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Head: Prof. Giovanni Cioni

Clinical, Molecular and Imaging Study in Neuromuscular Disorders in Developmental age: contribution to the genotype-phenotype correlation

Tutor	Candidate
Prof. Giovanni Cioni	Dr.ssa Guja Astrea

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Ad Angelina,

detta Evangelina

e a tutte le stelle

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e sulla terra

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Abbreviations

MRI Magnetic Resonance Imaging
dHMN distal Hereditary Motor Neuropathies
dSMA distal Spinal Muscular Atrophies
CMT Charcot Marie Tooth
CM Congenital Myopathies
CMD Congenital Muscular Dystrophy
LGMD Limb-girdle muscle disease
DMD Duchenne Muscular Dystrophy

Overview of the Thesis

Inherited neuromuscular disorders represent a heterogeneous group of human diseases with a large clinical and genetics overlap and affecting almost invariably muscle strength and size. Oftentimes, clinicians face a brain teasing mixture of clinical and laboratory findings and combine them like in a puzzle to offer a certain and complete diagnosis. Thus, this category of human disorders represent a diagnostic challenge in clinical neurology for which a high level of medical, molecular and imaging knowledge is needed to reach a final diagnosis and offer patients an adequate rehabilitation management or appropriate prospective follow up and therapies.

My interest in neuromuscular disorders goes back to the latest years of my residency in Pediatric Neurology in IRCCS Stella Maris and the initial work on the characterisation of a novel pattern of muscle MRI in a rare progressive myopathy with characteristic pathological findings (**Astrea et al., *Neuromuscular Disorders* 2009**). At that time, using clinical data and features at muscle MRI we contributed to discover the gene causing the reducing body myopathy, helping clinicians in recognising this rare muscular condition.

The experience gathering during residency years taught me how to use a “hand lens” to focus not only on clinical symptom and sign in children with neuromuscular disorders but also to integrate genetics and imaging approaches, to develop a true curiosity for the molecular basis of muscular dystrophies and to use a multidisciplinary approach when facing a young patient.

During my PhD course I also had the possibility to attend one of the most important European Neuromuscular Centre, the Dubowitz Neuromuscular

Centre (London, UK) under the direct supervision of prof. F. Muntoni and the Neuromuscular Department of the Catholic University in Rome (with the supervision of prof. E. Mercuri). Particularly, I could spend six months in London for a research fellowship training to improve my knowledge in muscle MRI examination and grading system. The specific training was crucial in detecting a well known pattern of muscle MRI associated with a congenital myopathy in two sisters whose clinical and immunoistological findings were misleading and unable per se to pinpoint the right diagnosis. The draft of the manuscript describing our findings in that family has been submitted to the *Neuromuscular Disorders* journal.
Furthermore, this professional training led me to a more exhaustive analysis of muscles involvement at MRI. With the aim of evaluating the sensitivity and specificity of a visual inspection of muscle imaging scans so as to identify clues able to differentiate distal neurogenic from distal myopathic diseases, I spent research time focusing on the identification of discrete alterations within muscle fibers because a simple visual pattern recognition could not be sufficient in neurogenic diseases. The presence of «islands» preserving muscle bulks in a background of fatty transformations was serendipitously —yet consistently — noticed and it could represent a “red flag” for neurogenic conditions, regardless of the genetic defect.
The pattern of involvement within muscles, that is not something normally captured by the standard approach adopting the so termed “Mercuri grading” or reviewing pattern analyses reported in the literature, seems to be the most intriguing and useful diagnostic marker of late changes in distal neurogenic clinical conditions.

Finally the collaboration with Dubowitz Neuromuscular Centre offered me the possibility to study a large series of cases presenting a clinical diagnosis of CMD (congenital muscular dystrophy) in a second tier molecular genetic laboratory in UK, as well as the chance to draw interesting observations in genotype-phenotype correlations and relative prevalence of CMD and allelic limb-girdle muscular dystrophies in large population.
Equally important during the PhD research years were the opportunities to collaborate closely with the Molecular Medicine and Neurogenetic Laboratory of IRCCS Fondazione Stella Maris (in collaboration with Drs. F.M. Santorelli and C. Fiorillo). Thanks to such a collaboration, we could discover and characterise a new form of lower limb spinal muscular atrophy (SMA) in two patients and the related mutations in the *TRPV4* gene (***Neurogenetics,* 2012**), and to integrate the clinical, genetics and imaging findings in an original article focused on the description of the pattern on muscle involvement at myoimaging (**Astrea et al, *Neurology,* 2012**). The still-growing number of new genetic aetiology for lower limb neuromuscular disorders was an important opportunity for embedding clinical management and pattern recognition on muscle MRI giving aid to the definition of pathogenicity of a new mutation and further clinical correlation. An additional manuscript will be shortly finalized on the clinical genetics and imaging characterization of a new form of SMA with lower limb predominance (under submission). Using the same approach, it was also possible to describe a presintomatic form of lipidic myopathy due to

neutral lipid storage disorder (***Biochem Biophys Res Commun*****, 2013**).
Finally, to obtain and even wider comprehension of neuromuscular disorders, I have participated in the study design, obtaining of data and interpretation of results in an original study with the goal to verify the presence and characteristics of literacy deficits in children with Duchenne Muscular Dystrophy (DMD) and the analyses of their neuropsychological profiles associated with reading abilities. This was done comparing DMD patients with similarly aged dyslexic children. Such a study explains how a multi-component cognitive deficit may contribute to specific literacy difficulties in DMD with rapid access to lexicon and phonological processing being the core deficits in our subjects. As a practical consequence, our study suggested that a careful evaluation of specific reading abilities should be part of periodic clinical evaluations of DMD children in pre-school years and prompt correct rehabilitation of those components that appear deficient. The manuscript describing the study and the results has recently been submitted to ***PlosONE*** journal.

To summarize, my doctoral thesis has a wide scope, resulting from a research effort put on better characterization of genotype-phenotype correlations in different forms of neuromuscular disorders, highlighting the usefulness of muscle MRI in differential diagnosis and to expand its use for a better depiction of pathogenic mechanisms in inherited muscle diseases.

Chapter 1
Introduction

1 Neuromuscular Disease in Children: diagnostic challenges

Neuromuscular diseases are disorders caused by an abnormality of any component of the lower motor neuron system: anterior horn cell, peripheral nerve, neuromuscular junction, or muscle (Figure 1).

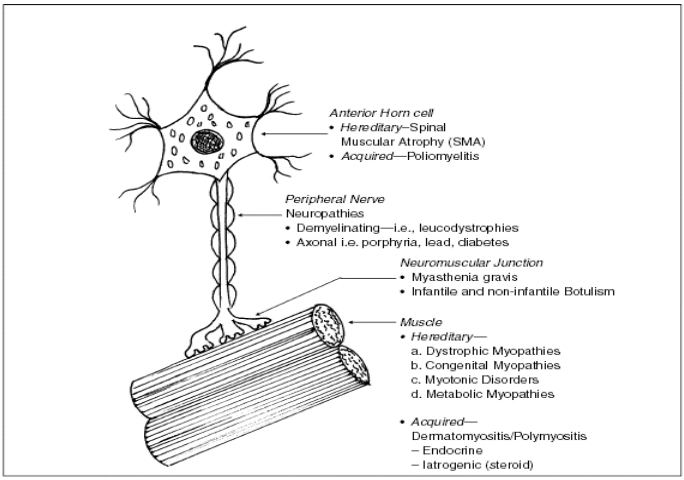

Figure 1 Anatomic Breakdown of Disorders of the Lower Motor Neuron.

The given condition can be either genetic or acquired. From the practical point of view, the anterior horn cell has got two separate disorders. Spinal muscular atrophy is the genetically determined condition of the anterior horn cell, whereas poliomyelitis is acquired. The genetic disorders of the peripheral nerve are named as hereditary sensory motor neuropaties, which could be

seen with mendelian and mitochondrial DNA inheritance. These are either axonal or demyelinating. The acquired demyelinating neuropathy is autoimmune based. If the latter condition is axonal, then toxic causes should be investigated. Myastenias overall encompass about 2% of neuromuscular disorders in childhood with majority being the congenital forms. Anatomically, congenital myasthenic syndromes are classified according to the site of the abnormality being pre-synaptic, synaptic or post-synaptic. Slow channel syndrome is seen less frequently than the other forms. The disorders of the primary muscle tissue can be divided into muscular dystrophies, congenital myopathies, metabolic conditions and acquired diseases. Juvenile dermatomyositis, iatrogenic myopathies and endocrine myopathies are typically acquired, where as the remaining are hereditary.

These group of disorders are diagnostically challenging as many of them also pose other systemic findings. One example would the commonly seen mental retardation in Duchenne muscular dystrophy. A detailed family history is mandatory. Physical stigmata such as localized atrophy or hypertrophy must be noted along with the functional evaluation, although the overlap of clinical phenotype among different categories, which includes common and not specific symptoms, makes the diagnosis tricky.

Recently the standard diagnostic pathway has largely moved on from muscle biopsy analysis to genetics, even though mutation analysis remains complex owing to the large numbers of genes involved and the challenges of the latest generation of sequencing techniques, which are still not yet in routine use and

which produce vast amounts of data that are impossible to analyse without guidance from the phenotype, as the large numbers of variants found in every patient require interpretation of clinical relevance to discover the pathogenic change. Muscle imaging, especially magnetic resonance imaging, is exceptionally helpful and depict often the most important tool in this task.

1.1. Distal hereditary motor neuropathies

The distal hereditary motor neuropathies (dHMN) are a phenotypically and genetically heterogeneous group of diseases affecting lower-motor-neurons and characterised by slowly symmetrical progressive distal limb weakness (1). They are also termed distal spinal muscular atrophies (dSMA). There is frequently an overlap of clinical findings in dHMN and other neurogenic conditions, like hereditary motor and sensory neuropathy or proximal spinal muscular atrophy. Many of dHMN are, in fact, allelic with subtypes of Charcot Marie Tooth 2 (CMT2) disease with the principle distinction being mild to moderate sensory nerve involvement in CMT2 (1).

The cardinal feature of dHMN is usually a very slowly progressive length-dependent condition often starting in the first two decades; onset in the third decade is, however, not uncommon. Poor performance in sports at school and insidious progression are useful clues, whereas a short, de novo history in middle age should prompt a search for an acquired aetiology. Bulbar involvement, other than the recurrent laryngeal nerve, is rare in dHMN. The examination, as expected, usually confirms distal wasting and weakness with reduced or absent reflexes, and neurophysiology highlighted reduced motor amplitude potentials associated with EMG changes suggesting chronic distal predominant denervation (2).

Using this approach, a significant proportion of patients classified as dHMN will be ‘sporadic’ with no obvious family history and in this case a dominant mutation is assumed. Once the hereditary nature of the disease is established, delineation of clinical and

neurophysiological phenotype is the most efficient way to approach genetic tests. Neurophysiological studies are useful to differentiate CMT2 from dHMN; electromyography is not only used to confirm denervation but also in differentiating dHMN from a distal myopathy. However, the yield remains low, as more than 80% of patients with dHMN have mutations in undiscovered genes; early detection of these condition is so not so easy and more gene have to be test before perform a correct diagnosis, with negative implication in genetic counselling (2). The causative genes have implicated proteins with diverse functions such as protein misfolding (*HSPB1*, *HSPB8*, *BSCL2*), RNA metabolism (*IGHMBP2*, *SETX*, *GARS*), axonal transport (*HSPB1*, *DYNC1H1*, *DCTN1*) and cation-channel dysfunction (*ATP7A* and *TRPV4*) in motor-nerve disease (2). At present, we are able to recognise seven categories of dHMN and other four forms with associated specific features. These conditions are inherited in autosomal dominant or recessive pattern and only one form is X-linked; some genes are associated with sensitive involvement and are linked to CMT disease (2). Type I and II are typical distal motor neuropathies beginning in the lower limbs and presenting in either childhood or adulthood, respectively. Both can be due to mutations either *HSPB1* or *HSPB8*, demonstrating that these phenotypic categories are genetically heterogeneous. If sensory involvement is present, the disease is termed CMT2F if it is due to mutations in *HSPB1* and CMT2L if the mutations reside in *HSPB8*. dHMN with pyramidal signs can be due to mutations in *BSCL2* and *SETX* and they have also been linked to three separate loci 9p21.1p12

(HMN-Jerash), 7q34q36 and 4q34.3q35.2. *BSCL2* is also allelic to spastic paraplegia type 17 (*SPG17*, Silver syndrome). Type V is characterised by upper-limb onset and can be due to mutations in *BSCL2* or *GARS*: if it is due to a mutation in *GARS* and there is also sensory involvement, it is termed CMT2D. Type VII is associated with vocal cord paralysis and can be due to mutation in *DCTN1*, *TRPV4* or in an yet unidentified gene on chromosome 2q14.6. dHMN types III, IV and VI are autosomal recessive distal motor neuropathies. Type III and IV have been linked to the same loci and are chronic forms of dHMN. They are differentiated by the presence of diaphragmatic palsy in type IV. Type VI occurs in infancy and it is characterised by distal weakness and respiratory failure. It is due to mutations in the gene IGHMBP2 (1-2).

The overlap between clinical and genetic findings makes difficult the differential diagnosis in this heterogeneous group of diseases.

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1.2. Congenital Myopathies

The congenital myopathies (CM) are a distinct and markedly heterogeneous group of muscle disorders which are clustered into subtypes based on the predominant pathological features revealed on muscle biopsy.

The clinical presentation includes congenital hypotonia, delayed motor milestones, feeding difficulties, muscle weakness, facial muscle involvement, and ophtalmoplegia, although certain features are more frequently associated with distinctive conditions. Serum creatine kinase values are generally normal or slightly raised and the course of the disease is essentially static. In addition to congenital onset, there is heterogeneity in the onset as well as the clinical course of the disease, even among affected members from the same family. Together with progressive or slowly progressive weakness there are patients with a more severe course, and still others with late onset or almost asymptomatic presentations (1).

In the last decade, several new genes and proteins responsible for individual forms of CM have been identified and the classification has expanded considerably. Moreover some advances have also started to explain the pathological mechanisms of these disorders, providing the basis for therapeutic strategies (2, 3).

More specifically it has been found that:

1) mutations in the same gene can produce different pathological features, affecting different pathogenetic pathways within muscle sarcomere. For example, mutations in alpha−skeletal actin can cause nemaline myopathy, myopathy with cores and congenital fibre

type disproportion (CFTD). Mutations in alpha−tropomyosinSLOW can result in both nemaline myopathy and CFTD. Mutations in beta−tropomyosin can result in nemaline myopathy and cap disease, and mutations in selenoprotein N can result in multiminicore disease and CFTD, as well as dystrophic pathology.
In addition, the same mutation can cause different pathological features also in members of the same family or in the same individuals at different ages, suggesting that modifyng genes or environmental factors may influence the pathological appearance of the muscle.
2) the same clinical−morphological phenotype can be associated with mutations in different genes. Nemaline myopathy, for example, is currently associated with seven known genetic loci. Mutations in several different genes can cause core pathology and CFTD.
Therefore, although clinical symptoms together with the muscle biopsy and the imaging of the muscle group degeneration pattern are able to define an increasing number of specific conditions, the genetic confirmation of the protein defect is essential for definitive diagnosis and reasonable taxonomy.
As such, a new classification based on the morphological−genetic correlation has been proposed (2, 3):
−Myopathies with protein accumulation
−Myopathies with cores
−Myopathies with central nuclei
−Myopathies with fibre size variation

However, most of the disorders are genetically heterogeneous, firm genotype−phenotype relationships

remain as yet unclear, and further genes have to be found.

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1.3. Congenital Muscular Dystrophies

Classically the term congenital muscular dystrophy (CMD) includes a group of genetically, clinically and biochemically distinct entities sharing clinical and pathological features such as early presentation of weakness and hypotonia and dystrophic features on muscle biopsy. In the last few years the identification of several new genes responsible for different forms of CMD has not only expanded the spectrum of the known forms of CMD but has produced exciting progresses in the understanding of the mechanisms underlying this group of disorders.

The incidence and prevalence of CMD in populations is not sufficiently known. Part of this uncertainty stems from the limited diagnostic means available in the early 1990s at the time when patients were initially ascertained for some of the larger surveys. The range of point prevalence that can be gleaned from these studies ranges from 0.68 to 2.5 per 100,000, which could still be an underestimate (1-4). The relative frequency of individual types does vary in different populations depending on the respective genetic background. For instance, in Japan the most commonly diagnosed form is Fukuyama CMD caused by a founder mutation in fukutin, followed by collagen VI deficient CMD (5), whereas fukutin mutations are very rare in other populations (4).

Most of these studies were performed before the recent advances and identification of new genes that have changed the scenario of CMD.

To date over 20 genes responsible for CMD forms have been identified. These forms can be classified into five

main groups according to clinical, pathological and genetic data:

1. CMD due to mutations in genes encoding structural proteins of the basal membrane or extracellular matrix of the skeletal muscle fibers. These include forms due to mutations in the genes encoding collagen VI, laminin alpha 2 (merosin) and integrin alpha-7 and integrin alpha-9.
2. CMD due to mutations in genes encoding putative or demonstrated glycosyltransferases, which affect the glycosylation of alpha-dystroglycan. These include FCMD, MEB and WWS, and other forms with normal brain MRI, with and without mental retardation.
3. CMD with rigidity of the spine, due to mutations in the SEPN1 gene, which encodes selenoprotein 1, an endoplasmic reticulum protein of unknown function.
4. CMD due to abnormalities of nuclear envelope proteins (LMNA and nesprin),
5. CMD with mitochondrial structural abnormalities (CMDmt).

This classification only includes forms of CMD in which the primary genetic defect has been identified, but there are several other forms with distinctive phenotypes in which the underlying defect has not yet been identified.

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2 Muscle MRI: a useful tool for differential diagnosis of muscular disorders

In the past few years, muscle Magnetic Resonance Imaging (MRI) has become an important tool in the detection and characterization of pathological alterations of skeletal muscle that cause changes in muscle signal intensity, becoming an additional tool for diagnosing inherited neuromuscular disorders (1). Following the description of a short muscle MRI protocol to be used in young patients, MRI has become suitable also for paediatric population (2).

The use of this technique has mainly been devoted to identifying patterns of muscle involvement and has revealed patterns of selective involvement that in a number of cases are fairly disease-specific. Exactly why certain muscles are spared from pathology despite the fact that the proteins involved are ubiquitously expressed is a question that has still not been adequately answered. Nevertheless, the characterization of these patterns has helped to improve the diagnostic work-up of patients with neuromuscular disease, guided genetic testing and helped in the differential diagnosis of muscle disorders with overlapping of clinical features (1); the identification of a typical muscle MRI pattern aids also the often challenging task of assigning pathogenicity to those novel genetic variants with uncertain significance.

More recently, the new challenge has become the possibility to use muscle MRI not only for diagnosis but as an outcome measure, trying to quantify muscle size, signs of inflammation, fatty replacement or, using fat suppression, other causes of abnormal signal (3). Other

studies have also explored whether the correlation between muscle histology and MRI changes could aid to better understand the alteration arising in the muscle during the course of the disorder and to acquire more specific information useful for clinical trial (4).

Although muscle MRI can provides an excellent non-invasive visualization of anatomical structures, such as nerves, MRI studies have been focused on a limited series of CMT patients including cases with CMT1A duplication (5) or CMT2 associated with *DNM2* mutations (CMT2/*DNM2*) (6) or CMT2A associated with *MFN2* mutations (CMT2A/*MFN2*) (7). Too few studies have been carried out on evaluation of specific pattern in dSMA (8) or on identification of different markers able to differentiate myopathic forms from neurogenic ones.

The knowledge in this field are rapidly improving and new information are likely to be achieved using more advanced MRI techniques.

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Chapter 2
Clinical and genetic studies

1 Clinical and genetic characterization of distal spinal muscular atrophy

Inherited forms of slowly progressive neurogenic weakness and wasting may be labeled, on the basis of nerve conduction involvement, as distal spinal muscular atrophy (dSMA), when nerve conduction function is spared, distal hereditary motor neuropathy (dHMN), when the involvement is only motor, or axonal Charcot-Marie-Tooth (CMT2), when both sensory and motor nerves are affected. However, in the “exome era”, the rapidly growing list of mutated genes (http://neuromuscular.wustl.edu/), high levels of allelic and genetic heterogeneity, and overlapping of conditions are tending to limit the usefulness of precise clinical-nosographic labels.

The recent identification of mutations in the gene encoding transient receptor potential vanilloid 4 (TRPV4) in distal spinal muscular atrophy (dSMA) prompted us to screen for mutations a small group of children with a compatible phenotype.

1.1 TRPV4 mutations in children with congenital distal spinal muscular atrophy

by Chiara Fiorillo, Francesca Moro, **Guja Astrea**, Claudia Nesti, Giacomo Brisca, Zoltan Balint, Andrea Olchewsky, Christian Guelly, Michaela Auer-Grumbach, Roberta Battini, Filippo M. Santorelli and Claudio Bruno

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Introduction

Congenital distal spinal muscular atrophy (dSMA) (MIM 600175) is a lower motor neuron disease with prenatal onset, autosomal dominant inheritance, and variable clinical severity ranging from muscle weakness predominantly affecting the lower limbs to more severe neurogenic muscle weakness and arthrogryposis. A form of dSMA is associated with mutations in transient receptor potential vanilloid 4 (*TRPV4*) — the gene encoding a calcium-permeable ion channel of the vanilloid subfamily — that operate through a loss-of-function mechanism (1). *TRPV4* also determines two allelic disorders, scapuloperoneal SMA (SPSMA, MIM 181405) and hereditary motor and sensory neuropathy 2C (HMSN2C, MIM 606071) (2, 3, 4, 5). Although also dominantly transmitted and involving preferentially the distal legs, they show unique features: SPSMA presents also scapular weakness and atrophy, whereas HMSN2C combines motor and sensory involvement, diaphragm and intercostal muscle weakness, and hearing loss. However, overlap phenotypes and intrafamilial variability between and within kindred harboring the same mutation have been described (1-4). On top of this, dominant mutations in

TRPV4 also cause clinical conditions affecting the bones — without signs of primary muscle weakness — ranging from mild brachyolmia and spondylomethaphyseal dysplasia to lethal neonatal metatropic dysplasia (6). Type of the mutations and location within the protein domains are believed to account for this wide clinical heterogeneity.
Reviewing our pooled databases of dSMA patients, we screened six apparently sporadic patients for variants in *TRPV4* and detected two mutations, including a novel variant.

Material and methods

Three girls and three boys (age range 2-21 years, median age at onset 2±2, median age at latest examination 15.5±7) were selected because their clinical characteristics satisfied the clinical and electrophysiological criteria of dSMA. In particular, there were neurogenic muscle weakness and atrophy in lower (in all patients) and upper limb (in two), congenital arthrogryposis (in three cases), scoliosis (in four), and vocal cord paralysis (in a single child).
EMG and muscle biopsy examinations confirmed a neurogenic damage of muscle and nerve conduction studies were normal in all. Respiratory and cardiac functions were normal. No patient displayed involved of CNS nor sensation defect (Table 1). Mutations in the *SMN1* gene had already been ruled out.

Table 1
Clinical characteristics of patients with distal spinal muscular atrophy (dSMA) screened for mutations in *TRPV4*

Patient	Age	Molecular analysis	Phenotype	Clinical Features
1	12 yrs	c.290 C>G (p.P97R)	dSMA and vocal cord paralysis	Congenital arthrogryposis. Vocal cord paralysis. Severe scoliosis.
2	2.5 yrs	c.694 C>T (p.R232C)	dSMA and Platispondilia	Congenital arthrogryposis. Shoulder amyotrophy. Hoarse cry. Platispondilia, short phalanges, dysmorphic femoral heads.
3	19yrs	wild-type	dSMA	Pes cavus. Independent walking
4	10yrs	wild-type	dSMA	Distal leg weakness. Scoliosis. Independent walking
5	21yrs	wild-type	Scapulo-peroneal SMA	Congenital arthrogryposis. Shoulder amyotrophy. Ophtalmoplegia.
6	19 yrs	wild-type	dSMA	Pes cavus. Bladder urgency. Pyramidal signs.

The coding exons and flanking introns of *TRPV4* were amplified by PCR and bidirectionally sequenced using the BigDye v3.1 chemistry (Applied Biosystems Foster City, CA), on an ABI3500 DNA analyzer. Chromatograms were analyzed and aligned against the reference sequence using SeqScape v2.6 software (Applied Biosystems). For the description of the mutations we used the latest conventions of the Human Genome Variation Society nomenclature. New variations were systematically evaluated *in silico* to predict their effects on protein function (Polyphen analysis, genetics.bwh.harvard.edu/pph/; ESEfinder, www.rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi; www.fruitfly.org/seq_tools/splice.html). Multiple alignment with *TRPV4* orthologs used ClustalW (www.ebi.ac.uk/clustalw) to evaluate the degree of conservation of missense variants.

Immunofluorescence analysis of skin and muscle sections and fibroblasts was carried out using standard protocols. Briefly, serial 7-µm thick sections of the patients' skin and skeletal muscle biopsies were obtained from isopentan frozen samples. Human fibroblasts were obtained from patient skin biopsy and grown in DMEM medium supplemented with 10% foetal bovine serum and 4.5 g/L glucose. Tissue sections were pre-incubated with PBS+4%BSA for 10 minutes. Cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min, then blocked for 1 hour in 10% FBS. Sections and cells were then incubated overnight at 4°C with a rabbit polyclonal anti-TRPV4 antibody (1:100; Sigma-Aldrich, St. Louis, MO). diluted 1:1000 . The day

after sections and cells were incubated with a fluorescein isothiocyanate (FITC) labeled goat anti-rabbit IgG Alexa Fluor 555 (Cell Signaling-Millipore, Billerica, MA) diluted 1:1000 for 1 hr at room temperature. Nuclear counterstained was performed using DAPI (Sigma-Aldrich, St. Louis, MO). Coverslips were mounted in mounting medium and visualized with fluorescence microscope Axio Imager M2(Carl Zeiss, Oberkochen, Germany). No labeling was observed in control experiments in which primary antibody was omitted.

Cloning TRPV4_ wt and mutant TRPV4_P97R constructs in expression vectors, heterologous expression in HeLa cells, measurement of intracellular calcium concentration ($Ca^{2+}i$), and patch-clamp recordings were performed as previously described (1). Values were determined as means ± s.e.m. of number of cells. Intergroup differences for Ca^{2+} imaging and electrophysiology were assessed by a factorial analysis of variance using Fisher's least-significant-difference as *post hoc* analysis. Statistical significance was set at $p < 0.05$.

Results

In two children we identified mutations in *TRPV4*. Case 1 is a 11-years-old girl born by C-section for podalic breech. At birth, clubfoot and congenital hip dysplasia were evident, together with marked floppiness prevalent in lower limbs. There was also a bone fracture in the left femur. The patient was never able to stand up and walk without support. Her cognitive development was normal as well as facial and upper limb muscles. Sensation was never affected during a 9-year follow-up. At age 5

years, the patient developed scoliosis whereas hoarse voice manifested at age 7. Paralysis of vocal cords was documented by video-endoscopy of upper respiratory tract. Neurophysiological studies documented muscle denervation on EMG with polyphasic potentials and fibrillation at rest whereas sensory and motor conduction velocities were normal. Serum creatine kinase (CK) levels were mildly elevated (221 U/L, normal <170 U/L). A skeletal muscle biopsy confirmed a neurogenic damage of muscle, showing hypertrophy of type 1 fibers and type grouping in a severely substituted/atrophic muscle. Spine and pelvis X-ray images documented marked thoraco-lumbar scoliosis and right hip dysplasia. The latest neurological examination showed a bright young girl confined to a manual wheelchair. She daily used a static stander with ischial support and wore a brace for scoliosis awaiting spine surgery. Lower limbs were plegic with ankle, knee and hip contractures. Upper limbs appeared to be spared, though there was mild weakness in the shoulder girdle. Deep tendon reflexes were absent. Cranial nerves were unaffected but the patient spoke with a feeble and hoarse voice which worsened during minimal exercise. Laringeal stridor and suprasternal retraction were occasionally reported during more intense physical activity. Basal pulmonary function test showed nearly normal forced vital capacity (92%) and only a mild reduction of forced respiratory volume and peak expiratory flow (88% and 83%).

Case 2 is a 2.5-year-old boy born at 39 weeks with bilateral talipes and fractures of right clavicle and left femur. At 3 months he underwent surgery for partial correction of foot deformation. The patient was able to

control his head and could sit without support but he was never able to stand nor to walk. Gastrostomy was performed for severe gastroesophageal reflux at age 2 years. The latest neurological examination when he was age 2.5 years showed severe involvement of lower limbs with complete paralysis and moderate atrophy. Only distal movement of toes were possible. Initial knees retraction were present. The child could not raise his legs from bed and sat with a kyphotic posture. There was minimal shoulder weakness and bilateral tremor of hands. Deep tendon reflexes were reduced in all limbs. Cognition appeared to be normal; the boy was alert and smiling. Full range of ocular movement was possible. Crying sounded hoarse. EMG study revealed severe neurogenic damage. Nerve conduction studies, EEG and brain and spine MRI studies were all normal. CK was only mildly elevated (211 U/L, normal <170 U/L). A first muscle biopsy at age 12 months showed diffuse fatty replacement whereas a second biopsy at age 18 months detected variability of fibre size with selective atrophy of type 2 fibres, consistent with denervation. X-rays imaging documented vertebral abnormalities consistent with platispondilia, short and stubby phalanges and dismorphic features of the femoral head. By direct gene sequencing, we identified a novel heterozygous c.290C>G variant in exon 2 of *TRPV4* in case 1. The mutation replaces arginine for a highly conserved proline at residue 97 (p.P97R) in the cytosolic N-terminus of human *TRPV4*. The mutation was not detected in 200 control chromosomes and it was absent in genomic DNA from the healthy parents and a younger brother. In case 2, we detected an already reported c.694 C>T (p.R232C) in exon 4. This

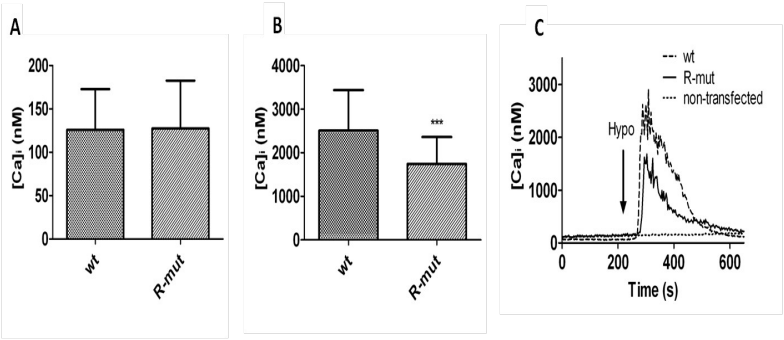
mutation has been described in nine patients from three families, and associated with dSMA and vocal cord paralysis in one family, dSMA and SPSMA in another kindred, and a HMSN2C clinical phenotype in the third family (4).

None of the remaining cases presented disease-related variants in *TRPV4*.

In muscle sections from case 1, we observed a faint and diffuse TRPV4 immunoreactivity alike control muscles. In skin sections from both patients, there was a strong staining in keratinocyte layer, endothelium and nerve endings as expected (7) without significant differences between patient and control. No relevant differences of expression or signal localization were detected between patients and controls in cultured skin fibroblasts.

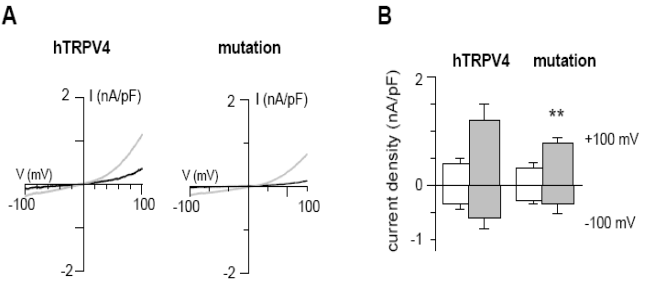
To evaluate the possible functional consequences of the novel p.P97R mutation, we expressed heterologously both mutant and wild-type alleles in HeLa cells. This system was preferred because HeLa miss detectable levels of the endogenous protein (1). Having confirmed genuine human *TRPV4* expression (1), we found that basal calcium levels did not significantly differ among transfected cells whereas TRPV4_P97R had a significantly lower response to intracellular calcium increase when challenged with hypo-osmotic solution ($p<0.001$) (Figure 1). Also, the mutant channels yielded significantly smaller currents (90% reduction) in patch-clamp studies when subjected to a hypo-osmotic challenge with reduction of extracellular osmolarity from 320 to 200 mOsm (Figure 2) ($p < 0.001$).

Figure 1



Intracellular calcium changes of *TRPV4*-transfected HeLa cells. (A) There was no difference in the basal calcium level of the transfected cell. (B) The hypoosmotic solution–induced calcium response was significantly reduced in the mutant compared to wild-type. (C) Original traces show calcium response during hypoosmotic challenge in HeLa cells transfected with wild-type or mutant *TRPV4* channels (dashed line, wild-type hTRPV4; continuos line, R-mut). Control (dotted line) indicates a mock-transfected cell. (*** $p<0.001$, nexp = 7, ncells = 37 for the wildtype, ncells = 35 for the mutant). wt, wildtype construct; R-mut, construct presenting the p.P97R variant.

Figure 2



Effect of substitutions on TRPV4 activation by hypo-osmotic swelling. (A) Effect of hypo-osmotic challenge on wild-type hTRPV4 or mutant p.P97R -transfected HeLa cells in response to a ramp protocol. (B) Pooled data from the same series as shown in A. Average basal inward and outward currents at −100 mV and +100 mV of HeLa cells expressing hTRPV4 and mutants p.P97R ($n \geq 4$). ** indicates significant differences compared to cells expressing hTRPV4 ($p<0.01$). Basal values are shown by white bars.The values of the hypo-osmotic challenge are indicated by gray bars. Mock-transfected cells were used as a control

Discussion

Upon screening of *TRPV4* in a small group of dSMA children, we identified a child bearing a reported p.R232C mutation in the ankyrin repeat domain (ARD) — a region thought to be important for oligomerization and proper trafficking to the plasma membrane (7) — and a novel variant in the N-terminus of the protein. The pathogenicity of the new p.P97R is sustained by the following considerations: i) the mutation arose *de novo* in the family and it was not found in a large set of control chromosomes; ii) it possibly interferes with the nearby proline-rich region (PRD), though to be involved in mechanosensation and interaction with other proteins (8); iii) when expressed in mutant HeLa cells, the p.P97R causes a statistically significant loss of function mechanism when challenged by hyposmotic solution. Conversely, there was no abnormal expression or mislocalization of the mutant protein when tested in patient's tissues suggesting that proline 97 might be relevant for correct response to different stimuli.

The present report adds to the genetic heterogeneity of *TRPV4*-pathies but has also relevance in terms of genotype-phenotype correlations. It is of interest that case 2 in this work, who carried a mutation in the ARD domain, also presented a well defined primary bone pathology documented by X-ray scans. This finding is seldom seen in children with distal muscular weakness and it is rather associated with dominant mutations outside the ARD domain (9). Contrariwise, case 1, bearing a mutation outside the ARD domain, manifested unspecific skeletal abnormalities such as congenital fracture, hip dysplasia, scoliosis and fixed joint contractures without sustaining images. Thus, the

first conclusion that can be drawn is that a clear-cut molecular correlation between type of mutation, position of the variant in protein domains and the resulting phenotype in *TRPV4*-pathies can be hardly proposed. It seems more reasonable to hypothesize that global effects on the many channel functions — yet to be explored—account for the clinical presentation. Other genetic or epigenetic factors might account as well for inter- and intrafamilial variability.

In our work, both children having mutations in *TRPV4* presented laryngeal stridor, with a documented vocal fold paralysis in the eldest and more severe patient. None of the remaining cases screened for *TRPV4* variants showed vocal cord involvement nor hoarse voice or cry. Involvement of laryngeal muscles is not frequent in hereditary motor-neuropathies but it is seldom associated with recessive mutations in the ganglioside induced differentiation-associated protein 1 gene (10), with dominant variants in the early growth response 2 gene (11), dynactin 1 (12), and in a form still awaiting gene cloning on chromosome 2q14 (13). Clinical differences between the aforementioned phenotypes and dSMA or SPSMA are easyand straightforward (14). A second conclusion of the present work is that presence of complete vocal cord paralysis or hoarse voice should alert on a possible *TRPV4*-pathy, especially when a subtle or manifest bone involvement is present. Interestingly, *TRPV4* channels are also expressed in the epithelium of upper respiratory tract airways including trachea and larynx (15) but it remains uncertain the relationship with vocal cord paresis.

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1.2 A novel mutation in DYNC1H1 bridges the gap between motor neuropathy and neuronal migration defect

by **Guja Astrea**, Chiara Fiorillo, Francesca Moro, F.M. Santorelli, R. Battini

manuscript in preparation

Introduction

Mutations in *DYNC1H1*, encoding the heavy chain protein of the cytoplasmic dynein 1 motor complex, which plays a key role in retrograde axonal transport, have recently been reported in a large autosomal dominant axonal CMT (CMT-2O) (1) family and in three families with congenital SMA-LED (2). *De novo* variants have also been detected in two unrelated cases with severe intellectual impairment, abnormal lobe gyration, and cortical dysplasia (3).

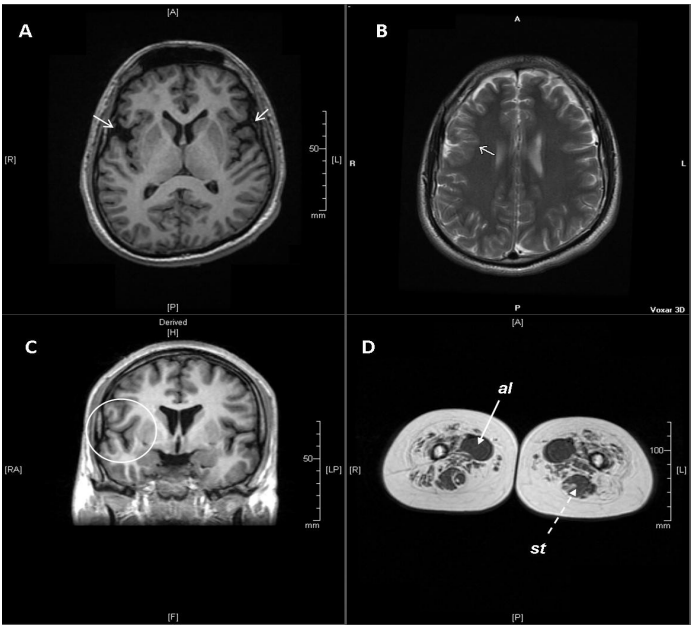
We are going to describe clinical, molecular and imaging findings in a young man with features of congenital dSMA, global developmental delay, and abnormal lobe gyration. Due to recent clinical findings and mice model observations, we performed full gene scanning of *DYNC1H1* discovering a novel mutation.

Case report

This 19-year-old patient presented at birth with bilateral foot malformation and amniotic bands affecting the lower limbs. Global development was delayed: he walked independently at 22 months and spoke his first words at 36 months. Neurological examination at 5 years disclosed lower limb weakness with waddling gait and positive Gowers' maneuver. Tendon reflexes were generally brisk with ankle clonus, while patellar reflexes

were reduced. The plantar response was extensor. Electromyography showed increased amplitude and frequency of motor unit potentials, whereas nerve conduction velocity (NCV) studies were normal. A muscle biopsy confirmed the presence of neurogenic damage. The latest neurological examination, at age 19 years, showed waddling gait with *pes cavus*. KAFO orthoses were worn to support ambulation. Hyperlordosis and right curve scoliosis were also present. The lower limbs were weak and distally atrophied, and appeared disproportionately short compared to the trunk and upper limbs, which were spared. Patellar reflexes were reduced, ankle clonus was present bilaterally and plantar responses were mute. The patient reported painful paresthesia. Vibratory but not touch sensation was severely impaired in the lower limbs. Mild cognitive impairment was detected, especially in verbal skills. Depression and generalized anxiety disorder were also present. An electroencephalogram showed nonspecific abnormalities, although the patient has never manifested seizures. NCVs were again normal. Brain MRI revealed bilateral dilatation of anterior sylvian areas as due to widening of the temporal opercula, and abnormal gyration of the right frontal lobe (Figure 1A-C). Muscle MRI strongly resembled previously reported dSMA cases (4) showing a pattern of diffuse atrophy of the thigh muscles with relative hypertrophy of the adductor longus and semitendinosus (Figure 1D).

Figure 1



Brain MRI in the patient. (**A**) Axial T_2-weighted, (**B**) Axial T_1-weighted FLAIR and (**C**) coronal T_2-weighted brain neuroimaging features in a patient presenting a novel mutation in *DYNC1H1* show slight frontal cortical atrophy, bilateral dilatation of anterior sylvian areas as due to widening of temporal opercula (arrows), and abnormal gyration of the right frontal lobe (circle). (**D**) Transverse T1-weighted MRI of thigh muscles shows extensive fatty atrophy preserving the medial compartment with hypertrophy of the adductor longus (*al*) and semitendinosus (*st*).

Molecular alterations in known genes associated with motor neuropathies as well as in *SMN1* had been ruled out during the disease course. We performed full gene scanning of *DYNC1H1*, and identified a heterozygous c.3581A>G/p.Q1194R variant. The novel mutation lies in the N-terminus tail of cytoplasmic dynein 1; it was not detected in 500 control chromosomes nor in large SNP databases, and it arose *de novo* in the family. Alamut 2.1 (Interactive Biosoftware, Rouen, France) and Polyphen2 analyses predicted the mutation to be deleterious to normal protein function.

Discussion

Cytoplasmic dynein 1 is a multisubunit motor protein that is driven along microtubules by energy derived from ATP hydrolysis. It is involved in a variety of fundamental cellular processes, including organization of the mitotic spindle, positioning of many intracellular organelles and retrograde axonal transport (5). The dynein heavy chain protein, encoded by *DYNC1H1*, forms the core of the complex, homodimerizing via its N-terminal tail domain. As in SMA-LED and CMT-2O, the p.Q1194R is located in the tail domain, also thought to serve for binding associated proteins. One such protein is LIS1 whose haploinsufficiency causes severe human neuronal migration disorder (6). Thus, the combination, documented in our patient, of neurodevelopmental abnormalities and a distinctive pattern of muscular atrophy seems to encompass the different features of the peripheral and the central nervous system (CNS) involvement to date associated with mutated *DYNC1H1* in human and mouse models.

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2 Identification of a new form of Neutral Lipid storage Disease

Neutral lipid storage diseases (NLSDs) are multisystem lipid disorders due to a defect either in the adipose triglyceride lipase (ATGL) or in the alpha/beta-hydrolase domain-containing protein 5 (ABHD5) (1,2). ABHD5 activates ATGL, which catalyzes the first step in the hydrolysis of triacylglycerol to produce free fatty acid and diacylglycerol. Mutations in PNPLA2, the gene encoding ATGL, cause NLSD with myopathy (NLSDM, OMIM # 610717) (3), whereas mutations in the CGI-58 gene, coding for ABHD5, cause NLSD with ichthyosis (NLSDI), also termed Chanarin–Dorfman syndrome (CDS, OMIM # 275630) (2,4).

The hallmark of both diseases is the presence of neutral lipid droplets storage in multiple tissues, including skeletal muscle and leukocytes.

Here, we report clinical, morphological, MRI and genetic findings of a boy carrying mutations in PNPLA2 and massive lipid storage in a muscle biopsy without overt muscle symptoms except for persistently markedly elevated serum CK levels. These findings show the importance to pay more attention on every clinical sign and confirm the role of muscle MRI for early muscular diagnosis.

2.1 Subclinical myopathy in a child with neutral lipid storage disease and mutations in the PNPLA2 gene

*by Chiara Fiorillo, Giacomo Brisca, Denise Cassandrini, Sara Scapolan, **Guja Astrea**, Maura Valle, Francesca Scuderi, Federica Trucco, Andrea Natali, Gianmichele Magnano, Elisabetta Gazzerro, Carlo Minetti, Marcello Arca, Filippo M. Santorelli, Claudio Bruno*

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Introduction

CDS/NLSDI is characterized by the presence of ichthyosiform nonbullous erythroderma. Slowly progressive weakness of proximal limb muscles with raised serum muscle enzymes can be detected in about 60% of cases. Hepatomegaly, various ocular symptoms (cataract, nystagmus, and strabismus), hearing loss, mild mental retardation, short stature, microcephaly and intestinal involvement are also described (4,5).

Patients with NLSM start complaining of limb weakness at the beginning of the third decade of life despite lipid accumulation most likely precedes the clinical symptoms (6,7). Serum CK is always highly elevated (up to 50-fold normal values) and occasionally can be the only sign in childhood as seen in an asymptomatic 18-year-old-girl (8).

The course of the disease is slowly progressive and cardiomyopathy may develop at later stages in half of the patients. No ichthyosis is present and neither the central nor the peripheral nervous systems are involved (1,8).

Case report

The propositus is a 14-year-old boy, the first child of healthy nonconsanguineous Italian parents.

He was born at term by normal delivery after an uncomplicated pregnancy. His motor milestones were normal.

At five years of age he was referred to our Center for additional evaluation of elevated levels of serum CK discovered during a routine blood test. Family history was negative for neuromuscular disorders. His younger brother is healthy.

General physical examination was normal and the patient denied the occurrence of myalgia or cramps. Neurologic examination did not reveal any muscle weakness or CNS impairment. Muscle tone, and strength were normal for age and there was no calf hypertrophy or contractures. Serum CK was 1200 U/l (normal values less than 150), while routine laboratory investigations and screening for metabolic disorders were normal. Electromyography of the anterior tibialis muscle showed myogenic signs, while nerve conduction studies were normal. EKG was normal.

In keeping with the national guidelines on the diagnostic approach to asymptomatic hyperCKemia (9), the patient underwent a muscle biopsy after obtaining parental informed written consent.

The biopsy specimen was taken from the quadriceps muscle and processed according to standard techniques for routine histology and histochemistry. We detected multiple neutral lipid vacuoles in both fibers type I and II. Neither necrotic or ragged-red fibers nor rimmed vacuoles were observed. Glycogen was normal.

Total and free carnitine dosage in blood and in muscle homogenate was normal. Carnitine palmitoyltransferase

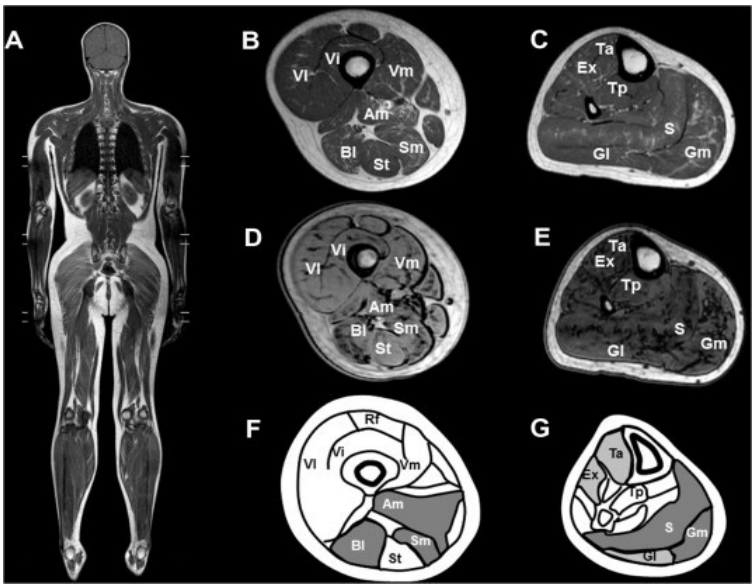
(CPT) determination and respiratory chain enzyme analysis in muscle homogenate were normal.
In the following years, the patient had variably, but persistently elevated serum CK levels. His clinical picture remained stable and muscle performances were in keeping with his peers.
Routine laboratory investigations, including cholesterol and triglycerides, were again normal. Peripheral blood smear showed the presence of lipid droplets in the patient's leukocytes. Liver and heart ultrasound were normal, as well as brain MRI spectroscopy. Oral test glucose was normal whilst euglycemic clamp showed a severe hepatic and a mild peripheral insulin resistance.
MRI of skeletal muscle, including whole body coronal T1-weighted, axial T1-weighted sequences in lower girdle muscles, thighs and lower legs, and in-phase and out-of phase scans of the legs, was performed on a 1.5-T MR system (Achieva Intera, Philips Medical System, Eindhoven, Nederland) using standard protocols (7,10).
Genomic DNA was obtained from peripheral lymphocytes using a standard salting-out technique. The entire coding regions and the exon–intron boundaries of the PNPLA2 gene were PCR-amplified, and directly sequenced using BigDye 3.1 chemistry on a multicolor fluorescence-based DNA analysis system (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA).

Results and discussion

A muscle biopsy performed in an asymptomatic child with elevated levels of serum CK when he was 5-years-old, showed a lipid storage myopathy. Dosage of free and total carnitine, carnitine palmitoyltransferase and respiratory chain enzymes were all normal.

At that time, the clinical condition remained undiagnosed. In the following years, the identification of the PNPLA2 gene (3) prompted us to revaluate the diagnostic approach. A simple peripheral blood smear revealed the presence of lipid accumulations in granulocytes (the so-called Jordans anomaly) leading to a possible diagnosis of NLSM. Moreover, muscle MRI images in our patient were similar to previous report (7) and consistent with a prevalent fatty degeneration of gluteal muscles and posterior thigh and leg muscles with a relative sparing of the anterior compartments, further contributing to define a selective pattern of muscle involvement in NLSM (Figure 1).

Figure 1



(A) Coronal T1-weighted slices showed prominent fatty degeneration in gluteal muscles, posterior thigh and posterior leg muscles with sparing of upper girdle muscles. (B) At thigh level, MRI showed predominant involvement of the posterior compartment with marked fatty infiltration of adductor magnus (Am), semimembranous (Sm) and biceps femoris long head (Bl). Quadriceps muscles (Rf, Vl, Vi, Vm) and semitendineous (St) appear to be spared. (C) At lower leg level moderate fatty infiltration of medial gastrocnemius (Gm) and soleus (S) in the posterior compartment was evident with hypotrophy of lateral gastrocnemius (Gl). Mild hyperintense signal changes were present in anterior compartment muscles, tibialis anterior (Ta) and extensor longus digitorum and hallucis (Ex) with sparing

of tibialis posterior (Tp). (D and E) Using out-of phase imaging, at thigh and leg level fatty degeneration is reflected by loss of signal intensity in the same muscles. In anterior compartment of the leg, more evident abnormalities in anterior compartment (Ta, Ex), compared to T1 sequences, were present. (F and G) Patterns of muscle involvement in neutral lipid storage disease with myopathy (NLSDM), based on the literature data and our experience. The replacement of muscle tissue by fat is reflected by the dark gray color of the muscle. Initial and/or variable involvement of the muscle is represented by the light gray color. The white color means spared muscles.

However, in our child, minimal changes in anterior compartment of the leg were already detectable in an early and asymptomatic stage of the disease. These findings were highlighted using out-of-phase scans so underlying the importance of this technique in lipid storage myopathies (11). Expectedly, analysis of PNPLA2 in the patient's genomic DNA detected two heterozygous mutations, a nonsense C>T mutation at nucleotide 865 (c.865C>T) in exon 7, predicting premature protein truncation at codon 289(p.Q289X) on the paternal allele and a novel missense mutation at nucleotide 424 (c.424A>T, p.N142Y) in exon 4 on the maternal allele (Fig. C). The latter variant was scored to be predictably deleterious to protein function in silico (Polyphen2, genetics.bwh.harvard.edu/pph/), and was absent in 200 healthy Italian chromosomes or in large SNP databases (NHLBI Exome Sequencing Project, evs.gs.washington.edu/EVS/).
To the best of our knowledge, NLSM has been reported in 22 cases, and 17 mutations have been identified

throughout the entire PNPLA2 gene (3,7,8,12-19). On clinical ground, the NLSDM phenotype is characterized by proximal, often asymmetric, muscle weakness in both upper and lower girdle muscles starting in early 30's, a presentation highly resembling limb girdle muscular dystrophies (LGMD). Distal involvement of finger extensors and foot flexors can manifest later in the disease course. In all the patients serum CK is elevated (around 5-fold) and laboratory investigations might reveal hypertriglyceridemia. A few cases develop diabetes mellitus. To date, one case of NLSM has been reported at young age, namely in a 18-year-old-girl that was asymptomatic for myopathy presenting only elevated CK levels (8). Here we report a NLSDM case detected in childhood without clinically evident myopathy but only hyperCKemia. This finding may further support the notion that hyperCKemia is the early sign of NLSDM in childhood preceding the manifestation of clinical overt myopathy.

PNPLA2 gene encodes for ATGL, a hormone-sensitive lipase that catalyzes the initial step in triglyceride hydrolysis in mammalian adipose tissue. At its N-terminal, ATGL contains a 'patatin domain' common to the acyl-hydrolases while at the C-terminal it displays an hydrophobic region responsible to bind the lipid droplets (3). Most of the reported mutations of PNPLA2 gene are nonsense and affect the C-terminal domain of the gene (residues 252–504) and particularly the hydrophobic region (lipid binding domain residues 309–391). Interestingly, our patient carried a novel missense mutation in the patatin-like phospholipase domain (PLPD) (residues 10–179), and a nonsense in the C-terminus. The latter has been previously reported

in homozygosis in an Algerian family in association with typical late-onset lipid storage myopathy and classical distribution of muscular weaknesses, In contrast, only two mutations (8,12) have been identified in the PLPD domain which affects the lipase activity, Given the limited penetrance observed in our patient, and in the absence of more functional tests, we can only speculate that the new c.424A>T/p.N142Y alone or in combination with other yet unknown factors – including the patient's genetic background – could protect his muscle from being weak and atrophic notwithstanding the accumulation of lipids, possibly increasing the function of the ATGL protein coded from the non-truncated allele.

Although no effective therapy is currently available for NLSM, encouraging results have recently been showed in patients' cells upon treatment with a beta-adrenergic agent, thought to activate alternative pathway of triglyceride metabolism (7).

In conclusion, we have genetically characterized a 14-year-old boy with hyperCKemia and a subclinical myopathy due to neutral lipid storage.

Search for lipid droplets in peripheral leukocytes and the PNPL2 gene analysis should be added to the diagnostic work-up of patients with hyperCKemia (20).

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3 Difficult orientation approach in CMD-LGMD: the genetic point of view

Congenital muscular dystrophy (CMD) is a clinically and genetically heterogeneous group of inherited muscle disorders. Muscle weakness typically presents from birth to early infancy.

The main CMD subtypes, grouped by involved protein function and gene in which causative mutations occur, are laminin alpha-2 (merosin) deficiency (MDC1A), collagen VI-deficient CMD, the dystroglycanopathies (caused by mutations in POMT1, POMT2, FKTN, FKRP, LARGE, POMGNT1, and ISPD), SEPN1-related CMD (previously known as rigid spine syndrome, RSMD1) and LMNA-related CMD (L-CMD). Several less known CMD subtypes have been reported in a limited number of individuals. With the discovery of causative mutations in the last two decades, the concept of CMD has evolved from a narrowly defined clinical diagnosis (onset in the first months of life) and histologic diagnosis (dystrophic muscle on biopsy) to a more inclusive group of subtypes defined by genes in which causative mutations occur. No complete or satisfactory classification system exists; furthermore, phenotypes overlap both within CMD subtypes and among the congenital muscular dystrophies, congenital myopathies, and limb-girdle muscular dystrophies. Nonetheless, the umbrella term CMD remains useful by providing a framework for the diagnostic approach to the infant or young child with muscle weakness.

The assumptions here below are a part of a collaboration project involving Dubowitz Neuromuscular Centre (London).

3.1 Genetic breakdown in a large cohort of Congenital Muscular Dystrophies: update of the Mutation Spectrum

Astrea Guja, *Feng Lucy, Mein Rachael, Robb Stephanie, Natalie Chandler, Muntoni Francesco*

manuscript in preparation

Introduction

Congenital muscular dystrophies (CMD) are a highly heterogeneous group of conditions caused by alterations in more than 12 genes involved in several and not completely understood pathogenetic mechanisms (1).

CMD are characterized by muscle weakness since birth, or shortly after, and variable clinical manifestations affecting the eye and the central nervous system. Some of these disorders are fatal in the first year of life, whereas others have a milder course, and progression into adulthood (2).

The diagnosis of CMDs requires a multidisciplinary expertise (neurology, morphology, genetics, neuroradiology) that is available in a few centres worldwide with sufficient experience in the different CMD. Currently, achieving a full molecular diagnosis in CMD is of paramount importance not only for improved phenotype-genotype correlations, better genetic and prenatal counselling, prognosis and aspects of management, but also because of the imminent availability of clinical trials and treatments (2).

There is no complete and satisfactory classification in CMD. Following the initial definition of forms based on clinical features and country of origin, it was soon recognized a significant overlap of features and an

ample genetic heterogeneity. A more modern molecular classification recognizes forms on the basis of the involved protein function and the gene in which causative mutations occur. Thus, we consider the following subtypes: laminin alpha-2 (merosin) deficiency (MDC1A), collagen VI-deficient CMD, the dystroglycanopathies (caused by mutations in *POMT1*, *POMT2*, *FKTN*, *FKRP*, *LARGE and POMGNT1*), *SEPN1*-related CMD (previously known as rigid spine syndrome, RSMD1) and *LMNA*-related CMD (L-CMD) (3). Several less known subtypes have been reported in a limited number of individuals. However, even with current molecular tools, approximately 25-50% of patients remain without an identifiable genetic mutation and further heterogeneity is expected (4). In addition, information on incidence and prevalence of CMD is scanty because of the lack of diagnostic genetic confirmation before the identification of the several causative genes. Few studies, however, have defined a prevalence ranging from 0.68 to 2.5 per 100,000, which is probably underestimated (5). Moreover, founder mutations are known to occur, such as the insertion of a 3-kb retrotransposon element in the 3' untranslated region of fukutin (*FKTN*) reported in Japanese patients with Fukuyama-type CMD (FCMD) (6). All these data, however, provide only a limited information rather than a real figure of incidence or prevalence. With several genetic ethologies being offered in diagnostic neurogenetic laboratories, it is possible to draw more precise figures of the molecular prevalence of CMD.

This retrospective review reports the genetic breakdown in a CMD population referred for a genetic work-up at the Dubowitz Neuromuscular Centre. Our aim is to

describe the relative frequency of CMD subtypes in this patient population and the relative frequency of different type of mutations among different genes, commenting on novel mutations and summarizing the genetic variation that occurs in the CMD-associated genes.

Patients and methods

Patients

The Dubowitz Neuromuscular Centre is the UK National Commissioning Group referral centre for CMD.

About 600 DNA samples obtained from peripheral blood from UK patients regularly assessed by the Dubowitz Neuromuscular Centre or forwarded to us for a confirmatory molecular diagnosis of 'possible CMD' have been analysed between 2001 and 2011 and represented the whole study cohort. We focus our attention to data from 331 of 600 (55%) index cases in 289 unrelated families for whom a pathogenetic mutation in know CMD genes was detected. When more than one affected member of a family was available, we focus our analyses only on the proband.
Written informed consent was obtained from each family prior to testing, and this project was approved by the relevant local ethics committee.

Molecular genetic studies

Molecular genetic analysis of CMD genes was provided by Guys and St Thomas' Trust, London (part of CMD NCG service) and the NCG Referral Centre for LGMD at the Institute of Human Genetics at Newcastle University. The entire coding regions of the 11 known CMD genes (namely, *LAMA2*, *COL6A1-A2-A3*, *POMT1*,

POMT2, *POMGNT1*, *FKRP*, *FKTN*, *LARGE*, *ISPD*, *SEPN1*, *LMNA*) including splice sites, were sequenced in blood DNA in all patients.

For the patients in whom we identified mutations, we also tested available family members for the specific mutations and any variants of unknown significance (VUSs) by direct Sanger sequencing.

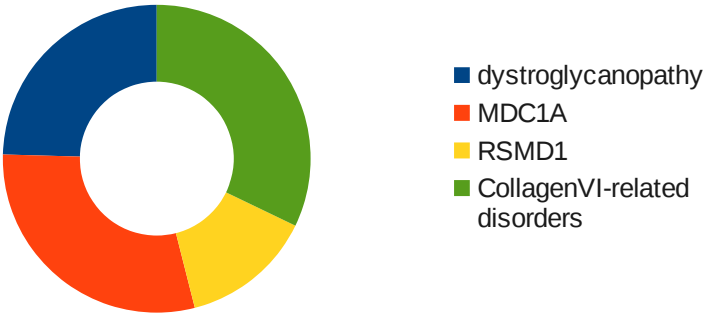
All the variants identified were investigated *in silico* using Alamut v1.5 (Interactive Biosoftware, http://www.interactivebiosoftware.com/). This software incorporates several prediction algorithms including SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer, as well as variant scoring methods — namely, PolyPhen2, SIFT, and Align GVGD. Alamut v1.5 also examines the conservation of both the nucleotide and amino acid residue across 11 species, and includes a search of previously reported variants in the literature.

Based on *in silico* findings, literature searches, and revision of data in dbSNP (http://www.ncbi.nlm.nih.gov/snp/), all variants were divided into “mutations,” “polymorphisms,” or “VUSs.” Variants were classified as “polymorphisms” if they were listed in dbSNP with an MAF (minor allele frequency) of 5% or greater in a sufficient number of control chromosomes. Variants were classified as VUSs if there was no convincing evidence that they had a causative effect or were polymorphic. VUSs included both synonymous and nonsynonymous changes not listed in dbSNP; variants listed in dbSNP with a MAF <5% and also variants where the allele frequency was derived from testing only a very small number of chromosomes. VUSs also included some intronic variants that were not predicted to affect splicing. We classified novel variants

as “mutations” if any of these conditions occurred: they affected a moderate to highly conserved nucleotide, or a highly conserved residue, and if the resulting physiochemical difference of the wild-type and mutant amino acid was at least moderate.

Results

In the 289 CMD families included in this study (331 patients), the most common diagnosis was collagen VI related –CMD (32,18%), followed by merosin deficient -MDC1A (29,41%), dystroglycanopathy (24,56%) and selenoprotein 1-RSMD1 (13,84%) (see Figure 1).



Collagen VI mutations

The COL6 protein is composed of three different subunit α-chains encoded by the 3 genetically distinct genes *COL6A1* (on chromosome 21q22.3), *COL6A2* (22q23.3) and *COL6A3* (2q37).

The *COL6A1* gene spans 29 kb and contains 35 exons. The encoded protein measures 1028 amino acids with a predicted molecular mass about 108kD.

The *COL6A2* gene spans 36 kb and contains 30 exons. The encoded protein is 998 amino acids long with a calculated molecular mass about 140kD.

The *COL6A3* gene spans 90 kb and contains 43 exons. The encoded protein is made of 3177 amino acids with a calculated molecular mass of > 300kD.

Mutations in *COL6* genes may cause three muscle diseases: Ullrich congenital muscular dystrophy (UCMD), Bethlem myopathy (BM) and autosomal recessive (AR) myosclerosis myopathy. UCMD was initially regarded as an exclusively AR condition; however, a few years later, patients were reported harbouring heterozygous mutations in *COL6A1* and *COL6A2*. BM is a relatively milder autosomal-dominant (AD) disorder linked to all *COL6* genes but recent studies identified recessive mutations in *COL6A2* associated with the BM phenotype. It is current opinion that UCMD and BM cannot be considered as separate clinical entities, but they rather represent a continuum ranging from severe UCMD to mild BM phenotypes with patients showing intermediate phenotypes considered to have either “mild UCMD” or “severe BM” (7,8).

Mutations spanning the *COL6A1-A2-A3* genes have been identified in 104 index cases (31,42% of total) from 93 families manifesting a spectrum ranging from

the most severe UCMD to the mildest BM. Forty families carried mutations in *COL6A1*, 24 in *COL6A2*, 29 in *COL6A3.* Both AD and AR inheritance were detected with AD more common in *COL6A1* and *COL6A3* (31/40 and 23/29, respectively) and AR in *COL6A2* (18/24 kindred).

COL6A1

In a total of 44 patients (40 families), molecular analyses identified (31 families) with pathogenic heterozygous mutations in *COL6A1* whereas 9 cases harboured homozygous mutations. Whilst a clear AR pattern of inheritance was documented in a single kindred, a parent-to-son transmission was seen in 4 families only, with an asymptomatic parent transmitting the mutated allele to his affected son in a single instance. In a single family, two allegedly severe mutations (c.859G>C/p.Gly287Arg and c.931-1G>C/p.Ala420Glyfs) were found in cis and related to a different clinical expressivity between a father and a son.

We identified a total of 21 putatively dominant mutations, 16 of which were novel. Among recurrent mutations, the described c.1056+1G>A was found in three unrelated families (four patients) and the 868G>A*/p.Gly290Arg and 850G>A/p.Gly284Arg was seen* in four families each. Of 16 novel mutations, two were a deletion affecting the splice site sequences, 9 were substitutions affecting the canonical sites for splicing, and 5 were missense. Among the latter, c.877G>A/p.Gly293Arg was found in three putatively unrelated families (a total of 5 patiens). Mutations were widely scattered in all exons and introns, though exon

10 (n= 5/21 mutations) and intron 13 (n= 4/21) were predominantly involved. Splice site substitutions and missene substitutions were the most common mutations reported in our cohort (10/21 and 9/21, respectively). Revision of available clinical data of 22 patients suggested that 13 could be classified as UCMD, 7 as BM, and 2 had an intermediate phenotype. Seven recessive mutations were identified, 6 of which were novel, and included deletions (n= 4, 1 in frame, 3 frameshift) and splice site substitutions (n=2). The c.1272+1G>A was found in three unrelated families and the *c.1660delG/p.Asp554fs* in two not linked families.
Mutations were widely scattered in all exons and introns. Deletion and splicesite substitutions were the only mutations reported in our cohort. All the patients for whom clinical charts could be reviewed (n= 6) were UCMD.

COL6A2

In a total of 25 patients (24 families) we detected mutations in *COL6A2*. Dominant mutations occurred in 6 families but a clear AD pattern was seen in 5 only. Conversely, we found recessive changes in 18 kindred (19 patients); an AR inheritance was documented in a single case.
Overall, we discovered 6 novel, putatively dominant mutations (5 splice site substitution or deletion and 1 missense), of which 4 were in intron 5. Fifteen of 19 likely recessive mutations were novel and included deletions (n=10, of which 5 were in frame and 2 large deletions), missense (n= 3) and nonsense (n= 2). The homozygous c.2329T>C/p.Cys777Arg recurred in two unrelated patients whereas the homozygous

c.2839_2850del/p.Leu947_Gly950del was found in four unrelated kindred and the *c.1660_1668del/p. Lys554_Glu556del* was homozygous in one and in compound heterozygosity in another family. Recessive mutations were scattered throughout the gene with exon 26 (n= 3) and exon 28 (n= 5) being more affected. Deletions appeared more common than other types of changes in COL6A2.

COL6A3

In a total of 35 patients (29 families) we detected mutations in *COL6A3*, dominant in 29 patients (23 families) and recessive in 6 cases. We could documented an AD inheritance in 7 families, and an AR pattern in 5 families.

Eighteen of 21 putative dominant mutations were novel (6 deletions, 3 splice site mutation and 9 missense). The described c.6210+1G>A *and the new c.6212G>A/p.Gly2071Asp* recurred each in two families. Mutations were widely scattered in all exons and introns, though exon 17 was involved predominantly, missense and deletions were the most common in our cohort (9/21 and 6/21, respectively). Eigh novel mutations (1 frameshift deletion, 1 deletion at splice site, 4 missense, and 2 nonsense) were allegedly recessive with the 7447A>G;*/p.Lys2483Glu* being more frequent (three unrelated kindred).

Mutations were prevalent in exon 36 and 38 (each with 3 variants) and missense substitutions were relatively more frequent in our cohort (4/8).

LAMA2 mutations

The laminin alpha-2 gene (LAMA2) encodes the heavy α2 chain of the laminin 211 isoform (α2/β1/γ1) also termed merosin, it is localised on 6q22-23, spans about 633 kb of genomic DNA and contains 65 exons. *LAMA2*-deficient CMD has an autosomal recessive inheritance and is a severe form presenting with an absence of laminin alpha-2 (merosin) around muscle fibres, high serum creatine kinase (CK) levels especially in early life, no independent ambulation due to weakness and contractures, and a respiratory insufficiency which often requires tracheotomy (9). Most patients have normal intelligence but some show moderate mental retardation and epilepsy. In contrast to less common cases with partial merosin deficiency cases, MDC1 forms a clinically homogeneous subgroup. Diagnosis is usually made by the clinical features and a muscle biopsy examination (complete LAMA2 deficiency can also be determined using a skin biopsy) (10). In a sample from north-east Italy, the prevalence of CMDs has been estimated to be 0.7/100,000 (5). LAMA2-deficient CMD accounts for about 30% of the CMD cases in European countries, but only 6% in Japan (6).

In our cohort, we identified *LAMA2* mutations in 94 patients (28,40%) in 85 families. Forty-six families carried a compound heterozygous mutation while the remaining 39 had a homozygous mutation. Multiple affected sibs and a probable AR pattern of transmission was seen in 8 kindred. Out of 81 different mutations, we found 32 novel variants, including 6 missense, 11 nonsense, 7 frameshift deletions, and 7 spice site substitutions. We also detected a single synonymous

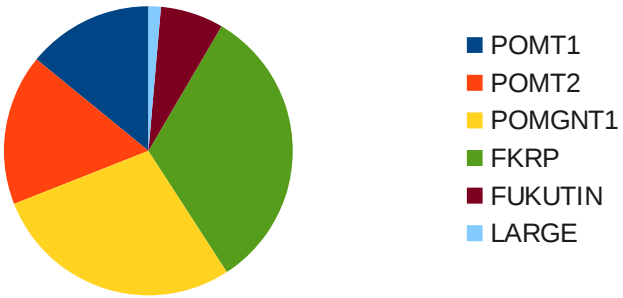
mutation (c.4860G>A) predictably affecting splicing. There were also several recurrent mutations. These included the reported 2049_2050delAG variant — that was homozygous in 3 families and compound heterozygous in 6 —, the 5562+5G>C and the c.2556delT/p.Phe852fs (both found at the heterozygous state in 6 and 7 families, respectively). The novel c.3283C>T/p.Arg1095* and the c.1306+2T>G change were detected in two unrelated families (one in homozygousity).

Mutations were widely scattered throughout the gene and nonsense and splicesite substitutions were more common (28/81 and 20/81 respectively). Interestingly, 3 patients from 2 unrelated families had merosin deficiency, linkage data compatible with linkage to the 6p22.23 region were *LAMA2* resides, and yet not detectable mutations in coding exons and flanking intronic sequences.

Mutations in genes related to dystroglycanopathies

The groups of CMDs characterized by abnormal glycosylation of alpha-dystroglycan (aDG) are commonly termed alphadystroglycanopathies (aDGpathies) and include FCMD (MIM 253800), muscle-eye-brain disease (MEB; MIM:253280), Walker–Warburg syndrome (WWS; MIM:236670), congenital muscular dystrophy 1C (*FKRP*-related or MDC1C; MIM:606612), and congenital muscular dystrophy 1D (*LARGE*-related or MDC1D; MIM:608840) as well as the allelic LGMD presentations for most of these genes. The underlying genetic defects in this group of disorders are mutations either in known or

putative glycosyltransferase enzymes and cooperating proteins involving aDG-*O*-mannosyl-linked glycosylation or subsequent *LARGE*-dependent glycosylation, aDGpathies. The mutation frequency in the 6 more common genes in aDGpathies has been examined in two independent large series and ranges from 34% to 53% indicating that approximately half of the patients with an aDGpathy harbour mutations in still undiscovered genes (4, 11). Less studied are mutations in *ISPD*, usually associated with MEB, WWS, as well as cerebellar cysts, but seem less common (1).
In our cohort we identified 86 patients (25,98%) in 71 kindred of which 23 harboured mutations in *FKRP*, 20 in *POMGNT1*, 12 in *POMT2*, 10 in *POMT1*, 5 in *FKTN*, and 1 in *LARGE* (Figure 2).



FKRP

The Fukutin-related protein gene (*FKRP*) maps on chromosome19q13.3, spans less than 12.5 kb and contains 4 exons, but only exon 4 is translated. The encoded protein is 495 amino acid long, has a significant similarity to fukutin and it is a putative glycosyltransferases probably localized in the Golgi apparatus. The range of phenotypic severity due to FKRP-mutations is large and includes MDC1C, (MIM 606612), LGMD2I (MIM 607155), CMD, mental retardation and brain abnormalities (12,13), Muscle-Eye-Brain disease (MEB) and Walker-Warburg syndrome (WWS), as well as asymptomatic hyperCKemia without overt muscular involvement.

We found gene variants in 26 patients in 23 families but only in 4 we had a clear AR pattern of transmission. Homozygous mutations were seen in 11 kindred and compound heterozygous changes in 12. A total of 18 different mutations were identified including 10 novel. The recurrent c.826C>A/p.Leu276Ile change was homozygous in 9 families and was seen in compound heterozygousity in 8. Novel mutations included 7 missense, 2 nonsense and 1 frameshift. The new c.1223G>A/p.Ser408Asn was found in 2 unrelated families. Missense substitutions were the most common type of gene variants.

POMGNT1

The O-mannose beta-1,2-N-acetylglucosaminyltransferase protein gene (POMGNT1) maps to 1p34-p33 and codes for a 660 amino acid protein that participates in O-mannosyl glycan synthesis. *POMGNT1* gene is divided into 21

coding exons and produces a type II transmembrane protein. Mutations in *POMGNT1* cause WWS, MEB, and a milder form of LGMD.
Molecular studies identified 22 patients in 20 families with recessively inherited mutations, with homozygous changes in 10. A typical AR segregation could be shown in 2 kindred only. In our cohort we collected 23 different mutations, 13 of which were novel. Of the recurrent mutations, the c.1539+1G>A variant was homozygous in two families and heterozygous other two kindred. Of the 13 novel mutations, 5 were missense, 1 was nonsense, 3 were frameshift deletion, and 1 each duplication, in-frame deletion, insertion, and deletion-insertion. The new c.1832T>C/p.Leu611Pro was homozygous in 3 patients (2 unrelated families). Mutations were unevenly distributed with 2 variants each in exon 16, intron 17, and exon 17. Missense (n= 10) and splicesite (n= 4) substitutions were the most common.

POMT1

The O-mannosyltransferase protein gene (POMT1) maps to 9q34.1 and encodes for an enzyme that catalyzes O-mannosylation of proteins, an important protein modification in eukaryotes that is initiated by an evolutionarily conserved family of O-mannosyltransferases. The gene contains 20 exons and spans about 20kb. The encoded protein measures 725 amino acids with a calculated molecular mass about 82.5kD. Mutation in *POMT1* cause WWS, MEB, and LGMD2K.
Mutation analysis in our cohort identified 10 families with recessively inherited *POMT1* mutations. Three

families carried a compound heterozygous mutation whereas the remaining 7 harboured a homozygous mutation. A clear recessive segregation could be proved in a single family. Twelve different mutations were identified including 7 novel (2 missense, 2 nonsense, and 1 frameshift deletion). No recurrent variants were seen in our sample. Mutations were widely scattered in all exons with deletions and missense substitutions being the more common (5/12 each).

POMT2

The O-mannosyltransferase 2 protein gene (POMT2) maps to 14q24.3 and encodes for an integral membrane protein of the endoplasmic reticulum that participates in O-mannosyl glycan synthesis. The POMT2 gene is divided into 21 exons and spans 46 kb. The encoded protein measures 750 amino acids. Mutation in *POMT2* cause MEB, WWS, and LGMD2N with mental retardation.

Mutation analysis identified 12 families with recessively inherited *POMT2* mutations. Eight families carried a compound heterozygous mutation whereas the other 4 were homozygous, with a clear recessive segregation documented only in two families. We detected 15 different mutations, including 4 new changes. The described c.1997A>G/ p.Tyr666Cys was found at the homozygous state in 2 families and at heterozygous state in two others. Out of the 4 new mutations, 3 were missense and one located at the donor splice-site. Mutations were widely scattered in all exons and missense substitutions (12/15) were the most common reported.

FKTN

The fukutin gene (*FKTN*) maps to 9q31.2 and encode a secreted protein expressed in various tissues in normal individuals. *FKTN* gene is made of 10 exons and spans over more than 100 kb. The encoded protein measures 461 amino acids. Mutation in *FKTN* can cause a severe congenital form with brain and eye anomalies (formerly designated FCMD), WWS, MEB, and LGMD2M. A form of dilated cardiomyopathy is also associated with mutations in *FKTN*.

We discovered 5 families with recessively inherited *FKTN* mutations. Two families carried a compound heterozygous mutation whereas the other 3 had a homozygous mutation. Six different mutations were identified, including 2 novel (a missense and a nonsense change). The described c.1167dupA variant was heterozygous in two unrelated. Three variants were detected in exon 10.

LARGE

The like-acetylglucosaminyltransferase protein gene (*LARGE*) maps to 22q12.3 and encodes a bifunctional glycosyltransferase that alternately transfers xylose and glucuronic acid to aDG, to form a polysaccharide that confers the ability to bind ligands (Inamori et al. 2012). The *LARGE* gene contains 16 exons and spans more than 664kb. The encoded protein measures 756 amino acids.

Mutation in LARGE cause WWS, MEB, and a less severe congenital form with mental retardation, formerly designated congenital muscular dystrophy type 1D (MDC1D).

A single patient in our cohort carried mutations in

LARGE: a described missense substitution in exon 9 and a novel duplication in exon 4.

Selenoprotein 1 mutations

The Selenoprotein 1 gene (*SEPN1*) maps to chromosome 1p36, encodes selenoprotein N, a new member of the selenoprotein family, which function is still unknown. *SEPN1* contains 13 exons including the selenocysteine insertion sequence (SECIS) element, a secondary structure located in the 3′ UTR of the transcript that allows selenocysteine incorporation at a UGA codon. The TGA stop codon is in exon 3 and of TGA selenocysteine stop codon in exon 10. The gene spans about 18Kb of genomic sequence, encodes a 678 amino acid-long protein with a calculated molecular mass about 70k, and it produces a glycoprotein-localized within the endoplasmic reticulum. Mutations in *SEPN1* cause a congenital muscular dystrophy with rigid spine (formerly designated RSMD), the "classical" form of multiminicore disease, a desmin-related myopathy with Mallory body-like inclusions, and a form of congenital fiber-type disproportion.

We detected recessive *SEPN1* mutations in 47 patients (14,20%) from 40 families. Twenty families carried a compound heterozygous mutation while the remaining harboured a homozygous mutation. Only 7 kindred presented with multiple affected sibs and a sure AR transmission. Of 32 different mutations identified, 9 changes were novel. The described recurrent c.943G>A/p.Gly315Ser change was found homozygous in 5 families and compound heterozygous in 8. Out of 9 novel mutations, 2 were missense, 1 was a duplication,

3 were frameshift deletions, 1 was an insertion, and 2 were splicesite substitutions. Among those, the c.1-11_81del92 mutation affected for the first time the 5'UTR sequences. No mutations affected the SECIS sequences. Mutations were widely scattered in all exons and introns but exons 3 and 8. Missense substitution were the most common (15/32).

Discussion

We present here the genetic breakdown in a large cohort of UK patients referred to the CMD NCG centre for genetic analyses in the past decade (2001-2011). A diagnosis was genetically confirmed in 48% of the cases (289/600). Within this group, collagen VI related myopathy was the most common diagnosis with 93/289 patients (32,18%), followed by *LAMA2* with 85 (29,41%) and the heterogeneous dystroglycanopathies (71 patients; 24,56%). Twenty-three patients harboured mutations in *FKRP* and 20 in *POMGNT1* and represented the most common dystroglycanopathies. A diagnosis of RSMD1 was made in 40/289 cases (13,84%).

Our results agree with previous reports (4) where a genetic diagnosis was reached in about 46%, and it is higher than a previous Australian study where a definitive genetic diagnosis could be made in 24% (14). In the latter study, the dystroglycanopathies represented the most common group with 25% of cases, and collagen VI abnormalities were found in 12% (14). In our present as well as in the previous study (4) collagen VI related myopathy was the most common diagnosis. These data are also in agreement with those of a Japanese population where collagen 6-related disorders were the second most frequent CMD, following FCMD due to a founder mutation in *FKTN*.

Different from data in our previous study, mutations in *LAMA2* represent about 28% of our cohort but the difference may be due to the previous more strict inclusion criteria that excluded cases with merosin deficiency whose classical presentation make the diagnosis less challenging also by first level or second level Centres. Genetic test is however possible only on a third level centre and this can explain the difference between the two reports.

Alpha-dystroglycan related dystrophies represented the third group for frequency in our study. Mutations in *FKRP* (32%), followed by *POMGNT1* (28%) are the most common, with *FKRP* mutations more frequent in the LGMD.
The relative frequency of *POMGNT1* mutations is higher than the 10% figure gathered in an Italian cohort (11) and the 15% seen in another French study in type II lissencephaly (15).
Considering the frequency of *POMT1* and *POMT2* mutations, we have identified 10 (14%) and 12/71 families (17%), respectively. These relative frequencies are diametrically opposite to data observed in the French and Italian studies (11, 15) where *POMT1* appeared to be more common.
Our present study also confirmed a relatively high frequency of mutations in FKTN, though not as high as in Japan where a found mutation account for the significant proportion of FCMD (49.2% of CMD cases in one series) . On the other hand, mutations in LARGE were extremely rare in our as in previous studies (4, 11, 15).

An additional consideration emerging from our analysis relates to the frequency of the different aetiologies and the amount of new mutations in the CMD-associated genes.

COL6A

We have identified 40 of 93 families carrying mutations in *COL6A1*, 24 in *COL6A2* and 29 in *COL6A3*, data that are partially in contrast with previous reports where a high frequency of mutations in *COL6A1* and *COL6A2* was reported (7).

In particularly, in *COL6A1* we have identified a significant number of new mutations, both dominant and recessive.
The relatively larger number of dominant compared to recessive mutation is in part in agreement with initial reports indicating an association between AD mutations in *COL6A1* and BM (7). However, in our sample we identified several AD UCMD (13/31) suggesting that *COL6A1* are a major cause in both BM and UCMD. The high number of new mutations could also be related to a more extensive gene mutations search.
Consistent with previous reports (7), most dominant mutations in our cohort (12 of the 21) were splice site mutations of which 4 involved intron 13 (33%) and 2 intron 14 (14%). A missense mutation was only found in one case, in cis with a splice-site mutation and making more complex understanding the individual contribution to the resulting phenotype. This was made even more complex if one considers the possibility for reduced penetrance and different expressivity seen in two families. Seven different recessive mutations were identified, and 4 were deletions, all associated with UCMD.
In *COL6A2* we have identified 6 causative dominant mutations and 19 recessive mutations in 24 families.
The relatively larger number of recessive compared to dominant changes may be explained by the fact that *COL6A2* seems more associated with AR inheritance in UCMD patients (17). Consistently, our cases harbouring mutations in *COL6A2* were mainly UCMD. Alike previous reports (7), most dominant mutations in our cohort (5 of the 6) were splice site substitution and deletions (83%).

In patients with recessive inheritance, 10 were novel deletions (52,6%), and two were large deletions.
To date, only 18 deletions in 255 mutations are recorded in Leiden mutation database, and our data contribute to enlarge this subgroup of variant *further corroborating the importance of searching for deletions when suspecting collagen related disorders*. Interestingly, the novel *c.2839_2850del/p.Leu947_Gly950del* recurred in four unrelated families and the *c.1660_1668del/p.Lys554_Glu556del* in two.
In *COL6A3* we have identified 21 causative dominant mutations and 8 recessive mutations in 29 families.
The relatively larger number of dominant changes could be related to the fact that the majority of patients (13/17) for whom clinical data were available were BM. Most dominant mutations in our cohort (9 of 21, 43%) were missense, similar to data present in mutation database (328/338 variants detected in COL6A3 were substitution).
COL6A3 dominant mutations were widespread across the entire gene but apparently localized in the first part of the gene.
Of the 8 recessive mutations identified, 4 were missense (50%) and the c.7447A>G/ p.Lys2483Glu recurred in 3 unrelated families with muscle MRI pattern consistent with BM.
COL6A3 recessive mutations are widespread across the entire gene but apparently localized in the second part of the gene.
Altogether 67 of the 82 mutations detected in *COL6A* genes were novel, 38 dominant, and 29 recessive. The high number of new mutation may reflect not only the methods used in our laboratories, but also the extensive

supportive investigations, including analysis of collagen VI in fibroblasts and muscle MRI imaging performed in our Centre to help in directing genetic investigations. In fact, the highly polymorphic nature of the *COL6A* genes makes it difficult to definitely assign pathogenicity to some variants, especially missense outside the triple-helix domains of the protein.
This report highlights the difficulty on assigning genotype-phenotype correlations and the extensive research of gene mutations needs considering the not obvious linkage between a kind of inheritance and the phenotype. This awareness represents the winning corps of our approach.

LAMA2

The exact prevalence of MDC1A is unknown. The regional prevalence is variable: early-onset *LAMA2* muscular dystrophy accounts for about 30% of the CMD cases in European countries, but only 6% in Japan (18). We have identified 81 causative recessive mutations in 85 families, 32 of which novel. The relative low number of novel mutations detected could be ascribed at ample array of already described mutations (1495 reported in the Leiden mutation database). In keeping with data reported in the Leiden mutation database, we identified 34,5% nonsense substitutions and almost 25% splicesite substitutions. Our cohort also included 16/81 (19,7%) frameshift deletions. The recurrent c.2049_2050delAG, was found in a total of 12 alleles whereas the homozygous Kenyan founder mutation, c.7881T>G, associated with a variable clinical phenotype (10) was found in two families in heterozygous state and in one in homozygous state.

The Italian founder mutation c.2901C>A (19) was not detected.
Mutations were distributed throughout the gene; different from those reported in the Leiden database where the largest number of mutations occur in exon 38. Although it was not a specific aim of our report, our findings suggest lack of significant genotype-phenotype correlations and variability in clinical presentation in merosin-deficient CMD.

FKRP

We found 18 different mutations in our study, 13 of which (72%) were missense, as in the Leiden database. Among those, the recurrent c.826C>A/p.Leu276Ile occurred in 17 unrelated families, although there was no obvious correlations between the nature of the mutation and the severity of the disease.
According to previous reports, no patients harboured two *FKRP* null alleles suggesting that the complete lack of FKRP might be embryonically lethal. The two nonsense mutations found in our cohort in two unrelated patients were in compound heterozygosity with the recurrent missense mutation. Reviewing the clinical notes of 5/6 patients with CMD and not carrying the c.826C>A/p.Leu276Ile further substantiate the impression that this particular protects individual from a severe form of the disease.

POMGNT1

Twenty-three pathogenic recessive mutations were found in 20 families 13 of which novel and 10 (43,5%) missense. The recurrent variant c.1539+1G>A, in intron 17, reported by Diesen et al (2004) (20) as a founder

mutation in the Finnish population was present in 4/23 patients. The high number of novel mutation reported in our cohort contribute to enlarge also the group of micro/macro rearrangements and nonsense mutations, that were mostly located towards the last part of the coding region.

POMT1

Of the 12 different mutations identified in our study, 5 (41,6%) were missense, though the recurrent variant c.1598G>C/p. Ala200Pro was found only once. Again, our analyses contribute to enlarge the relatively small number of deletions reported in this gene (so far about 10% of total changes).

POMT2

We have identified 15 causative recessive mutations in 12 families, 4 of which novel, and about 80% missense. The recurrent variant c.1997A>G/p. Tyr666Cys was found in roughly one fourth of the cases. Variant were scattered in all exon and intron; we can only found a mild prevalence for exon 21 (3/15-20%).

FKTN

In *FKTN* we identified 6 recessive mutations in 5 families, 2 of which novel. The recurrent c.1167dupA, was heterozygous in two families. Contrary to data reported in the Leiden database where insertion (359/577) are the prevalent mutations, we found several missese changes, including the c.920G>A/p.Arg307Gln already described in the Italian cohort (11).

LARGE

A single patient harboured 2 mutations, including a novel duplication in exon 4 adding to the list of mutations in *LARGE*.

SEPN1

In *SEPN1* we have identified 32 causative recessive mutations in 40 families 9 of which are novel. Of the 32 different mutations identified in our study, 15 (46,8%) were missense, 7 deletion, 4 splicesite, 3 duplication, 1 insersion and 2 nonsense. The recurrent variant c.943G>A/p. Gly315Ser was found in 13 kindred. Among novel variants the c.1-11_81del92 mutation affected also the 5'UTR for the first time.

Different from previously reported cases, where most variants were nonsense, microdeletions or insertions leading to frameshift, as well as splice-site mutations predicting an aberrant pre-mRNA splicing, we found a high rate of missense mutations (15/32) not necessarily around or at the potential catalytic site located in exon 10.

Conclusion

In summary, data presented in this study represents the largest series of CMD from a referring molecular genetic laboratory in UK, if not worldwide. Our data offer the opportunity for several considerations:

collagen VI-related disorders represent the most common form of CMD in our cohort, accounting for about one third of total. Patients with MDC1A follow closely behind accounting for about 29% whereas dystroglycanopathies and RSMD seem relatively less common (roughly 25% and 14%, respectively).

Among dystroglycanopathies, mutations in *FKRP* represents the most common and are associated particularly with a LGMD clinical presentation. Mutations in *POMGNT1* , represent the second common aetiology. There seem that there are no clear-cut genotype-phenotype correlations. It appears that neither clinical presentation can predict the gene/mutation affected nor that individual genes display distinctive features to be used in clinical practice.

The level of allelic heterogeneity is extremely high with a wide array of novel mutations being detected. This implies that full gene testing (eventually adopting modern, fully automated high-throughput methodologies) are needed to define the genetic breakdown of the CMD population.

Finally, several data are in contrast with those reported in previous study concerning single series of patients, but largely agree with those reported in general mutation database. This is a further significance of our large cohort that correlates well not only with the frequency regardless a particular geographic areas but also the frequency of CMD-LGMD population worldwide.

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Chapter 3
Muscle MRI studies

1 Muscle MRI: what we learn by pattern recognition analysis

Until recently the use of muscle magnetic resonance imaging (MRI) in neuromuscular disorders was limited to inflammatory myopathies. Few systematic studies on muscle MRI in inherited neuromuscular disorders have been published, mostly to highlight the selectivity of muscle involvement in relation to other diagnostic investigations performed. During the past few years, however, there has been increasing evidence that muscle MRI, in combination with a detailed clinical assessment and appropriate biochemical studies, can identify specific patterns of muscle involvement and help in the differential diagnosis of neuromuscular disorders in children and adults. We report our experience on the clinical value of muscle MRI in the diagnosis of patients with inherited muscle disorders, with a focus on practical aspects related to establishing mutations pathogenicity or differential diagnosis.

1.1. Muscle MRI in TRPV4-related neuromuscular disorders

by Astrea Guja, Brisca Giacomo, Manganelli Fiore, Pisciotta C., Fiorillo Chiara, Santoro Lucio, Bruno Claudio, Santorelli Filippo Maria, Battini Roberta

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Introduction

As described in chapter 2 mutations in the transient receptor potential vallinoid 4 (TRPV4) gene may cause diverse autosomal dominant neuropathies including hereditary motor and sensory neuropathy type 2C (HMSN2C), hereditary motor neuropathy (HMN), scapulo-peroneal spinal muscular atrophy (SMA) and congenital distal SMA (1-3). Genotype-phenotype correlation is not yet define and mutation in the same domain of the receptor could be determinate alternately distal SMA or CMT conditions (4). It is also described an intrafamilial variable penetrance (5). So in case of early genetic detection is too difficult make a judgment on the prognosis only on base on mutation.

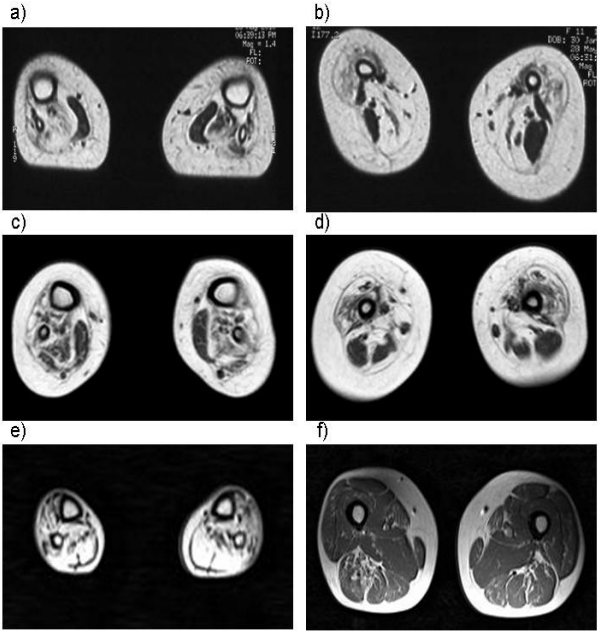
Patients and methods

We studied the pattern of muscle MRI in two patients with congenital distal SMA (a 2-year-old boy and an 11-year-old girl — showing marked weakness in proximal and distal muscles, atrophy of distal legs and clubfoot with an electromyography and muscle biopsy compatible with neurogenic conditions) and in one patient (a 63 years men) affected by CMT2C. The three patients was harbouring three different mutation in TRPV4 (p.P97R; p.R232C; p. R315W).

Results and Discussion

A similar pattern of muscle MRI was evident either in patients with congenital distal SMA than in patients with CMT2C with a predominant alteration of anterior calf muscle (tibialis and peroneus groups) with relative sparing of medial gastrocnemius. Nevertheless, in congenital distal SMA, at thigh level there is an extensive and widespread fatty atrophy, with selective spread of biceps femoris, semimtendinosus and vastus lateralis, fewer evident in CMT2C. This finding suggest the presence of a specific and selective pattern with a continuum of skeletal muscle involvement in the legs between phenotypes in TRPV4 gene-mutation-related skeletal muscle disorders. The predominant involvement of thigh's muscle in distal SMA could be helpful in distinguish this form in situation of overlap of clinical finding, helpful in make consideration on natural history.

Figure1



Transverse T1-weighted MRI of thighs and calf muscles in dSMA patients harboring mutations in TRPV4 (a-d) and in CMT2C patient (e-f). An extensive fatty atrophy preserving biceps femoris and medial gastrocnemious is evident in dSMA and fewer, although also evident, in CMT2C patient. This pattern is different from non-TRPV4 patients i.e. *DYNC1H1* patient presented in chapter 2 where the medial compartment at thighs – with hypertrophy of adductor longus – and anterior muscles at calf level – tibialis anterior – are spared.

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1.2. Discovery of a novel RYR1 mutation through the use of muscle MRI

*by **Astrea Guja**, Munteanu Iulia, Cassandrini Denise, Lillis S, Trovato Rosanna, Cioni Giovanni, Mercuri Eugenio, Muntoni Francesco and Battini Roberta*

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Introduction

Congenital myopathies (CM) due to mutations in the skeletal muscle ryanodine receptor gene (*RYR1*) are the most common forms of CM with an ever increasing array of mutations being associated with a wide set of clinical syndromes, including central core disease (CCD), susceptibility to malignant hyperthermia, multi-minicore disease (MmD) (1, 2), centronuclear myopathies (3), 3-OH methylglutaconic aciduria (4) and core-rod myopathies. Both autosomal dominant (AD) and autosomal recessive (AR) patterns of inheritance are possible regardless of the underlying clinical phenotype. Heterogeneity in terms of clinical and genetic findings is paramount. On one hand, there is a wide spectrum of clinical severity ranging from children who never achieve independent walking, to individuals with easy fatigue and fully-blown muscular atrophy, to patients with susceptibility to malignant hyperthermia and little or no muscle weakness. On the other hand, muscle biopsy, the gold standard for a definitive diagnosis of CM, often presents unspecific pathological findings that are unable *per se* to indicate the responsible genetic alteration. Also, allelic heterogeneity does not allow precise locus-driven genotype-phenotype correlations.

Multi-centre experience and current literature suggest that muscle Magnetic Resonance Imaging (MRI) is able to recognize characteristic patterns of selective muscle involvement in many neuromuscular disorders, including those associated with mutations in RYR1 (5, 6, 7), and might help narrowing the differential diagnosis prior to muscle biopsy (8; 9), if not as an alternative to it.
We present the case of two sisters with clear clinical findings of CM co-morbid with AR cystinuria type B. Despite a slight intra-familial clinical variability and unremarkable muscle biopsy findings, the recognition of a characteristic muscle pattern on MRI allowed the genetic diagnosis with the identification of a new mutation in *RYR1*.

Case report

Two sisters (F.T. & F.A.), coming from southern Italy, were referred to the Developmental Neuroscience Department of IRCCS Stella Maris at the age of 13 and 6 years respectively.
Both were born at term by cesarean section due to breech presentation, to healthy consanguineous parents. Pregnancies had been remarkable for reduced fetal movements in both cases. Past medical history in the mother was significant, with one miscarriage in the first trimester of pregnancy and one stillbirth.
Patient 1 (F.T.) is a 16 year-old girl who was floppy at birth and had feeding and respiratory difficulties and delayed development. She presented at birth with bilateral clubfoot, congenital scoliosis and poor suction; she is able to walk with ortosis since age 6. The younger sister (F.A.), now aged 6, had a similar though milder clinical phenotype: she achieved the sitting

position at 13 months and independent walking at 36 months.

The clinical course was slowly progressive in both children. Cardiac, respiratory and cognitive functions as well as routine laboratory investigations (including CK levels) were all normal.

An electromyography (EMG) of the lower limbs in patient 1 at the age of 5 years showed a myopathic pattern. Muscle biopsy indicated marked dystrophic changes without any additional features. Interestingly, both girls also present with kidney stones and elevated urinary levels of cysteine with childhood onset. A homozygous c.605-3C>A mutation in the *SLC7A9* gene corroborated the clinical diagnosis of cystinuria type B. Parents were heterozygous for the mutation, which impairs the *SLC7A9* gene splicing.

At first examination, F.T. and F.A. showed generalized hypotonia, myopathic facies with high arched palate and triangular mouth. Extra-ocular muscles were spared. Deep tendon reflexes were diminished in both girls but there were neither amyotrophies nor pes cavus. In F.T. the latest neurological examination was significant for a prevalent weakness of lower limbs, hyperlordosis, dorsal and lumbar scoliosis and joint hyperlaxity. She could not walk without orthesis. F.A. had prevalent weakness at scapular girdle; she could walk independently but with waddling gait and bilateral foot drops and she was unable to run.

Neurophysiological examinations, including motor action potentials and nerve conduction studies, were normal in both girls. Muscle MRI of the thighs using 1.5 T MR System (Signa Horizon LX, Healthcare GE, USA) and a conventional T1 weighted spin eco images showed a

typical pattern of RYR1 myopathy with the selective involvement of vasti, sartorius, adductor magnus and the relative sparing of the rectus, gracilis and adductor longus (Figure 1a and 1c). In addition, within the calf muscle, there was selective involvement of soleus, gastrocnemii and peroneal group with relative sparing of the tibialis anterior (Figure 1b and 1d).

"Capillary" Sanger sequencing of the coding exons and flanking introns of *RYR1* using the BigDye 3.1 chemistry uncovered a homozygous c.14928C>T mutation (p.F4976L) in patient 1. The mutation was also homozygous in her younger sister, heterozygous in the healthy parents, and it was not found in 500 healthy control chromosomes. Computational predictions using the Polyphen-2 (genetics.bwh.harvard.edu/pph/) and PMut (mmb2.pcb.ub.es/pmut) bioinformatic tools suggested that the new p.F4976L was "probably damaging".

Discussion

The MRI pattern of selective muscular involvement in patients with central core disease due to *RYR1* mutations was initially reported in 2004 (5). The initial findings have been subsequently confirmed independently in patients with dominantly-inherited mutations (6) and, more recently (7), in a larger sample with different degrees of clinical severity and different patterns of inheritance. Mutations in *RYR1* are associated with a wide range of phenotypes, including central core disease (CCD), malignant hyperthermia susceptibility and distinct subgroups of multi-minicore disease (MmD) (1, 2), centronuclear myopathy (3) and core-rod myopathy (4). Because of the impressive

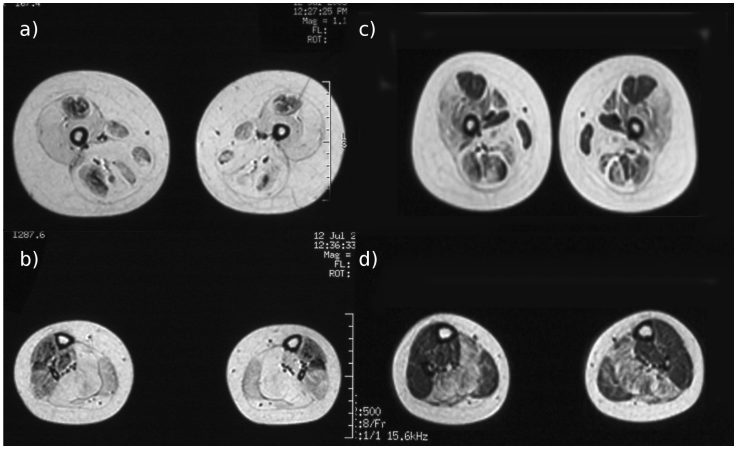
overlap of clinical and pathological findings between different CMs and of the ever increasing genetic heterogeneity, selection of the correct gene to analyse remains a difficult task, especially in the absence of cost-effective functional assays. In this context, the muscle MRI appears to be a useful “biomarker”, as use of this tool together with the correct result interpretation may allow an informed prioritization of gene analysis in several CMs.

Our report of a new *RYR1* mutation discovered following suggestive MRI changes lends further support to this hypothesis, especially considering the unspecific muscular findings and the dissimilar clinical presentation in the two sisters.

Although a recent report suggests that the spectrum of RYR1-related myo-imaging is broader than originally reported and that there seems to be a gradient of muscular hypo-intensity (7), there remain sufficient distinctive fundamental features which turn out to be crucial when clinical and pathological data cast doubts onto performing time-consuming gene analysis. As suggested by others (7), detailed examination of involvement (or sparing) of selective muscular groups, such as the extra-ocular muscles, might represent an adjunctive decisive factor prior to embarking on the testing of the large *RYR1* gene. Likewise, it is appealing that mutation screening of *RYR1*, which is highly polymorphic, with more than 300 disease-related variants and a similar number of “possibly” neutral changes (Human Gene Mutation Professional Database, www.hgmd.cf.ac.uk/; latest accession March 2012), would benefit of a relatively less costly

“functional” MRI investigation to substantiate the predicted deleterious effect of novel sequence variants.
In conclusion, this study presents a novel *RYR1* genetic variant whose pathogenic significance was reinforced by a RYR1-specific pattern on muscle MRI. Muscle MRI is an important diagnostic tool, particularly for myopathies associated with a wide phenotypic spectrum, such as those RYR1-related.

Figure 1



T1 weighted transverse scans performed through thigh and calf in F.T at 13 yrs (1a and 1b) and in F.A at 6 yrs (1c and 1d). Both scans show the selective involvement of vasti, sartorius, adductor magnus and relative sparing of rectus, gracilis and adductor longus (1a, 1c) and the selective involvement of soleus, gastrocnemii and peroneal group and relative sparing of the tibialis anterior within the lower leg (1b, 1d).

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2 Expanding the use of muscle MRI: from overall pattern to pattern within muscle

The assumptions here below are a part of a collaboration project involving Dubowitz Neuromuscular Centre (London).

2.1 Can muscle MRI help distinguish myopathic from neurogenic genetic causes of distal weakness?

*by **Astrea Guja**, Battini Roberta, Gunny Roxana, Manzur Adnan, Mercuri Eugenio, Morrow Jasper, Muntoni Francesco, Reilly Mary, Tarek Yousry*

manuscript in preparation

Introduction

In the past decade there has been increasing evidence that muscle MRI may play an important role in identifying genetically different conditions. Specific patterns of muscle involvement in series of patients, including primary myopathies or muscular dystrophies, have been consistently outlined demonstrating that muscle MRI has the power to direct genetic tests in a number of neuromuscular conditions (1).

On the other hand there has been limited focus on the role of muscle MRI in distal myopathies and spinal muscular atrophies (2) and, so far, no studies have specifically compared the ability of MRI to distinguish myogenic and neuropathic disease processes.

Distal myopathies refer to inherited or sporadic disorders characterized by progressive weakness and

atrophy beginning in hand or feet muscles and by myopathic changes in skeletal muscle biopsies (3). The increasing number of new genetic aetiologies for neuromuscular disorders has permitted to identify novel forms of distal spinal muscular atrophies, including SMA-LED and *TRPV4*-related myopathies (4,5), though the associated myoimaging features were described in a limited number of patients (6), making difficult to draw clinically relevant correlations.
In principle, the use of a muscle MRI study could be of interest in differential diagnosis of conditions presenting distal muscle weakness and atrophies where the primary muscular or neurogenic involvement is at times difficult on clinical and neurophysiological grounds alone and in the presence of a high level of genetic heterogeneity. Although some clues such as proximal-distal gradient or more marked involvement in myopathies than in peripheral neuropathies have been proposed, they have not systematically confirmed in large scale studies. Moreover, current studies mostly ignore pattern presentation within individual muscles, though this seems very helpful also in these conditions, as in Bethlem myopathy (1).

We here studied muscle MRI images in a group of patients with a pattern of distal weakness and affected by either inherited neurologic conditions of anterior horns or distal myopathies. Our aims were:
1) to verify the ability of myoimaging in specifically detecting the neurogenic or miopathic condition;
2) to recognize in the two conditions possible distinctive characteristics;

3) to elucidate the mechanisms underlying muscle specific distribution of changes in different disorders.

Methods

We reviewed muscle MRI scans performed in the last 10 years at GOSH and Hammersmith Hospital (London, UK) and at the IRCCS Stella Maris (Pisa, Italy) obtained from patients included in a regular clinical follow up and consecutively seen at the three centres.

Patients were included in the study group if they met the following criteria: i) presence of at least one muscle MRI scan of thigh and leg muscles performed at one center; ii) absence of weakness in limb-girdle muscles; iii) available clinical vignette, neurophysiology data, levels of serum creatine kinase (CK), genetic information when available, and, eventually, information from muscle or nerve biopsies.

With these inclusion criteria, we selected MRI scans from a total of 38 patients, including

22 patients with a genetically confirmed myopathic disorder or with a genetic condition affecting anterior horn cell, and not related to the common SMN1-associated form of SMA;

16 patients with the same condition but not yet genetically defined.

All MRI scans were performed according to the protocol previously used by our groups using conventional T-1 weighted spin echo. Non contrast transverse images were obtained in the legs, selecting the axial plane with respect to the long axis of the body (7).

This study was approved by the ethical committees of the participating Institutions upon receiving patients' written informed consent.

Image Analysis

All the scans collected were standardized in format and were reviewed blindly to genetic and clinical data by two expert neurologists (J.M., A.M.) and two neuroradiologists (T.Y., R.G.). We initially designed a validation arm of the study. Experts assessed data from 12 patients with known genotype by obtaining a group full consensus. Seven patients presented a molecularly-confirmed myogenic disease (5 harbored mutations in *MYH7*, 1 in *NEB* and 1 in *MYH2*) and 5 a genetically confirmed neurogenic disease (4 had mutations in *DYNC1H1* and 1 in *DYNACTIN2*). In this first part of the study, all the examiners were asked to provide expert impression of the gestalt of an image and a visual understanding of, based on the following characteristics: a) quality of images; b) presence of atrophy or hypertrophy of muscles bulks; c) quantitative degree of normal or abnormal signal intensity within each muscle according to the described Mercuri gradient; d) possible presence of a distal versus proximal gradient of muscle involvement; e) analysis of texture within each individual muscles.

Results of the "gist of a muscular scene" from each examiner were collected in a score sheet and subsequently analyzed in order to find a marker capable to distinguish the two clinical disease categories (myopathies versus neuronopathies).

A non-parametric test (Mann-Whitney test) was used to evaluate if any differences between muscle involvement were evident at thigh and calf level in each groups. Significance was set at $p < .05$. The Wilcoxon test was used to correlate differences between thigh and calf muscles in each group, independently. Significance was set at $p < .05$.

Having completed this validation task, examiners were asked to apply the consensus scoring system blind to the final diagnosis to an additional set of images from 26 patients (5 each genetically confirmed neurogenic and myopathic disorders, and 8 each yet molecularly undefined neurogenic and myopathic conditions).

Results

Table 1 summarizes clinical and genetic data in our patients.

Pts	DOB	Age at exam.	Gene	Neurop.	Bio.	Clinical finding	A/NA
C.L.	04/03/1992	15y	DYNC1H1	N	M	Arthrogryp. predominant lower limb weakness with thinning of lower legs	A
B.S.	06/11/1972	38y	DYNC1H1	N	N	Very mild distal weakness	A
J.A.	26/02/1999	8y3m	DYNACTIN2	N	M	Scapular, peroneal and hip girdle weakness	A
B.T.	23/12/2000	10y3m	DYNC1H1	M	M	Predominant lower limb weakness with thinning of lower legs	A
J.J.	12/01/1988	11y2m	DYNC1H1	M	NP	Marked distal muscular atrophy and weakness	A
M.L.	24/11/1993	16y9m	MYH7	M	NP	Mild scapulo-peroneal weakness	A
B.A.	08/06/1992	15y	MYH7	M	D	Mild scapulo-peroneal weakness	A

F.A.	13/11/1995	15y6m	MYH7	M	M	Pes cavus, mild distal weakness	A
P.V.	03/03/2001	8y6m	MYH2	M	M	Mild scapulo-peroneal weakness	A
H.N.	20/12/2002	6y6m	NEB	M	M	Severe distal weaknes, bilateral foot drop	A
M.C.	02/03/1958	42y	MYH7	N	M	Mild scapulo-peroneal weakness	A
J.M	13/07/1996	16y	MYH7	N	NP	Predominant lower limb weakness with thinning of lower legs	A
T.G.	30/11/1999	10y6m	TRPV4	N	N	Arthrogr. predominant lower limb weakness	NA
P.R.	22/02/1988	19y	BAG3	M	NP	Severe weakness of lower limbs with foot-drop.	A
H.L.	15/06/1994	5y8m	BICD2	N	NP	Mild scapulo-peroneal weakness	A
H.P.	04/07/1962	39y3m	BICD2	M	M	Mild scapulo-peroneal weakness	A

V.I.	08/10/2001	8y9m	BICD2	N	M	Arthrogryp. reduced muscle bulk in lower limbs more marked distally	NA
W.J.	16/05/1997	12y2m	BAG3	N	M	Longstanding weakness of lower limbs with foot-drop	A
S.K.	28/12/1995	15y1m	BAG3	N	M	Bilateral pes cavus and tiptoe walking very minimal distal hand weakness	A
McC	01/10/1989	12y	TPM3	normal	M	Mild distal myopathy	A
B.S.	27/02/2002	3y5m	DYNC1H1	N	N	Arthrogryp.	A
S.C.	27/09/1977	23y	NEB	M	M	Distal myopathy with footdrop	A
P.D.	25/07/1985	17y2m		N	normal	Arthrogryp. reduced muscle bulk in lower limbs more marked distally	
C.M.	13/08/2007	2y6m		N	NP	Congenital talipes muscle bulk reduced	A

S.A.	20/10/2003	7y5m		N	N	Arthrogryp. reduced muscle bulk in calves but	A
P.J.	24/11/2011	14y11 m		M	M	Mild distal weakness	A
B.G.	03/03/2001	10y10 m		M	M	Mild distal weakness	A
M.R.	15/01/2000	10y9m		N	N	Distal myopathy with footdrop, congenital talipes equinovarus bilaterally	A
W.A.	07/06/1965	41y 2m		N	NP	Distal myopathy with footdrop	A
C.S.	25/02/2000	10y6m		NP	D	Marked distal weakness	A
R.K.	19/09/1965	36y5m		M	M	Marked distal weakness	A
B.F.	20/07/1994	14y		M	M	Distal myopathy with footdrop	A
M.D.	11/01/1997	13y4m		M	M	Scapular, peroneal weakness	A
R.S.	03/01/2004	4y6m		M	M	Distal myopathy; footdrop	A

T.C.	20/03/1993	17y		N	M	Distal myopathy with footdrop	A
D.A.	18/12/1989	9y8m		N	D	Arthrogryp. very severe distal involvement in the limbs	A
L.E.	07/04/1988	14y4m		N	D	Arthrogryp. mild weakness in the lower limbs	A
W.An	17/12/1987	11y3m		N	NP	Mild weakness in the lower limbs	A

Age at exam.: age at examination; **DOB:** date of birth; **Neurop.:** neurophysiology; **Bio.:** biopsy; **N:** neurogenic; **M:** myopathic; **NP:** not performed; **D:** dystrophic; **A:** ambulant; **NA:** non-ambulant; **Arthrogryp.:** Arthrogryposis.

Data analysis in the validation study

a. Image quality

All images, except one, had a good quality: at thigh level 7/12 images scored 0 (“no artifacts”) whereas the remaining scored 1 (“presence of artifacts, but all muscles were gradable”). At the level of the calves 9/12 scored 0, 2/12 scored 1 and only 1/12 had a score of 3 (“strong artifacts in several muscles, cannot be analyzed”). The latter image was not included in statistical analysis.

b. Muscles bulks

For what concern muscles bulk at thigh level, neurogenic conditions showed normal bulk (2/5) or hypotrophy/hypertrophy (3/5), whereas myopathic conditions consistently were either normal (n= 3) or hypotrophic (n= 4). There were no differences between anterior or posterior compartments except for hypertrophy of adductor longus in neurogenic condition (3/5) that seems to be a prevalent feature in most of them and not evident in myopathic conditions (0/7).
At the level of calves, both neurogenic and myopathic disorders had most of muscles bulks in the range of hypotrophy (3/5 and 4/7, respectively), regardless of the involved compartment. The bulk in the remaining muscles were in the normal range. No muscle hypertrophy was evident at this level.

c. Appraising the values of Mercuri gradient

From a descriptive point of view, several muscles analyzed in neurogenic conditions were in the range of

high Mercuri score (score 4), in particular, at thigh level (49/120 muscles at thighs and 25/70 at calves). Conversely, no muscles (both at thigh and calf) had a score as 4 in myopathic conditions, with most of them presenting a score of 3 at the level of tibialis anterior, extensor digitorum longus (EDL) and soleus. From a statistical point of view, median score values were higher in the group of neurogenic conditions than in myopathic disorders but for the adductor longus muscle where scores were similar and EDL where scores were higher in myopathic conditions, although with a large variation.

Median value difference between the two groups was significance both at thigh ($p<.005$) and at calf level ($p.<018$) (Table 2).

Moreover, we could detect at thigh level in neurogenic conditions only a prevalent involvement of anterior respect to posterior compartment, with a relative sparing of adductor longus. There was a significant prevalent involvement in all anterior muscles in neurogenic diseases (vastus intermidius $p<.01$; vastus lateralis $p<.003$; vastus medialis $p<.018$). Muscles in posterior compartment were not significantly different between the two clinical condition with the important exception of the capus longus of bicepes femoris ($p<.018$). In medial compartment, we observed a significant difference in fewer specific muscles: gracilis ($p<0.1$), sartorius ($p<.005$) and, marginally, right adductor magnus ($p<.048$). At calf level differences could be find only in the posterior compartment (in particular in lateral and medial gastrocnemius $p<.003$), whereas the anterior compartment was predominantly involved in myopathic conditions.

Table 2 Median value difference between neurogenic and myopathic conditions

Conditions	Thigh		Calf		Significance
	Median	Interquar. range	Median	Interquar. range	
Neurogenic	5	2,75	4,5	2,25	P<.005
Myopathic	1	1	2	2	P<.018

d. Presence of a disto-proximal gradient

A prevalent calf involvement could be detected in myopathic conditions as highlighted by the analysis of Mercuri gradient (see above) though this did not reach statistical power (p<.066). Four out of 7 myopathic patients had distal more than proximal high median scores, with the remaining 3 cases having similar values. There was no significant differences in neurogenic conditions (p= 0.85) with median score values proximally higher in 3 cases and more pronounced distally in 2.

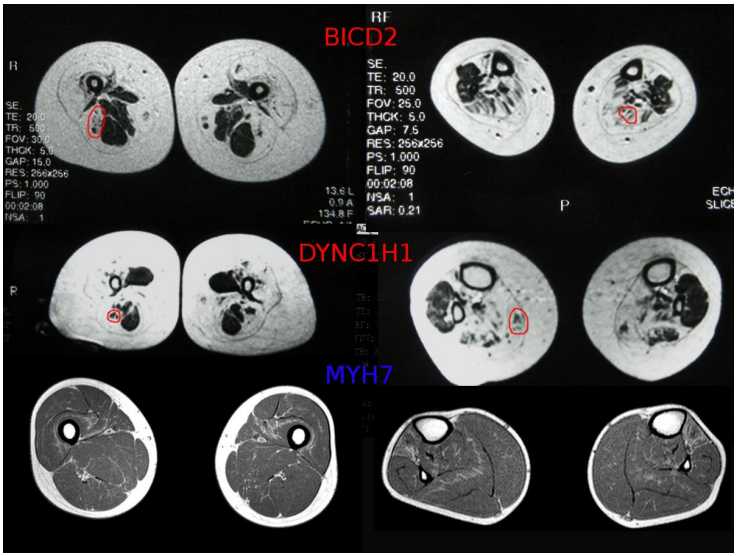
e. Qualitative analysis in individual muscles

Considering texture pattern, experts occasionally identified an alteration that was termed “islands” in those muscles completely replaced by fat infiltration but with evident sparing of a little parts of muscle bulks. This alteration was evident in 5/5 neurogenic conditions but in 0/7 myopathic conditions (Figure 1).

Data analysis in an additional set of 26 unknown patients

A blind review of muscle MRI scans from an additional set of 26 patients adopting the above mentioned criteria and evaluations gave a high inter- and intra-rater sensitivity and specificity (95%).

Figure 1. Muscle MRI at thigh and calf level in two neurogenic conditions (DYNC1H1 and BICD2) and in one myopathic condition (MYH7). Note the islands in the neurogenics ones (red circle).



Discussion

The rationale of this study was to evaluate the sensitivity and specificity of a visual inspection of muscle MRI scans to identify clues useful to differentiate distal neurogenic from distal myopathic disease.

Our results suggest that muscle MRI could be a powerful predictor of neurogenic involvement in patients with a pattern of distal weakness.

Some clues could be represented by the degree of muscles involvement that appear disproportionally higher compared to the clinical functioning, and the predominant involvement at thighs in contrast to the distal symptoms and signs. At calf level, the prevalent involvement of anterior compartment in myopathic conditions compared to those observed in neurogenic diseases could be another important discriminating factors, as well as the disto-proximal gradient of involvement, evident in myopathic conditions but no in neurogenic ones. It should be noted that no analyses from patients presenting peripheral neuropathies (i.e. CMT syndromes) were included in the study. However, in this conditions differential diagnosis could be made by nerve conduction studies that are unequivocally altered in CMT and normal in myopathic conditions.

Another clue factor could be the relative hypertrophy seen in neurogenic conditions (adductor longus in 3/5 cases), since in none myopathic disorders increases of muscle bulks was evident, but it is also possible that this relates to the specific gene defects.

Finally the pattern of involvement within muscles seems to be the most discriminating factor. The presence of «islands» preserving muscle bulks in a sea of fatty transformation was noted serendipitously but it appears

to be a red flag for neurogenic conditions, regardless of the genetic defect.

The pattern of involvement within muscles, that is not something normally captured by Mercuri grading or reported in the literature to date, seems to be the most intriguing factor, and a useful diagnostic marker of late neurogenic change in this clinical group. It is tempting to hypothesize that the presence of islands in neurogenic conditions could represent the effects of giant motor units of the few remaining axons, but this impression will require more detailed investigation. Also, larger studies are needed to define island as a strong marker of distal neurogenic disorders and useful clues in determining the severity of the disease.

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Chapter 4
The Brain involvement

1 The brain involvement in DMD patients

This section reports a neuropshycological study carried out in Duchenne Muscular Dystrophy patients so as to verify the presence and characteristics of literacy deficits in DMD, analyse neuropsychological profiles associated with reading and spelling abilities and compare them with patients with Developmental Dyslexia (DD) ones.

1.1. Duchenne Muscular Dystrophy and Developmental Dyslexia: comparison of reading and neuropsychological profile

*by **Guja Astrea**, Chiara Pecini, Filippo Gasperini, Giacomo Brisca, Marianna Scutifero, Claudio Bruno, Filippo Maria Santorelli, Giovanni Cioni, Luisa Politano, Annamaria Chilosi, Roberta Battini.*

manuscript submitted to PlosOne

Introduction

Duchenne muscular dystrophy (DMD) is a hereditary, X-linked recessive disorder affecting about 1 in 3500 males and clinically characterized by severe, progressive and irreversible loss of muscular functioning and strength (1). Brain involvement in DMD has been known since its original description (2), and a meta-analysis in 1,146 patients has showed that full-scale intelligence quotients were at more than one standard deviation (SD) below the mean of age-matched population, although frequency and distribution did not seem to differ significantly (3). As a result of this downward shift, 35% of DMD children had full-scale intelligence quotient of less than 70, that is, in

the “mental retardation” range and only roughly 5% were above normal average (3).
Notably, a significantly lower verbal intelligence quotient (VIQ) than performance intelligence quotient (PIQ) has been reported in DMD boys (3), in addition to an increased incidence of learning disabilities with major difficulties in literacy skills (4,5,6,7). However, problems in reading and writing are still largely uncharacterized. When reading skills in DMD were studied (7), impairments have been reported similar to those observed in children diagnosed with Developmental Dyslexia (DD), a condition characterized by a significant deficit in reading decoding skills with normal non-verbal cognitive abilities. In particular, Billard (1992) (8) has reported that 12 out of 24 DMD children had severe reading disabilities compared to a similar size group of children affected by different neuromuscular disorders (i.e., spinal muscular atrophy).
Research of reading deficits in DD have suggested that multiple neuropsychological factors may partially account for defects such as: phonological processing deficits (9,10,11); abnormalities in rapid access to long-term phonological representations, specifically from visual input (12); sluggish focused multimodal attention (13) or general automatization deficits (14). We have shown that phonological processing deficits characterized only one subgroup of DD subjects with a history of early language delay, whereas speed deficit in rapid automatized naming tasks (RAN) can be observed also in children without any history of language delay. This finding has suggested that a reduced speed in forming visuo-verbal associations could be the main cognitive marker of DD in a shallow orthography

language such as Italian (15,16,17,18). Keeping in mind the characterization of DD children, we reasoned that a detailed characterization of learning skills in DMD children and a direct comparison of both disorders in a similar experimental setting could help better define the type and extent of learning difficulties in DMD. Thus, the aims of our study were two-fold: i) to verify the presence and the nature of literacy deficits in DMD, and ii) to analyse the neuropsychological profiles associated with reading abilities in individuals when compared with different groups of DD children.

Patients and methods

We recruited 36 subjects older than 8 years of age in order to ensure acquisition of basic scholastic skills. 12 ambulatory DMD boys (mean age at examination 9 years and 10 months) and 24 DD males (mean age 10 years) were selected. Diagnosis for DMD children was based on onset of progressive weakness before 5 years, elevated serum creatine kinase levels (25-30x the normal limits), and confirmed by either molecular analysis of DMD gene or by significantly reduced expression of dystrophin in sarcolemma in muscle biopsy, or by both. All children lived at home, spoke Italian as a primary language and were willing to participate in the study. Additional inclusion criteria for participation were: i) general intelligence level within normal values (that is, within 1 standard deviation (SD) from the mean for their specific age group as assessed by Raven Colored Progressive Matrices' score); ii) good physical health (a part from impairment related to DMD); and iii) regular school attendance.

As a control group for reading disabilities, we selected 24 DD children who met the following criteria: i) general intelligence level within normal limits (defined as stated above); ii) impaired performance on standardized reading tests (a score two or more standard deviations below the mean of the normative sample on at least one of three reading decoding skill tests); iii) regular school attendance; iv) absence of adverse conditions in pre-, peri-, and post-natal clinical history and v) absence of neurological abnormalities established from a standardized neurological examination. According to a semi-structured interview, DD children were divided into two 12-subject groups, depending on a documented presence of Language Delay (LDD) or absence (noLDD). Children were labeled LDD if they manifested at least one of the following characteristics: i) no vocabulary burst before 24 months; ii) late (after age 30 months) combinatory use of words; iii) persistent grammatically incomplete sentences after 4 years; and iv) persistent phonological mispronunciations after 4 years (16).

All children underwent a battery of literacy and neuropsychological tests validated in a previous analysis of a DD population (15,18) (see supportive information). Data were collected by some authors (F.G., C.P., G.A.) at the IRCCS Stella Maris from January 2010 to November 2012; all subjects were individually assessed in a quiet room, for 4 to 8 hours, sometimes spread over two days. Subjects were given breaks if required.

Statistical analyses

Scores were considered mildly impaired if they fell between 1-1.5 SD below mean; a performance of 2 SD below mean of normal values was scored as a clear impairment.

One-way and Mixed ANOVAs were used to investigate literacy and cognitive profiles of DMD group and differences between groups on various literacy and neuropsychological tests. Significance was set at $p<0.05$ with Bonferroni post-hoc comparisons.

Correlation analyses were conducted by the Spearman test in order to evaluate the association between literacy skills and verbal and non-verbal neuropsychological abilities (significance at $p<0.05$).

Results

Table 1 summarizes clinical characteristics of DMD and DD patients and genetic features of DMD subjects included.

Case	DMD			NoLDD		LDD	
	Age	Schooling	*DYS* mutations	Age	Schooling	Age	Schooling
1	7y	2	p.m.	7y11m	2	7y	2
2	8y	3	Del 45-50	8y 5m	3	8y	3
3	8y	3	p.m	9y 1m	3	8y	3
4	8y	3	Del 46-47	8y 5m	3	8y	3
5	8y	3	Del 48-52	8y 4m	3	9y	3
6	9y	4	p.m.	9y 5m	4	9y	4
7	10y	5	Del 51-55	10y 11m	5	10y	5
8	10y	5	Del 45-50	10y 9m	5	10y	5
9	10y	5	Del 45-47	10y 9m	5	10y	5
10	10y	6	Del 51-52	11y 4m	6	12y	6
11	11y	6	Del 46-47	12y 1m	6	12y	6
12	11y	6	Del 49-52	12y 1m	6	11y	6

DMD: Duchenne Muscular Dystrophy; **noLDD:** Developmental Dislexia without language delay; **LDD:** Developmental Dislexia with language delay; ***DYS*:** Dystrophin; **DEL:** deletion; **p.m.:** point mutation.

Intelligence Profile

Mean Raven Coloured Progressive Matrices' score (CPM) was within normal in DMD group (z = -0.51, SD=1.20) and did not significantly differ (F (2.32) = 1.98, ns) from that of DD groups (z = 0.15, SD= 0.87 for LDD; z = 0.22, SD=0.84 for noLDD).

In the DMD group, mean Full Scale IQ at WISC-III was within borderline range (79.7, SD =15.9) but with a slight difference between performance and verbal quotients. Mean Performance IQ was within normal limits (PIQ=85.1) while Verbal IQ fell to within borderline range (VIQ= 78.8; F=8.56, p<.05). When considering each subtest, Information, Arithmetical Reasoning, Comprehension and Coding had the lowest mean group score (1SD below the population mean). The highest scores were observed for Figure Completion, Picture Arrangement and Block Design.

Reading and writing profile of DMD group

Individual scores as well as means and standard deviations for different literacy skills in DMD children are reported in Table 2.

Table 2. Individual scores and means and standard deviations (SD) for reading and writing skills in the DMD group.

Case	Reading speed			Reading accuracy			Text Comp	Writing	
	W	NW	Text	W	NW	Text		W	NW
1	0.23	-	-0.26	-5.50		-3.90	-2.05	-2.89	
2	-5.21	-2.37	-0.33	-1.14	-3.00	0.23	0.48	-	
3	0.18	-1.00	-0.94	-0.50	-0.20	0.26	0.06	-1.50	1.00
4	0.69	0.64	0.46	-0.50	0.20	-0.72	-1.15	-1.50	-0.33
5	-2.98	-1.33	-1.70	-4.25	-1.20	-2.62	-0.90	-5.50	-0.67
6	1.03	0.18	0.81	-0.67	0.00	0.40	-0.42	0.00	0.00
7	0.28	0.08	0.04	-0.86	0.02	0.90	0.10	0.21	1.37
8	0.33	0.62	0.00	1.00	0.25	-0.03	-0.50	0.00	1.00
9	-0.74	-0.38	0.98	-0.33	-0.50	0.31	-0.73	0.00	-0.67
10	-3.65	-3.25	-1.60	-0.97	-0.74	-0.34	-1.10	-0.85	0.73
11	-2.24	-1.75	-1.69	-0.89	0.55	-1.41	0.46	-2.70	-0.31
12	0.24	0.30	0.19	-0.22	0.27	-0.10	-1.27	1.00	0.00
Mean	-0.99	-0.73	-0.34	-1.20	-0.43	-0.58	-0.58	-1.25	0.21
SD	2.02	1.29	0.95	1.83	0.98	1.41	0.74	1.87	0.75

W: words; **NW**: non words; **Text comp**: text comprehension.

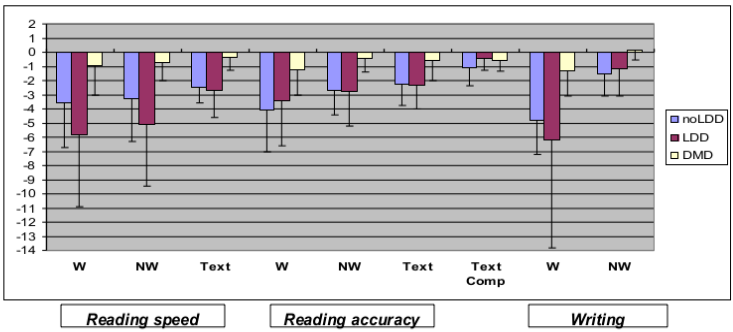
Most cases presented a mild-to-clear impairment in at least one literacy measure and only 4 subjects (individuals 6, 7, 8, and 9) had average values.
On a group level, all literacy measures, with the exception of non-word spelling, were below the population mean but only reading accuracy for words

and spelling for words fell below 1SD. Repeated ANOVA on word decoding (accuracy and speed), word spelling and reading comprehension did not show any significant differences among these measures (F (3.30) = 0.80, ns).

Comparison of reading and writing difficulties between DMD and DD groups

Means and standard deviations of z scores obtained in literacy measures of DMD, LDD and noLDD groups are reported in Figure 1**.**

Figure 1



DMD: Duchenne Muscular Dystrophy; **noLDD:** Developmental Dyslexia without language delay;
LDD: Developmental Dyslexia with language delay; **W:** words; **NW:** non words; **Text comp:** text comprehension

From a descriptive point of view, all groups had the worst scores in word reading, reading speed and accuracy, and in word spelling.
A two-way ANOVA, with group as unrepeated factor and literacy measure as repeated factor,–revealed a significant effect of the group ($F(2.27) = 7.3$, $p<0.005$) and an interaction between literacy measure and group ($F(3.81) = 2.47$, $p<0.05$). DMD children performed better than LDD in all literacy measures whereas they did worse than noLDD in text comprehension, though a post-hoc analysis revealed that these differences were not significant**.**

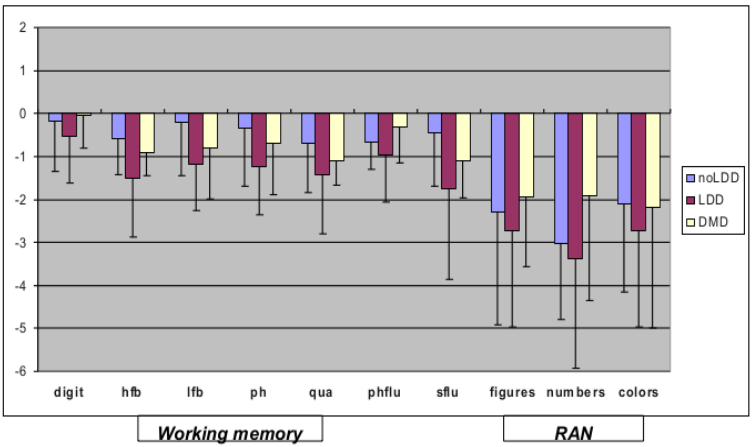
Neuropsychological profile of DMD and DD groups

Means and standard deviations of z scores obtained in verbal neuropsychological measures of DMD, LDD and noLDD groups are reported in Figure 2**.**

There was a clear speed deficit in all three Rapid Automatized Naming tasks (RAN tests) in DMD boys, each performed about 2SD below normal. On an individual level, 58-66% of subjects appeared to be severely compromised (< -2SD) depending on the type of stimuli (figures, numbers and colours). On a group level, a mild impairment was also found in semantic fluency and in short term memory (in particular in repeating quadrisillabic words).
Statistical analyses comparing the neurophsycological profile in verbal domain (RAN, Working Memory, Verbal Fluency, Digit Span) of DMD to both DD groups showed a significant measure effect ($F(5.90) = 6.9$, $p<0,0001$) whereas effect of group ($F(2.18) = 0.25$, ns) and the

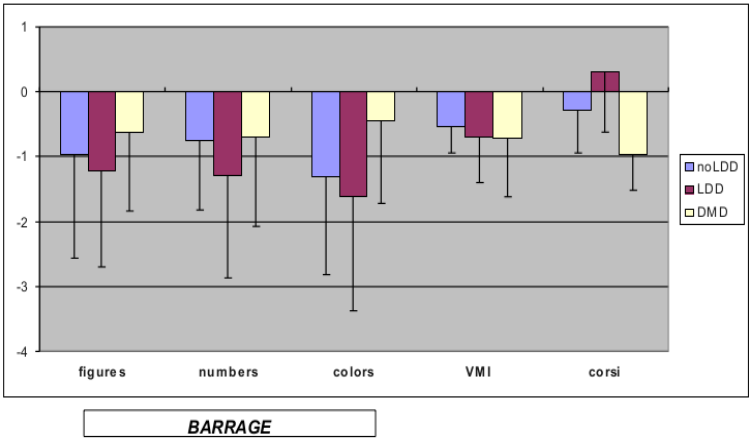
group by measure interaction (F (10.90) =0.92, ns) were not significant. All subjects in the three groups presented a clear RAN speed deficit.
Means and standard deviations of z scores obtained in the non-verbal neuropsychological measures of DMD, LDD and noLDD groups are reported in Figure 3.

Figure 2



DMD: Duchenne Muscular Dystrohy; **noLDD**: Developmental Dislexia without language delay; **LDD:** Developmental Dyslexia with language delay; **digit:** Digit span; **hfb:** two syllabe high frequency words; **lfb:** two syllabe low frequency words; **ph:** phonologically similar words; **qua:** four syllabe high frequency words; **phflu:** phonologic fluency; **sflu:** semantic fluency.

Figure 3



DMD: Duchenne Muscular Dystrophy; **noLDD:** Developmental Dyslexia without language delay; **LDD:** Developmental Dyslexia with language delay; **VMI:** Beery Developmental Test of Visual-Motor Integration; Corsi Block-tapping Test.

The group of DMD boys scored slightly below population mean on all non-verbal measures in speed visual scanning (barrage tests) and in visuo-motor integration tasks (VMI) with the worst performance being in visual memory (Corsi test). Comparing DMD and DD revealed that visual-scanning skills were

predominantly impaired in DD subjects, VMI was within normal limits in all groups, and there was a slight weakness in Corsi test for DMD individuals.
A Mixed Anova was conducted to compare DMD group to both DD groups in the non-verbal domain (Barrage, VMI, Corsi test). Measure ($F(3.54) = 0.78$, ns), group ($F(2.18) = 0.04$, ns) and group by measure interaction ($F(6.54) = 0.93$, ns) did not show any significant differences.

Neuropsychological measures correlated to reading and writing skills

Results from correlation analyses were similar between DMD and DD groups when analyzed together. In the DMD group, RAN speed was the only measure significantly associated with reading decoding speed (r_s ranging from 0.61 to 0.65) and accuracy (r_s ranging from 0.60 to 0.78). For text reading decoding accuracy, however, other significant concurrent predictors were found: PIQ ($r_s = 0.73$) and visual scanning speed (r_s from 0.79 to 0.82). Spelling was correlated to phonological memory measures (r_s from 0.67 to 0.68). These measures did not vary significantly for the DD group, where RAN speed (r_s from 0.53 to 0.61), together with phonological processing measures (r_s from 0.51 to 0.88), and visuo-spatial tasks (r_s from .48 to .64) predicted reading decoding speed. Reading decoding accuracy was correlated to RAN speed (r_s from 0.43 to 0.50) and phonological processing ($r_s = 0.53$). Results were also similar for spelling measures with RAN speed and phonological processing variables as concurrent predictors (r_s from 0.55 to 0.84).

Discussion

This study was designed to verify the presence and characteristics of literacy deficits in DMD children and analyse the neuropsychological profiles associated with reading abilities when compared to similarly aged DD children. DD individuals with and without a documented history of language delay were included because delayed verbal communications are associated with different cognitive and neurofunctional profiles (16,18).

Our DMD sample had non-verbal intelligence within lower limits of normal range and verbal intelligence within borderline range, although motor impairment typical of DMD was expected to interfere with non-verbal tasks. This profile is in agreement with earlier data reported by others (3) and closely resembles the patterns seen in DD children (19). Analysis of subtests through Wisc-III revealed the lowest performances in tasks involving arithmetic, verbal comprehension and processing speed although large inter-subject variability was noted. This profile is also in agreement with previous studies (7) and suggests that school-age DMD children have greater difficulties in maintaining and processing on-line information.

Given a normal non-verbal intelligence, a comprehensive evaluation of reading, spelling and text comprehension showed relevant weaknesses in all these abilities. The incidence of reading and spelling difficulties in DMD group was about 30% higher than expected value in the normal population. Nevertheless, a high inter-subject variability characterized the sample with 8 individuals being clearly deficient in more than one domain and four scoring better than normal

similarly age children. These results confirm previous data documenting the existence of literacy difficulties in DMD regardless of the presence of a clear cognitive impairment (7). Furthermore, our study adds evidence to the notion that learning difficulties in DMD individuals tend to be specific though not single-domain, as the impairment may affect both reading (speed or accuracy), writing and text comprehension. Considering that literacy difficulties found in DMD resemble those documented in DD children, we reasoned that only the direct comparison between children affected by these two disorders could clarify the possible cognitive causes of their impaired reading abilities. Although DMD scored better than the two DD groups, DMD and DD groups shared the worst performances in reading decoding and spelling. Moreover, the presence of a greater impairment in accuracy rather than in speed of decoding words and text, creates a DMD profile very similar to that described in LDD subjects (15,16).

The profile previously described could result from a compromised sublexical strategy of reading and from a deficit in phonological processing. DMD were clearly deficient in all RAN tests and had mild impairment in some measures of phonological and visuo-spatial processing. As in previous studies (15) and in our own sample, RAN deficit is a common feature in LDD and noLDD, and is closely linked to reading decoding deficits. In particular, it has been repeatedly hypothesised that difficulty in rapid access of lexical information from visual input can explain the deficit in reading speed. This finding sheds new light on literacy difficulties in DMD suggesting that besides mild impairment at the behavioural level, DMD is also

characterized by a clear cognitive deficit accounting for reading decoding difficulties. This hypothesis is further supported by correlation analysis highlighting a strong relationship between rapid access to lexicon and reading decoding both in DMD and DD males. It should be pointed out that RAN is a complex task presumably involving visual search processes, quick and automatized retrieval of phonologic codes from long-term storage, and precisely timed integration between visual and lexical codes and it is quite difficult to determine which components of the RAN task are critically involved in reading impairment (20,21,22).

Another fact emerging from our study is that DMD patients share with LDD only a mild impairment in phonological processing tasks requiring temporary storage and on line elaboration of phonological information. Thus, insufficiently stabilized phonological representations can also contribute to difficulties in decoding accuracy.

Finally, we also observed in DMD low performances in visual tasks requiring visuo-spatial elaboration while visual scanning procedures are mostly preserved. This impairment might be an additional contributor to explain literacy difficulties.

In summary, this study shows that a multi-component cognitive deficit may contribute to specific literacy difficulties in DMD with rapid access to lexicon and phonological processing being the core deficits in our subjects. As a consequence, a careful evaluation of these specific abilities should become part of periodic clinical evaluations of DMD children in preschool years and prompt rehabilitation of those components which could lead to deficiencies should be provided.

Although the exact brain function is largely unknown, it is tempting to hypothesize a direct role of dystrophin (or its cerebral isoforms) in the cortical circuits involved in reading as seen in DD. Analyses of larger sample groups and further studies on functional neuroimaging (fMRI) could help establish possible genotype correlations and more precisely verify learning deficits in DMD. This information would proved to be extremely useful in accurately evaluating preschool children before the overt appearance of muscular manifestations and promote a timely rehabilitation of weaker tasks.

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Conclusions and future directions

Conclusions

The PhD work summarized in this thesis aimed at a full integration of clinical competence and advanced diagnostic procedures that are needed to achieve a correct diagnosis in neuromuscular disorders (NMD).
The contribution outlined in several peer-reviewed articles and original data awaiting submission further suggests that muscle MRI could be an useful, non-invasive approach to select the more appropriate genetic and biochemical investigations. Thus, it can be used as an additional tool in the diagnostic flow chart for the improvement of the differential diagnosis. Muscle MRI analysis also plays a leading role on the assignment of a pathogenic value to new mutations as well as on the recognition of muscular involvement in pre-symptomatic or not completely defined phenotype. Last but not least, it also offers useful information for a better understanding of the related pathogenesis.
With this final aim and to go through its conventional use we have conducted a study focused on research of patterns not only related to a specific gene but different pathogenetic pathway. In fact, we have looked at markers capable to distinguish different forms in spite of an apparently similar clinical presentation. This innovative approach brought us to pinpoint discrete alterations of the inner muscle bulks and suggest pathogenetic mechanisms in different forms of NMD.
Additional studies will be necessary to validate data presented in the present work, eventually with a higher “zooming” on muscle structure for example by 7T apparatus.

Future directions

Most of the published studies on inherited NMD employed the short protocol previously described (Mercuri et al 2002). Although this has proved to be very effective for detecting patterns of muscle involvement in young patients, there are a number of other clinical and research issues that could be addressed with the use of a extended protocol in older patients. A more comprehensive protocol (e.g., with T2 and STIR sequences) would not only provide valuable information on the possible presence of oedema (which may identify early stages of muscular involvement as shown in Duchenne Muscular Dystrophy), but could also supply important clues as to the pathology of the muscles and the mechanisms of muscle damage. For example, STIR sequences may help to highlight pathology in muscles that appear normal on T1 images by removing the signal originating from fat, or contribute to clarify the nature of the abnormal signal in different muscle disorders due to different gene and protein defects, allowing for a pre-symptomatic diagnosis.

The recently introduced advanced and quantitative MRI methods including T2 relaxation time measurements, muscle fat quantification using the 3-point Dixon technique, magnetic resonance spectroscopy, and perfusion imaging are quite promising as well, but also represent a future challenge in terms of quantification and specification of the disease process and monitoring. Quantification of muscle volume by muscle MRI and advanced quantitative MRI methods may be used for controlling the safety and efficacy of (experimental) treatment. Its application in a number of the ongoing

therapeutic trials, where cross-centre comparability of results is a prerequisite, has been greatly facilitated by the development of standard operating procedures for these protocols by a multinational, multi-platform working group under the auspices of the international initiative TREAT-NMD.

The limited number of sensitive outcome measures in this field and the need to avoid invasive techniques in a patient population where muscle function is already compromised makes MRI particularly attractive in this regard, and this is reflected in an increasing interest in quantitative MRI outcome measures from a regulatory and pharmaceutical industry perspective.

Considering the paediatric population only, we have to exploit new diagnostic protocols associated with muscle MRI, however, in order to make the examination suitable and not long-lasting.

It could be of interest adopting a protocol of quantitative image analysis to evaluate muscle fat infiltration for grading different forms of muscle disorders, and avoiding needs for additional sequences that expand time of analysis.

Moreover new advanced techniques such as ultra-high-field MRI (UHF-MRI), with their greater signal-to-noise ratio and spatial resolution, could offer increased image quality and promoting new and earlier information on muscle damage.

Furthermore, the increased spectral resolution and the greater sensitivity to low-gamma nuclei available with UHF-MRI should facilitiate use of techniques such as 1H and 31P-MRS improving biochemical and physiological imaging of skeletal muscle.

Two project exploiting the use of UHF-MRI have been designed and submitted to national funding agencies in order to understand its feasibility and its possible use in clinical practice with the final aim to deepen our knowledge of pathogenesis and potential targets.
In particular, we have defined an original protocol to integrate data from ultra high deep-sequencing (next generation sequencing, NGS) technology of several cases presenting with limb-girdle muscle weakness and dystrophy and those emerging from UHF-MRI, followed by the dissection of the molecular pathways involved. NGS currently offers new opportunities to diagnose NMD, confirm the pathogenetic role of new gene variants, and evaluate better gene expression profiles and molecular interactions in primary muscle disorders. The use of UHF-MRI and gene expression studies might help to investigate the pathogenetic pathways underlining muscle pathology and to address differential diagnosis.
Moreover, this study design could identify novel molecular pathways and new potential therapeutic targets.

Finally, another possible future application of myoimaging in NMD would be to perform neurofunctional MRI acquisition for analyzing and comparing the alteration of neurofunctional circuit in different diseases with brain involvement, as in DMD for example.
Although its exact function in the brain is largely unknown, it is tempting to hypothesize a direct role of dystrophin (or its cerebral isoforms) in the cortical circuits involved in reading as seen in dyslexic patients

in our own neuropshycological study. Analyses of larger sample of DMD children and further studies on functional neuroimaging (fMRI) could help establishing possible genotype-phenotype correlations and more precisely verify learning deficits in DMD. This information has the lilihood to impact on evaluation of DMD toddlers in preschool years before the overt appearance of muscular manifestations and promote a timely rehabilitation of weaker tasks.

Publications and scientific communications

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