features of *HNRNPDL*-related muscular dystrophy were the typical clinical phenotype with late onset limb girdle weakness and distal upper limb and lower limb weakness. We also observed asymmetric scapular winging (Fig. 1a) and distal limb atrophy (Figs. 1b-c).

In our case, thigh MRI confirmed the presence of a common pattern with preservation of the *rectus femoris* and *adductor longus* after 18 years of disease activity (Fig. 1). In upper limbs, we observed a fat replacement of deltoid muscle bilaterally, and the pectoral, flexor-extensor muscles of the elbow associated with muscle bulk reduction (Figs. 1e-f). Whole body muscle MRI would be important to assess if a common muscular pattern might be identified in other muscular group such face or axial muscles.

The presence of frank respiratory muscle involvement with a diaphragmatic weakness leading to right hemidiaphragm elevation (Fig. 1d) is a novel finding in *HNRNPDL*-related myopathies, being previously described in a single case⁵. We therefore suggest an accurate respiratory workup including FVC in sitting and supine position, EGA, and nocturnal oximetry to detect respiratory muscle dysfunction, and to adopt NVI whenever appropriate.

Our patient presented bilateral cataract, a finding repeatedly reported by others³⁻⁵, and suggestive of a role for *HNRNPDL* in the molecular pathway related to opacity of the crystalline lens⁶.

The same variant detected in the Brazilian and Chinese family described in³ and⁴ was found. The identification of the same mutation in different ethnic backgrounds

leads us to speculate of a possible hotspot. However, the occurrence of a common Italian ancestor in the Brazilian family is also likely, due to the high rate of Italian immigration to Latin America in early 20th century. On the other hand, the identification of a different variant affecting the same codon (Asp378His) in a Uruguayan family⁵, is a further argument suggesting a possible hotspot in that position.

Conclusions

Our report describes the first Italian *HNRNPDL* mutated patient presenting proximo-distal muscular weakness and respiratory muscle involvement needing non-invasive ventilation.

Acknowledgements

We thank our Patient for her compliance.

Ethical statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Ethical standards

All procedures performed were in accordance with the ethical standards stated in the Declaration of Helsinki.

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MPV promote adherence to nocturnal NIV in a Duchenne patient

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We described a case of a patient 20 years old, affected by Duchenne dystrophy with obstructive sleep apnoea syndrome and severe nocturnal desaturation. He was not compliant to non-invasive ventilation (NIV) for claustrophobia and panic attacks. Mouthpiece ventilation was successfully used in this patient, who later accepted the nighttime NIV.

Key words: Mouthpiece ventilation, Duchenne dystrophy, NIV

Introduction

Non-invasive ventilation (NIV) is sometimes considered suboptimal in neuromuscular disease patients, due to excessive secretions in the airways, hypercapnia due to inadequate adherence, or lack of tolerance of the interface ¹. Interfaces that cover the nose and/or mouth and nose, are the most commonly used but they often cause skin lesions and some times even claustrophobia. Mouthpiece ventilation (MPV) was first described in 1953 to permit iron lung users without autonomous ability to breathe, to leave iron lungs during the day. The interface is provided by angled mouthpieces of 15 or 22 mm accessible to the mouth by means of a flexible support arm so that the patient can access them with the lips as needed. Patients can trigger the breath by creating a small negative pressure or by kissing the mouthpiece ². The main advantage over a nasal or oronasal mask is that mouthpiece produces less interference with language, no risk of skin breakage and claustrophobia. It is safer by allowing the use of glossopharyngeal respiratory ventilator failure or accidental disconnection from the ventilator.

Case report

In a 20 years old patient, affected by Duchenne dystrophy and hypokinetic dilated cardiomyopathy with moderate reduction of contractile function, and mild to moderate mitral and tricuspidal insufficiency, a sleep apnoea obstructive syndrome with severe nocturnal desaturation was diagnosed in the last year and use of CPAP was recommended to him. Though numerous interfaces were attempted to adapt him to the CPAP, the patient refused it for claustrophobia and the onset of panic attacks. One year later, the patient began to experience headache and morning tiredness; soon after he was admitted to the emergency room due to acute neurological symptoms, such as tremor, allucinations and dizziness. Clinical examination and blood test were normal. Blood gas analysis showed hypercapnia (52 mmHg) and mild hypoxemia (61 mmHg), requiring non-invasive mechanical ventilation. The patient was in a state of agitation, frightened and

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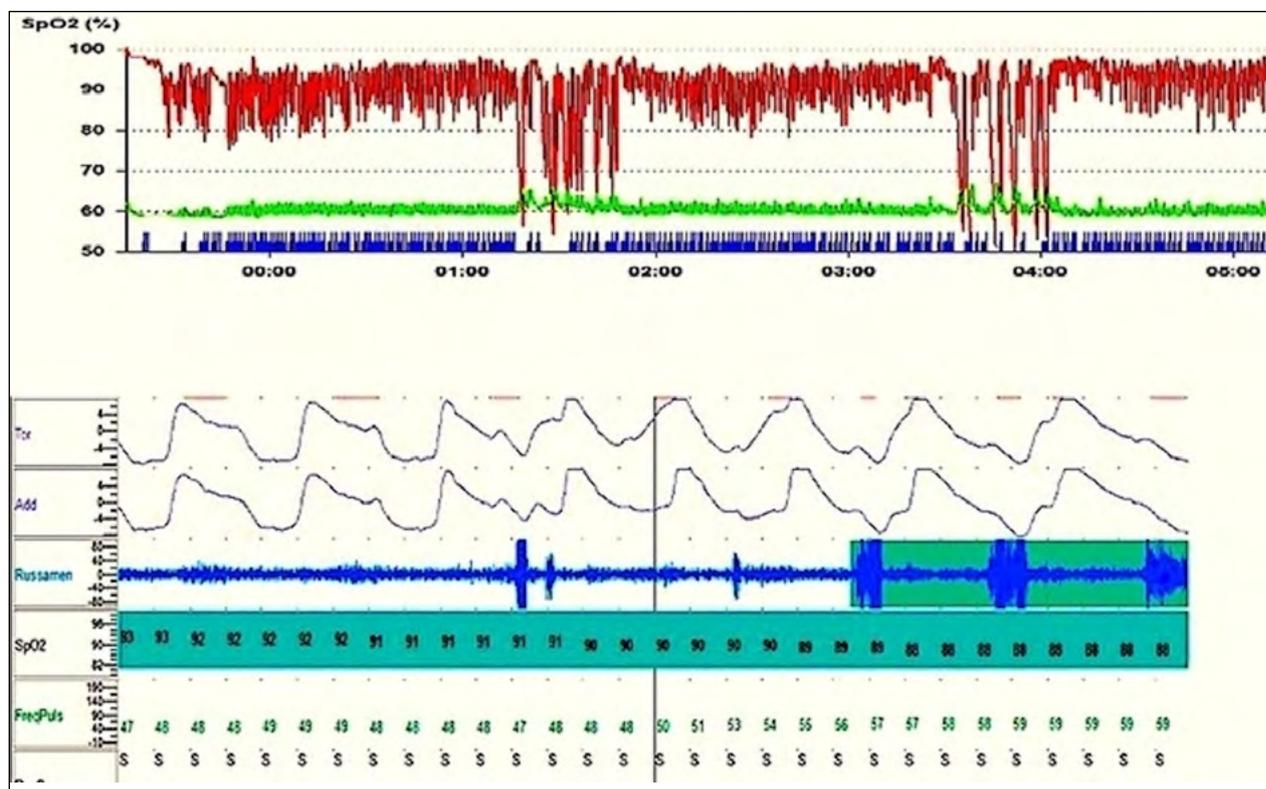


Figure 1. Overnight Polysomnography showing desaturation.

claustrophobic, but fully aware and refusing non-invasive mechanical ventilation with traditional interfaces. At this time, we proposed him MPV. The patient accepted to try the mouthpiece.

The inspiratory pressure was set between 12 and 14 mmHg, which obtained an optimal tidal volume between 8-10 mL/kg; No back-up rate was needed for daytime use, so no air blew into the patient's face; the inspiration time was set at 1.2 sec, EPAP 0, the rise time at 2/6. After only 1 hour, we observed an improvement in gas analysis values with normalization of CO₂ (46 mmHg) and oxygen (71 mmHg) values. The overnight polysomnography confirmed the presence of the severe obstructive sleep apnoea syndrome and prolonged hypoventilation (Fig. 1).

During the day, the patient selected MPV position with a rigid arm fixed on the bed or wheelchair. After only 2 days of MPV during daytime hours, the patient felt better and safer than before so that he accepted night ventilation with a nasal mask (Fig. 2).

He was adapted with mode ST-AVAPS, and parameters were the following: IPAP max 14 min 10, EPAP 6, Vt 650 mL; trigger flow 2.0 litres, 25% cycle, rise time 3, respiratory rate bk 10/min, Insp. Time 1.2 sec. Nocturnal pulse oximetry was performed, which confirmed the



Figure 2- The patient during MPV.

resolution of the desaturation events (Fig. 3). Blood gas analysis on awakening was normal.

Discussion

In this case report, the main goal of nocturnal NIV was achieved with the use of MPV. The use of MPV has been

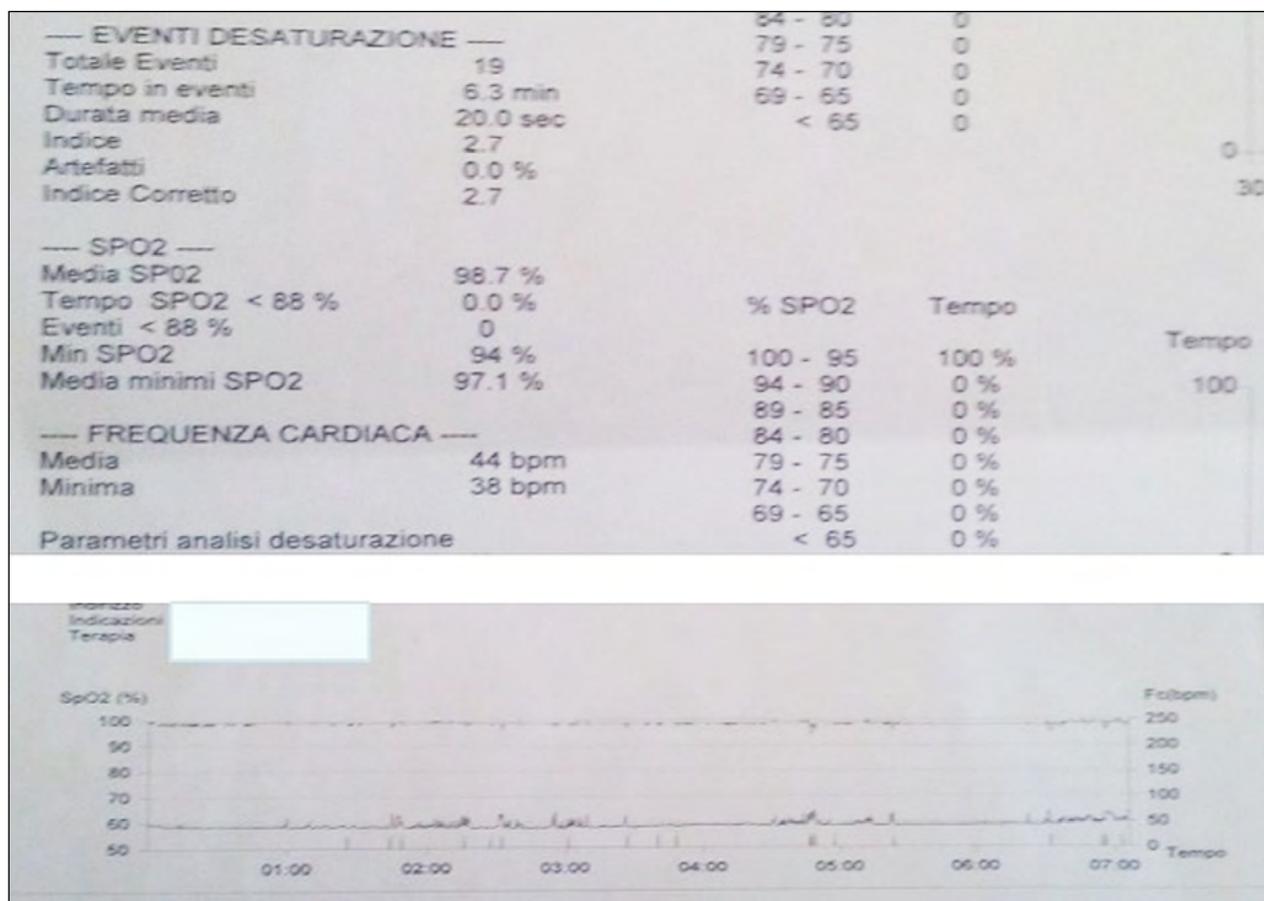


Figure 3. Pulse oximeter showing the correction of desaturation.

known for many years, but its diffusion has always been minimal until 2013 when models dedicated to commercially available portable ventilators were introduced. Despite this, still today, there is little knowledge of how to use it.

Bach et al.³ reported the sequential use of mouthpiece during the day and a nasal mask during the night. They also suggested the possible use of a standard mouthpiece with lip-seal retention or custom-moulded orthodontic bites for overnight use.

Positive expiratory pressure (EPAP) cannot be maintained for patients who use open NIV system, and is indeed rarely, if ever, necessary for these patients. Apnea alarms, when present, should be set at the highest threshold to avoid unnecessary activation and discomfort. The most common ventilator mode used is assisted volume- and pressure-controlled with no EPAP, low-pressure alarm set to apnea minimum and maximum duration⁴.

It is always necessary to carefully monitor the patient during the adaption phase since MPV requires true collaboration from the patient, and not all ventilators ensure rapid adaption to the patient's respiratory acts⁵. However, due to its specific features and disadvantages (air leaks,

etc.), MPV must be managed by expert hands and in a well-monitored way⁶. The use of MPV is likely limited to a few centres, for the longest time required to adapt and monitor the patient. The ventilator is easily activated by the mouth pressure of the patient. The mouthpiece is a preferable and comfortable alternative to NIV, but a more active participation is needed compared to the use of traditional masks.

It should always be considered for patients with chronic disease who need to start NIV; it is helpful to promote a positive approach to NIV. Patients reported a decrease in breathlessness and an increase in general and social productivity.

Our opinion is that it is necessary to know all the treatment possibilities to offer the best and personal therapy to each patient. It is also necessary to keep in mind that these patients often have psychological problems from seeing relatives or friends die of the same disease. Therefore, the time when NIV begins, that usually coincides with the progression of the disease, is a crucial time for the patients which often determines their quality of life and would require adequate psychological support⁷.

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NEWS FROM AROUND THE WORLD

AIM

The COVID-19 outbreak, exploded in Italy at the end of February 2020 and the subsequent quarantine lasted unevenly until mid-May 2020, forced everyone at home and blocked virtually all non-essential work activities. Therefore, both national and international meetings and conventions have been canceled and moved some at the end of the year, others even to 2021. Also the joint AIM/ASNP conference, to be held in Matera from 3 to 7 June 2020, was moved to December 2020, from 2 to 6. The program is being re-evaluated.

MSM

The 14th Meeting of the Mediterranean Society of Myology (MSM) the meeting is moved to spring 2021. Proposals to organize and host the event are welcome.

WMS

Below is the email communication received on 29/05/2020 from the Executive Board of the Society regarding the 25th Congress of the WMS.

“A decision has now been reached regarding the WMS 2020 Congress in Halifax, Canada, due to take place 30th September - 4th October 2020.

The COVID-19 situation has shown no sign of making the short/medium and longer-term future for events any clearer than it was a month ago and the Executive Board has therefore decided that we will proceed with a virtual congress, in this its 25th year, and plan to resume our face to face meeting next year with the 2021 congress in Prague, Czech Republic and then 2022 in Halifax, Nova Scotia, Canada.

A decision based on facts we want to make it very clear that we have made this decision based purely on the facts as we know them today and, importantly for the whole WMS team, based on the responsibility and genuine care we feel for all our members, and the neuromuscular community. We have tried today to take the best decision for the Society, and we are so thank-

ful for the support that has already been shown to us. We have thought long and hard in the past few weeks, consulting in depth with partners and stakeholders before making this announcement.

So, please keep the dates 30th September - 2nd October 2020 firmly in your diary as the WMS Virtual Congress, then watch this space for more updates as the programme, and the way in which we will connect and learn as a community for this year, is revealed. In fact the virtual platform for this year actually provides more opportunities for discussion.

Committed to serving our members We want you to know that we are fully committed to rearranging the meetings for next year and beyond with this year being a new and exciting experience for us all.

We will make the virtual 2020 congress free to attend so that as many of our members who might not have been able to travel to Canada this year as possible can join the WMS spirit of Education, Enjoyment and Excitement!

We will be in contact with all registered delegates, presenters, sponsors and exhibitors in the coming days to discuss refunds and transfer of registration/presentation to the virtual event etc. and anticipate they will all be processed within 30 days. Abstract notifications will be sent out next week and we hope to have the full virtual programme available on the congress website in July. Accepted abstracts will still be published in the journal this year. Late breaking abstract submission will open as usual.

For more information please contact Clare Beach, WMS Secretariat and 2020 Congress Manager: office@worldmusclesociety.org; website: www.wms2020.com

The WMS continue to add valuable information about COVID-19 and people with neuromuscular disorders: World Muscle Society position and advice to the website: <https://www.worldmusclesociety.org/news/view/150>

Once again, we thank all the attendees and our members for the support that has been shown to us and we are here to support others in the field as we all work through these difficult times”

The WMS Executive Board

FORTHCOMING MEETINGS

2020

June 6-9

The European Human Genetics Conference. Berlin, Germany. Information: website: [https:// www.eshg.org](https://www.eshg.org)

July 6-9

New directions in Biology and Disease of Skeletal Muscle Conference, New York, NY, US.

<https://myology.institute.ufl.edu/conferences/new-directions>

September 25-29

Muscle Study Group Annual Scientific Meeting, Washington, US. Information: website: <https://musclestudygroup.org/events/2020-annual-meeting>

September 30 - October 4

25th Congress of World Muscle Society. Halifax . Toronto, Canada. Information: website: www.worldmusclesociety.org

October 27-31

ASHG Annual Meeting. San Diego, CA, USA. Information: website: www.ashg.org

December 2-5

XX Congresso Nazionale AIM. Matera, IT. Information: website: <https://www.miologia.org>

2021

February 20

2nd Annual Conference on Genetics, Paris, France

June 12-15

The European Human Genetics Conference. Glasgow, United Kingdom. Information: website: <https://www.eshg.org>

September 21-25

26th Congress of World Muscle Society. Prague, Czech Republic. Information: website: www.worldmusclesociety.org

October 19-23

ASHG Annual Meeting. Montreal, Canada. Information: website: www.ashg.org

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, 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 4 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. Check that it represents the content of the paper and is not misleading. Also suggest a short running title.

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

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

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

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

PATIENTS IN PHOTOGRAPHS ARE NOT TO BE RECOGNISABLE

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

References. Reference numbers in the text must be in brackets. References in the list must be numbered as they appear in the text.

Standard journal article: Figarella-Branger D, Bartoli C, Civatte M, et al. Cytokines, chemokines and cell adhesion molecules in idiopathic inflammatory myopathies. *Acta Myol* 2000;19:207-8.

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

Please check each item of the following checklist before mailing:

- Three index terms, short title for running head (no more than 40 letter spaces) on the title page.
- Name(s) of the author(s) in full, name(s) of institution(s) in the original language, address for correspondence with telephone and fax numbers and email address on the second page.
- Summary (maximum 250 words).
- References, tables and figures cited consecutively as they appear in the text.
- Figures submitted actual size for publication (i.e., 1 column wide or 2 columns wide).
- Copyright assignment and authorship responsibility signed (with date) by all Authors.
- References prepared according to instructions.
- English style.
- Patients in photographs not recognisable.

For application or renewal to MSM

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