MARIA DO ROSÁRIO NETO DOS SANTOS

PERFIL GENÉTICO E EPIDEMIOLOGIA DE DISTROFIAS MUSCULARES EM PORTUGAL

GENETIC PROFILE AND EPIDEMIOLOGY OF MUSCULAR DYSTROPHIES IN PORTUGAL

Tese do 3º Ciclo de Estudos conducente ao Grau de Doutor em Ciências Farmacêuticas, especialidade de Biologia Celular e Molecular, apresentada à Faculdade de Farmácia da Universidade do Porto

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As distrofias musculares (DM) são um grupo de doenças neuromusculares, clínica e geneticamente heterogéneas, que se caracterizam pela perda progressiva de força e massa muscular, e com alterações distróficas na biópsia muscular. A idade de início, entre o período perinatal e a idade adulta, a progressão e o prognóstico são muito variáveis entre as diferentes formas e subtipos; a clínica manifesta-se entre mialgias ou ligeira miopatia, e hipotonia generalizada à nascença ou fraqueza muscular altamente incapacitante, muitas vezes resultando em falência respiratória e/ou cardíaca.

Presentemente são conhecidos mais de 80 genes ou *loci* envolvidos nas distrofias musculares, entre os quais se contam alguns dos maiores genes humanos e/ou produtos. O conhecimento crescente das proteínas deficitárias subjacentes, as suas localizações e funções, tem alterado a classificação das DM, que passaram do conceito fenotípico para uma categorização com base molecular. Este conhecimento também revelou que diferentes mutações num mesmo gene podem originar diversas doenças, tal como uma mesma doença pode dever-se a mutações em diferentes genes que codificam proteínas com funções similares.

Devido à sobreposição de fenótipos e genótipos entre as diferentes formas, em muitos casos é uma odisseia para se conseguir o diagnóstico correto. Além disso, a frequência das diversas formas varia entre populações, influenciando a abordagem do diagnóstico em cada país. De forma a determinar o melhor algoritmo de diagnóstico das distrofias musculares em Portugal, foram estudados 615 probandos/famílias, referenciadas de todo o país nos últimos 25 anos, com suspeita clínica das três principais formas com sobreposição fenotípica e genotípica: as distrofinopatias (*Duchenne/Becker Muscular Dystrophy* - D/BMD), as distrofias das cinturas (*Limb-Girdle Muscular Dystrophy* - LGMD) e/ou as distrofias musculares congénitas (*Congenital Muscular Dystrophy* - CMD).

Análises aprofundadas possibilitaram a caracterização molecular de 546/615 doentes (89%), permitindo um panorama epidemiológico realístico. A distribuição entre os três grupos foi: 284 D/BMD, 195 LGMD e 67 CMD, representando respectivamente 91%, 76% e ~75% dos casos rastreados para os genes candidatos de cada grupo de patologias.

Entre as D/BMD foram identificadas 39 novas variantes. A distribuição por tipo de mutação foi semelhante ao descrito noutras populações, havendo no entanto uma maior proporção de duplicações e deleções nos nossos doentes, devido aos estudos mais extensos que incluíram a análise de cDNA.

Entre as LGMD foram identificadas 44 novas variantes. Contrariamente ao descrito noutros estudos, o subtipo mais frequente foi o da γ-sarcoglicanopatia (~29%), seguido da disferlinopatia (~28%) e da calpainopatia (11%) – sendo estas últimas as mais frequentes na maioria dos países do ocidente. Não é expectável que a taxa de mutação nestes genes varie entre populações, pelo que os nossos resultados são influenciados por efeitos fundador; primeiro envolvendo uma mutação privada no γ-sarcoglicano (na etnia cigana Roma), e segundo devido a duas mutações novas na disferlina - uma privada e outra com um foco geográfico.

Foram identificadas 35 novas variantes nos genes associados às CMD. A maioria de doentes (~78%) era do subtipo MDC1A, com laminina-α2 (ou merosina) deficitária. Noutros países Europeus, estudos semelhantes registaram este subtipo em 20-40% dos doentes com CMD. A maior proporção de MDC1A nos nossos doentes com CMD deveu-se a uma nova deleção *out-of-frame*, detetada em 23% dos doentes com merosinopatia. Outro aspeto importante deste estudo foi o estabelecimento de novas correlações genótipo-fenótipo; particularmente, em dois doentes com uma forma associada à proteína *fukutin-related*, o que permitiu alargar o espétro mutacional e fenotípico, acentuando a dissipação da divisão clínica entre as LGMD e as CMD.

Uma das dificuldades do diagnóstico molecular é a averiguação da patogenicidade de uma variante que ainda não está descrita. A informação respeitante às 118 novas mutações detetadas no nosso coorte foi disponibilizada à comunidade científica através das respectivas bases de dados públicas, específicas de *locus* (*Locus-Specific DataBases* - LSDBs). Para este fim, foi necessário criar e implementar novas LSDBs para cinco *loci*, estando as mesmas ainda sob a nossa responsabilidade.

Foi também criado o Registo Nacional de Doentes Neuromusculares, em articulação com a base de dados global da organização TREAT-NMD (*Translational Research in Europe - Assessment & Treatment of Neuromuscular Diseases*). Este registo irá facilitar o recrutamento de doentes candidatos aos ensaios clínicos das novas terapias dirigidas a mutações específicas. Simultaneamente, o registo da apresentação clínica, progressão e respectiva mutação causal, ajudam a esclarecer as correlações genótipo-fenótipo. Numa perspetiva clínica, o diagnóstico diferencial de um subtipo específico de distrofia muscular permite refinar a vigilância médica, com o planeamento e a administração de medidas antecipatórias ajustadas e específicas para cada doença, proporcionando assim uma melhoria geral em termos de progressão, sobrevivência e qualidade de vida dos doentes.

Palavras-chave: distrofias musculares, heterogeneidade genética, heterogeneidade clínica, diagnóstico, LSDB, Registos.

ABSTRACT

The muscular dystrophies are a clinically and genetically heterogeneous group of neuromuscular disorders, characterized by progressive muscle wasting and weakness, sharing similar dystrophic changes on muscle biopsy. The age of onset, severity, rate of progression and prognosis vary widely among the various forms and subtypes; onset ranges from the perinatal period through to adulthood, and clinical manifestations range from myalgia or mild myopathy to generalized hypotonia from birth or highly disabling muscle weakness in childhood, often leading to respiratory and/or cardiac failure.

There are currently over 80 genes or *loci* known to be involved in muscular dystrophy, which include some of the largest known human genes and/or products. The growing knowledge of the underlying protein defects, their localization and function, has shifted the classification of the muscular dystrophies from a phenotype-driven towards a more molecular-based categorization. This knowledge has also revealed that mutations in the same gene can result in different disorders and, conversely, individual phenotypes can arise from mutations in different genes encoding proteins sharing similar cellular functions.

Because of the overlapping phenotypes and genotypes among the different forms, in many cases achieving the correct diagnosis can be an odyssey. Moreover, it has been seen that the frequency of some forms varies widely in different populations, influencing the diagnostic approach in different countries. In order to determine the most appropriate algorithm for the diagnostic work-up of muscular dystrophies in Portugal, a total of 615 unrelated probands/families were studied, referred from the entire Country over the past 25 years, with clinical suspicion of the three principal forms that present phenotypic and genotypic overlap: the dystrophinopathies (Duchenne/Becker Muscular Dystrophy - D/BMD), the limb-girdle muscular dystrophies (LGMD) and/or the congenital muscular dystrophies (CMD).

Extensive analyses enabled full molecular characterization of 546/615 cases (89%), providing a realistic epidemiological picture. Distribution among the three groups of disorders were 284 D/BMD, 195 LGMD and 67 CMD, respectively representing 91%, 76% and ~75% of the patients screened for candidate genes within each disease group.

Among the D/BMDs, 39 new variants were identified. The general distribution according to mutation type was similar to that previously reported for other populations, although in our group of patients slightly higher proportions of gross duplications and point mutations were detected, as a result of extensive studies that included cDNA analysis.

Among the LGMDs, 44 new variants were identified. Contrary to that described in other studies, the most frequent subtype was found to be the γ-sarcoglycanopathies (~29%), followed by the dysferlinopathies (~28%) and only then the calpainopathies (11%) – the latter being the most frequent in most western Countries. Mutation rates in each of these genes are not expected to differ between populations, so that our results were influenced by founder effects; in the first instance involving a private γ-sarcoglycan mutation (in the Gypsy Roma), and secondly involving two new dysferlin mutations – one private and the other with a geographical focus.

A total of 35 novel variants were identified in genes associated with CMD. The MDC1A subtype, with laminin-α2 (or merosin) deficiency, accounted for the majority of patients (~78%). This subtype reportedly comprises 20-40% of CMD patients, in similar studies carried out in other European Countries. The higher proportion of the MDC1A subtype among our CMD patients results from a founder effect involving a new out-of-frame deletion, which was found in 23% of the patients with merosinopathy. Another important outcome of this study was the establishment of new genotype-phenotype correlations; in particular, two patients presenting a fukutin-related proteinopathy revealed a wider mutational and phenotypic spectrum than had hitherto been recognised, and contributed towards further dissipation of the boundaries between the LGMDs and the CMDs.

One of the difficulties in genetic diagnosis is the ascertainment of pathogenicity of undocumented variants identified in the patients. Information on all 118 new pathogenic mutations identified in this cohort was made available to the scientific community via the respective locus-specific databases (LSDBs). To that end, it was necessary to create and implement new LSDBs for five additional *loci*, all of which are still curated by us.

A trial-ready National Patient Registry for Neuromuscular Disorders was also created, in articulation with the global database of the network TREAT-NMD (*Translational Research in Europe - Assessment & Treatment of Neuromuscular Diseases*). This will facilitate the identification and recruitment of our patients who are candidates for the various mutation-centred therapeutic trials. Concomitantly, the annotation of the clinical presentation, course and respective causal mutation, have led to a better understanding of genotype-phenotype correlations. From a clinical perspective, differential diagnosis of a specific subtype enables the refinement of patient surveillance, with the planning and provision of appropriate anticipatory disease-specific interventions, ultimately improving the clinical course, survival and quality of life of the patients.

Key-words: muscular dystrophies, genetic heterogeneity, clinical heterogeneity, diagnosis, LSDB, Registries.

INDEX

3'UTR…….. 3' untranslated region

6MWT……..… 6-minute walk test

ABD1 / ADB2……. Actin-binding domain 1 / actin-binding domain 2

AD…… Autosomal dominant

α-Db-2…… α-dystrobrevin-2

α-DG…… α-dystroglycan

α-DTN…….. α-dystrobrevins

α-SG……α-sarcoglycan

β-DG…… β-dystroglycan

β-SG…… β-sarcoglycan

γ-SG…….. γ-sarcoglycan

δ-SG……δ-sarcoglycan

AD-EDMD……Autosomal dominant Emery-Dreifuss muscular dystrophy

AFT grade……...Arm function test grade

AON…… Antisense oligonucleotides

AR…… Autosomal recessive

AR-EDMD……... Autosomal recessive Emery-Dreifuss muscular dystrophy

AUG codon……. Transcription initiation codon

BC……Before Christ

bp…… Base pair(s)

c. …… *Circa*

CAPN3……Calpain-3

CAT scan…… Computational axial tomography scan

CC domain……..Coiled coils domain

cDNA…….. Complementary DNA

CFTD……Congenital fibre type disproportion

CK……Creatine kinase

cM…… CentiMorgan

CMD……Congenital muscular dystrophy

CMD 1G…… Dilated Cardiomyopathy type 1G

CMD-3B……..X-Linked dilated cardiomyopathy

CMD-CD……..Dilated cardiomyopathy with conduction defects

CMH 9…… Familial hypertrophic cardiomyopathy type 1G

CMT…… Charcot-Marie-Tooth

CNS…… Central nervous system

DAG1…….. Dystroglycan gene

DAPI staining……..4',6-diamidino-2-phenylindole, dihydrochloride staining

D/BMD…… Duchenne / Becker muscular dystrophy

DCM……Dilated cardiomyopathy

DGC…… Dystrophin-glycoprotein complex

DGGE…….. Denaturing gradient gel electrophoresis

dHPLC…… Denaturing high-performance liquid chromatography

DM1……..Myotonic dystrophy type 1 (Steinert's disease)

Dp……Dystrophin gene products

DYS……..Dystrophin

DYSF……Dysferlin

EBS-MD…….. Epidermolysis Bullosa Simplex with Muscular Dystrophy

EDMD…….. Emery-Dreifuss muscular dystrophy

EF-hand site……... Helix-loop-helix motif

EM…… Electron microscopy

EMG…… Electromyography

FCMD…….. Fukuyama congenital muscular dystrophy

FPLD…… Familial Partial Lipodystrophy

FSHD……... Facio-scapulo-humeral muscular dystrophy

FVC……..Forced vital capacity

gDNA…….. Genomic DNA

GSGC score……Gait-stairs-Gowers-chair test

HGMD……. Human Genome Mutation Database

hnRNP……. Heterogeneous nuclear ribonucleoprotein

HUGO……..Human Genome Organization

HUMARA…….Human androgen receptor assay

IHC……...Immunohistochemistry

IQ……..Intelligence quotient

JHFT score…… Jebsen hand function test

kb……. Kilobase pairs (thousand base pairs)

kDa…….. Kilodalton

LAMP……...Lysosome-associated membrane protein

LBD1 / LBD2……. Lipid-binding domain 1 / lipid-binding domain 2

L-CMD……. Lamin A/C-related CMD

LGMD…….. Limb-girdle muscular dystrophy

LINE……Long Interspersed Nuclear Element

LOVD…….. Leiden Open Variation Database

LRG……..Locus Reference Genomic database

LSDB……Locus-specific database

MAPH…….. Multiplex amplifiable probe hybridization

Mb / Mbp…… Megabase pairs (million base pairs)

MB-DRM……. Desmin-related myopathy with Mallory body-like inclusions

MD …….. Muscular dystrophy

MDC 1C…….. Congenital muscular dystrophy, muscle hypertrophy, normal CNS

MDDGC……...Muscular dystrophy-dystroglycanopathy

MEB……. Muscle-eye-brain disease

MFM3…….. Myofibrillar myopathy type 3

MFM test……. Motor function measure test

MIM…… Mendelian Inheritance in Man

MLPA……...Multiplex ligation-dependent probe amplification

MR……Mental retardation

MRC……Medical Research Council

MRI…….. Magnetic resonance imaging

mRNA……..Messenger RNA

NGS…… Next generation sequencing

NHEJ……Non-homologous end-joining

NMD……Neuromuscular diseases

nNOS……...Neuronal nitric oxide synthase

OMIM…….. Online Mendelian Inheritance in Man

OPMD…… Oculo-pharyngeal muscular dystrophy

PAR-1b…… Polarity-regulating kinase 1b

pre-mRNA……...Pre-messenger RNA

RSMD…….. Rigid Spine Muscular Dystrophy

SB……Southern blot

SCAIP…… Single-condition amplification/internal primer

SCARMD……Severe Childhood Autosomal Recessive Muscular Dystrophy

SCPNK…… Neurogenic Scapuloperoneal syndrome, Kaeser type

SEPN1……Selenoprotein N, 1

SGC…… Sarcoglycan complex

SPN……..Sarcospan

SSCP……...Single strand conformational polymorphism

SYN……..Syntrophin

TGFBR2…….. Transforming growth factor-β receptor-2 gene

TMD…… Tibial Muscular Dystrophy

TP-PCR……...Triplet-primed polymerase chain reaction

TREAT-NMD…….. Translational Research in Europe; Assessment & Treatment of NMD

TRIM32…… Tripartite Motif-containing 32

UCMD…….. Ullrich congenital muscular dystrophy

WB…… Western blot

WES…… Whole exome sequencing

WGM scale…… Walton & Gardner-Medwin test

WW domain…… Domain with two conserved tryptophans

WWS…… Walker-Warburg syndrome

XLCM……...X-Linked dilated cardiomyopathy

XL-EDMD…… X-linked Emery-Dreifuss muscular dystrophy

X-linked…… Mapped to the X chromosome

ZZ domain…….. Zinc finger domain

Gene symbols and products may be found in Tables 1.1.4.1 and 1.1.5.1, and in Appendix I (7.1.1 and 7.1.2).

1. INTRODUCTION

1.1 DEFINITION AND CLASSIFICATION OF MUSCULAR DYSTROPHIES

1.1.1 Historical Background

The muscular dystrophies have been recognised to affect humans since primordial times, as evidenced by some relief paintings on the walls of Egyptian tombs [1, 2]. Drawings dating back to as early as *circa* 2800-2500 BC, witness the existence of club foot, lordosis, muscle pseudohypertrophy and *pes planus* (Figure 1.1.1.1). Over the years, these conditions have been portrayed in works of art, through the periods of Renaissance to Impressionism, as exemplified by Hieronymus Bosch's *Beggars and Cripples* or *The Last Judgment* triptych (c.1505), Raphael's *The Transfiguration of Christ* (c.1520) and the *Sick Boys* by Karl Schmidt-Rottluff (1915). While the first two examples are challenged as being speculative, in the latter the artist specifically refers the affliction of his subjects.

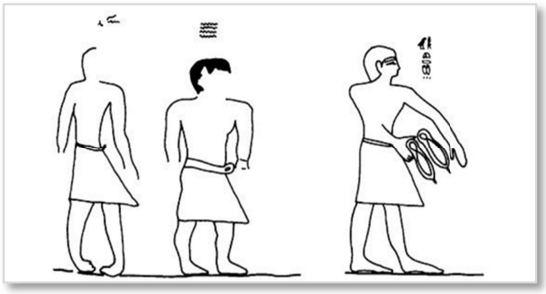

Figure 1.1.1.1
Drawings from a tomb at Beni Hasan.
The figure in the centre is believed to represent an individual with muscular dystrophy.
(In: *Duchenne Muscular Dystrophy,* 2nd Ed. AEH Emery. Oxford Medical Publications, 1993).

Nonetheless, firm knowledge of the early history of muscular dystrophy relies on the recorded clinical descriptions made by several physicians, since the beginning of the nineteenth century.

It is not consensual as to who first described the condition that we now designate as "muscular dystrophy". Many attribute this primacy to the work published by Sir Charles Bell, dating back to 1830 [3], in which he describes a young 18 year old boy with weakness and loss of muscle mass, affecting mostly the quadriceps. The disease had begun in the first decade, with *"…a weakness in the thighs, which disabled him from rising; and it is now curious to observe how he will twist and jerk his body to throw himself upright from his seat*". Sir Charles Bell pursued his studies in disorders of the nervous system, but six decades later this manner of rising from a chair or from the floor was again described in detail by Sir William Gowers, whose name, to this day, remains associated with these characteristic signs of proximal muscle weakness (see below).

Others recognise the first clinical description of muscular dystrophy as being that of Giovanni Semmola who, a year earlier (1829), had delivered a lecture at the Accademia Pontaniana, relaying the finding of two brothers with “muscular hypertrophy”. This, however, was only published in 1834 [4]. The same two cases were documented again in 1836 [5], by the Neapolitan physician Professor Gaetano Conte and his assistant. The brothers were described as having a disorder with onset in the first decade, aggravating over time. They had enlarged calves, progressive muscle wasting and weakness affecting mostly the lower limbs, and subsequently developed flexion contractures, particularly of the knees and hips. Sensory and mental functions were normal. Although unaided by details of muscle pathology, these clinical features are unequivocally consistent with muscular dystrophy, possibly of the common progressive type later eponymously associated with Duchenne de Boulogne (see below).

Further cases were described during the 1840s. Of note is the work by Dr. William John Little, a physician at the London Hospital, who provided some of the first descriptions of the muscle pathology observed in such patients. Essentially, he reported how the muscle tissue had been largely replaced by fat, on necropsy examination of the gastrocnemius and soleus muscles [6].

More reports followed, adding clinical and pathological details. However, the first complete and systematic study of progressive muscular dystrophy is credited to the British physician Sir Edward Meryon. In 1851, in a communication to the Royal Medical and Chirurgical Society of London, published the following year [7], he described the disease in 8 affected boys belonging to 3 families. The familial nature of the disease fascinated him, as well as the fact that the patients were all males. In postmortem exams he described degeneration of the muscle fibres versus the healthy state of the nervous system: “…*The chief structural change existed in the system of the voluntary muscles, which was throughout the entire body atrophied, soft, and almost bloodless; and, although the muscular fibres appeared to exist, yet were they not of that deep red colour as seen in the healthy and natural state*." From further microscopic examinations he stated: “…. *the striped elementary primitive fibres were found to be completely destroyed, the sarcous element being diffused, and in many places converted into oil globules and granular matter, whilst the sarcolemma or tunic of the elementary fibre was broken down and destroyed*.” This latter observation is particularly relevant; only 135 years later was it proven that the primary defect indeed resided in the muscle cytoskeleton (see below). But Sir Edward Meryon’s contribution towards understanding the disease, notably an X-inked form, was shadowed by the French physician Duchenne de Boulogne, 15 years later.

Guillaume Benjamin Amand Duchenne, born in Boulogne-Sur-Mer, became historically recognised as having contributed the most towards understanding the disease. Seen as a lone wolf during his life studies in Paris, he designed the protocol of the meticulous neurological examination, giving accurate descriptions of many neuromuscular disorders, including the muscular dystrophy to which his name is still attached.

Duchenne de Boulogne, as he became known, took an early interest in electrophysiology. He pioneered studies on electrical stimulation of muscles, having built his own machine for neuromuscular stimulation together with the development of surface electrodes. This he used to map all bodily muscles and to study their coordinating action in health and disease. He also invented the harpoon needle for percutaneous sampling of muscular tissue without anaesthesia - a forerunner of today's biopsy procedure – with which he obtained muscle biopsies from live patients. This enabled him to study biopsies from the same patient during different phases of the disease, thereby observing the progression of muscle degeneration.

In 1858 he observed his first patient whom he believed to be affected with “progressive muscular atrophy with degeneration”, as documented in 1861 [8]. He later coined the term “pseudo-hypertrophic muscular paralysis” to describe the disorder, as appears in his 1868 publication of 13 cases [9]. Here he defined the following characteristics: progressive weakness of movement, affecting firstly the lower limbs and later the upper limbs; a gradual increase in the size of the affected muscles; an increase in the interstitial connective tissue with the abundant production of fibrous and adipose tissue in the more advanced stages.

He also noted that this disorder, beginning in infancy or pre-adolescence, occurs more frequently in boys than in girls and sometimes affects several children in the same family. His name thus became associated with X-linked, early onset muscular dystrophy – now commonly known as *Duchenne muscular dystrophy*.

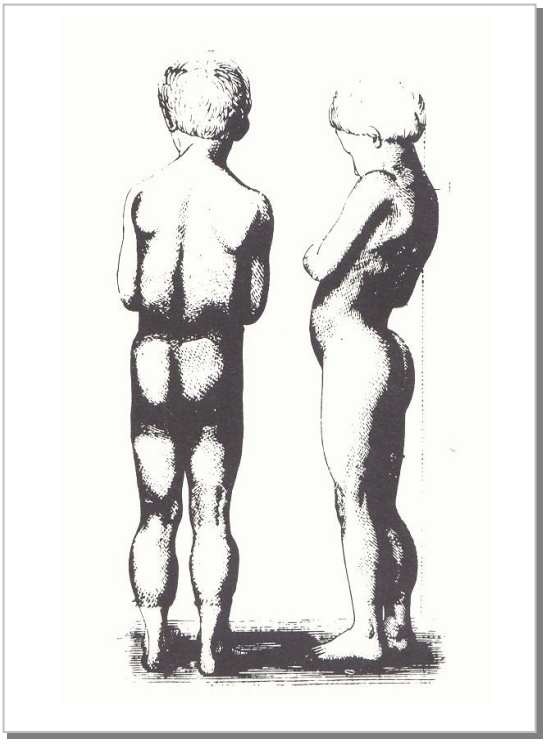

Figure 1.1.1.2
Duchenne de Boulogne’s sketch of his original case, emphasizing the calf enlargement and lumbar lordosis [9].

Another significant contribution towards the clinical identification of these patients was made by the academic neurologist Sir William Richard Gowers.

Gowers was an enthusiastic inventor, having devised several diagnostic instruments including the safety hypodermic syringe, the gridded blood cell counting chamber, the portable ophthalmoscope, the haemocytometer and the first practical haemoglobinometer. He also devised a chromatic reaction to measure glucose content in urine [10]. Being a habile stenographer and etcher facilitated his descriptive annotations on the patients. Besides his classical description of epilepsy (1881), he wrote and personally illustrated the atlas on *Medical Ophthalmology* (1897), but is probably best remembered for his two-volume *Manual of Diseases of the Nervous System* (1886 & 1888), to this day still considered "the Bible of Neurology". Amongst others, he gave his name to the bundle of nerve fibres in the spinal cord, now called the anterolateral fasciculus (Gowers' tract), to the weekly case presentation and clinical teaching sessions at London's National Hospital for Neurology and Neurosurgery (Gowers' Round) [11] and, in the muscular dystrophies, to the clinical sign of proximal lower-limb weakness (Gowers' sign, as described below),

In 1879 he published his studies on 220 cases of muscular dystrophy [12] where, besides other clinical signs, he described in detail the way in which the patients get up from the floor: placing their hands on the floor, spreading and stretching their legs, moving their hands back, supporting one hand on the knee and then climbing with their hands up their thighs until the torso is upright. This movement became known as *Gowers' sign*, or *Gowers' maneuver* (Figure 1.1.1.3).

He also highlighted the preponderance of affected males and the familial nature of the disease, pointing out that family history was always from the mother and never the father's side, and that a woman could have affected boys with different partners. He thus established a comparison with the hereditary pattern in hemophilia, known at the time as Nasse's law.

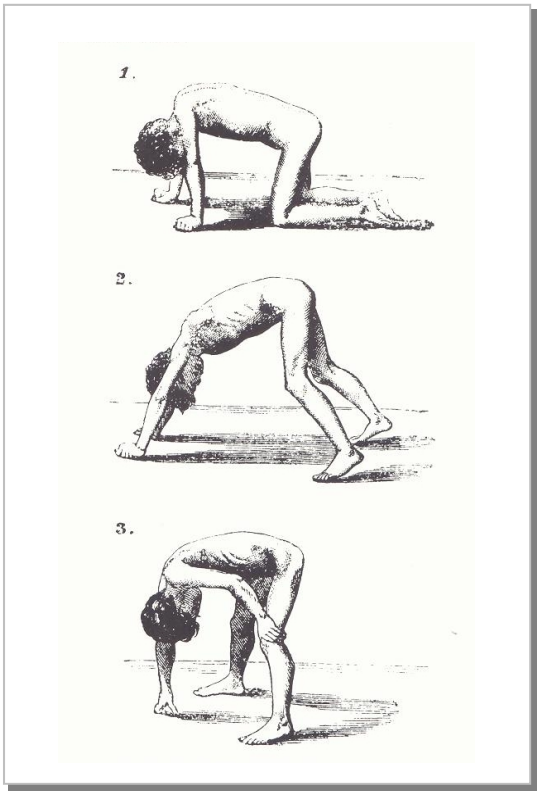

Figure 1.1.1.3

Gowers' maneuver: typical manner of rising from the floor [12].

Wilhelm Heinrich Erb, a physician of Internal Medicine working in Heidelberg, also made major contributions in the field of muscular dystrophy and in neurology, in general. Besides being the first to fully describe *tabes dorsalis* and myasthenia gravis (Erb-Goldflam disease), his name became associated with other neurological entities such as spastic spinal paralysis (Erb-Charcot disease), the Erb phenomenon (electrical instability of motor nerves, in tetany), the Erb point (in cardiologic electro-diagnosis), the Erb sign (degeneration reaction), Erb's Disease (progressive bulbar paralysis) and, most relevantly, Erb's dystrophy – a juvenile scapulo-humeral form, later grouped under the umbrella term "limb-girdle muscular dystrophy".

Erb described the first of these patients with juvenile weakness in 1884 [13], highlighting a clear difference from those described by Duchenne. In this case the disease presented mostly in the second decade of life, had a more benign course and was seen to affect both sexes. The patients had scapular winging, weakness and atrophy of the upper limb-girdle, with initial sparing of other muscles of the body but later also progressing to the lower limb-girdle.

He recognized the disease's close relationship with the previously described "pseudohypertrophy of the muscles" and "hereditary muscular atrophy", which led him to put forward the concept of a heterogeneous group of disorders, all resulting from a primary degenerative process involving muscle tissue. In his 1891 report [14] he wrote "…*on the basis of evident clinical coincidence I have bundled these three forms of disorders to a probable nosological entity for which I propose the name 'dystrophia muscularis progressiva'*." The theory that classed these disorders into the same group was generally well accepted and the term "progressive muscular dystrophy" remains in use to this day.

Erb also outlined the histopathology of affected muscle in his patients with progressive muscular dystrophy: the fibres were of abnormal number, size, shape and distribution, they were atrophic and occasionally hypertrophic, showed splitting, increased nuclei and vacuoles, interstitial fat cells were present and the tissue showed signs of necrosis, regeneration and reactive inflammation. These features reflect the loss of muscle and the necrotic nature of the tissue. In progressive disease, regeneration fails to keep pace with necrosis and the muscle is gradually replaced by connective and adipose tissue. This histological description has withstood the test of time, defining the dystrophic process that is regarded as the one unifying feature of the muscular dystrophies (Section 1.2.2.4).

With the successive detailed description of patients, the following decades saw several attempts to distinguish and classify the different forms of muscular dystrophy. In 1910,

Frederick Eustace Batten (considered the father of pediatric neurology), classified the muscular dystrophies into seven categories [15]: (i) simple atrophy, (ii) Duchenne pseudo-hypertrophic variety, (iii) Erb juvenile weakness, (iv) facio-scapulo-humeral dystrophy, (v) distal myopathy, (vi) myotonic dystrophy and (vii) mixed form. These distinctions, based on age at onset, group(s) of muscles involved at presentation and rate of disease progression, are still recognized today.

Earlier, in 1903, Batten had described 3 cases with "infantile myopathy" [16]. This form of muscular dystrophy was later termed *dystrophia muscularis congenital*. The designation “congenital muscular dystrophy” (CMD) now encompasses a heterogeneous group of genetically, clinically, and biochemically distinct entities, and in essence applies to a form presenting at birth, or within the first few months of life, with a static or slowly progressive muscle weakness, often associated with hypotonia and involvement of the respiratory and bulbar muscles. The CMDs are now further sub-classified on the basis of the presence or absence of structural involvement of the central nervous system (Section 1.1.5).

The category Batten referred to as “Erb juvenile weakness” comprises the currently designated limb-girdle muscular dystrophies (LGMD) – a term coined by Stevenson in 1952 [17] and further expanded by Walton and Nattrass in 1954 [18]. The nosologic entity of limb-girdle dystrophy was formally established by Walton and Nattrass, describing the disease as a progressive muscle weakness affecting both sexes, of autosomal recessive or autosomal dominant inheritance, with atrophy involving predominantly proximal muscles of the pelvis and shoulder, a widely variable age of onset, ranging from the late first to the fifth decade of life, and a generally slow clinical progression.

In this 1954 report on 105 cases, Walton and Nattrass proposed subdividing the muscular dystrophies, in general, into six groups based essentially on muscle weakness distribution (proximal or distal, presence or absence of facial muscle involvement) and on pattern of inheritance: (i) Duchenne, (ii) facio-scapulo-humeral, (iii) limb-girdle, (iv) distal, (v) oculo-pharyngeal and (vi) congenital. This classification was the most widely accepted, well into this century.

In the following year (1955), Emil Becker and Franz Kiener described a progressive muscular dystrophy clinically similar to Duchenne-type, also X-linked, but of later onset, milder and with a slower progression [19]. *Becker muscular dystrophy* (BMD), as it is now known, was thus long held under the umbrella of the LGMDs and thought to be entirely distinct from Duchenne muscular dystrophy (DMD). It wasn’t until the identification of the dystrophin gene, three decades later [20], that these were shown to be allelic and henceforth collectively designated as “dystrophinopathies” (Section 1.1.3).

In the ensuing years, the existence of LGMD as a separate entity was challenged several times. The development of physiological and histopathological means to assess muscular disorders made it evident that this group, as had been defined, included a variety of other, distinct neuromuscular disorders (e.g. spinal muscular atrophies, polymyositis and some endocrine, congenital, mitochondrial and metabolic myopathies). Since the late 1980s, the advancement of molecular biology and immunological methods saw the designation of LGMD subgroups based on the genes involved and/or the deficient components.

A workshop headed by Professor Katharine Bushby, in 1995 [21], reclassified the LGMDs according to mode of inheritance and chromosomal localization. At the time, only one dominant and four recessive loci had been identified and only one gene product was known ("adhalin", now designated α-sarcoglycan). Since then, many more forms have been identified at a rapid pace. The continuous identification of LGMD genes has evidenced a wide clinical and genetic heterogeneity, with mutations in different genes giving rise to similar phenotypes and, conversely, different mutations in the same gene resulting in distinct clinical manifestations (Section 1.1.4).

Around the same time, proposals arose to classify the muscular dystrophies, in general, according to the muscle groups affected at onset (Figure 1.1.1.4). This was extremely useful as a basis for research, and only recently did further elucidation of the molecular pathogenesis question the distinctions made between some of these forms. Besides clarifying their classification, these advances in the field of molecular genetics conferred an unprecedented improvement in the diagnostic accuracy of the muscular dystrophies.

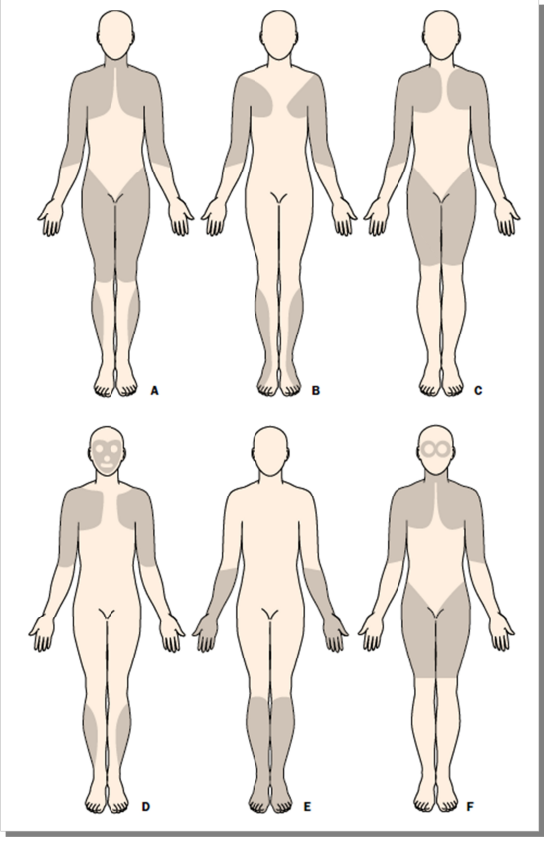

Figure 1.1.1.4
Distribution of predominant muscle weakness in the different main forms of muscular dystrophy: A - Duchenne and Becker type, B - Emery-Dreifuss, C - limb-girdle type, D - facio-scapulo-humeral, E - distal, F - oculo-pharyngeal [22].

1.1.2. The Genetics Era

In 1986 Kunkel and co-workers [20] identified the Duchenne muscular dystrophy gene, named “dystrophin” (*DMD*, MIM# 300377), in Xp21, and provided molecular genetic confirmation of the X-linked inheritance of the disorder. This accomplishment culminated from the efforts of several research groups that had narrowed down the target region on the X-chromosome, resorting to specimens from key patients, linkage analysis and a series of elegant molecular experiments. Based on the gene sequence, the protein product “dystrophin” (DYS) was then identified [23]. *DMD* thus become the first human gene to be identified by positional cloning (“reverse genetics”), whereby a causal gene is identified without prior knowledge of its product.

Significant developments emerged following the identification of the *DMD* gene, marking the transition to the genetics era across the field of neuromuscular disorders.

Shortly after the identification of dystrophin, biochemical studies revealed its tight association with other proteins of various molecular weights (Figure 1.1.2.1). Some of these were glycosylated and further co-purified with dystrophin as a highly stable complex; they co-localised with dystrophin at the sarcolemma and were of decreased abundance in DMD patients. It was concluded that dystrophin functioned as part of a hetero-oligomeric complex, henceforth known as the dystrophin–glycoprotein complex (DGC) [24, 25]. The DGC comprises transmembrane, cytoplasmic and extracellular proteins, providing a strong mechanical link and mediating interactions between the intracellular cytoskeleton and the extracellular matrix [25-27].

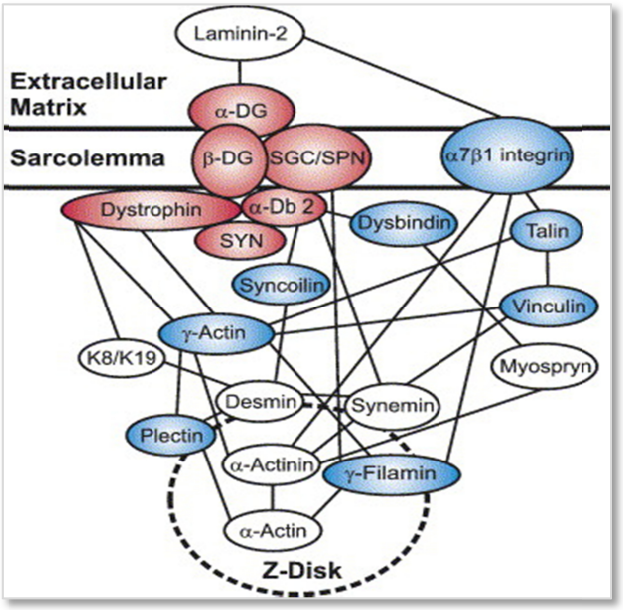

Figure 1.1.2.1
The DGC network. Components of the highly stable core DGC are shown in red: α- and β-dystroglycan (α-DG, β-DG), the sarcoglycan complex (SGC), sarcospan (SPN), α-dystrobrevin-2 (α-Db 2) and syntrophin (SYN). The directly interacting proteins and their binding partners are also shown. Those highlighted in blue are found in increased abundance when dystrophin is absent. (Adapted from [26]).

Since its description, the DGC has emerged as a critical nexus for the understanding of other muscular dystrophies arising from defects in several distinct genes [28]. Currently, over 350 unifactorial neuromuscular disorders are recognized, of which more than 80 are muscular dystrophies (http://www.musclegenetable.org; last accessed March 2015).

It was found that muscular dystrophy could result from mutations in genes encoding a disparate set of muscle proteins with various localizations, such as extracellular matrix, sarcolemma, sarcomere, cytosol, Golgi apparatus and nucleus (Figure 1.1.2.2). Many of the underlying pathogenic mechanisms began to be unraveled; they included physical fragility due to the loss of sarcolemmal structural proteins, perturbation of sarcolemmal repair mechanisms, abnormal post-translational modification, altered signal transduction, chromatin modulation and transcription regulation (Tables 1.1.4.1 and 1.1.5.1).

The extensive clinical and genetic heterogeneity of the muscular dystrophies also became evident; mutations in different genes can give rise to a similar phenotype and so too can mutations in the same gene give rise to highly disparate phenotypes. An example of the former is Emery-Dreifuss muscular dystrophy (EDMD), which may be caused by mutations in the *EMD*, *LMNA*, *SYNE1*, *SYNE2*, *FHL1* and *TMEM43* genes. The converse occurs with the *LMNA* gene, where mutations may cause at least 12 completely different phenotypes, including EDMD, LGMD1B, dilated cardiomyopathy with conduction defects (CMD-CD), Familial Partial Lipodystrophy (FPLD), Charcot-Marie-Tooth disease 2B1, mandibuloacral dysplasia, Malouf syndrome, Hutchinson-Gilford progeria and lethal restrictive dermopathy [29, 30].

A further feature which poses a diagnostic dilemma, with consequences for genetic counselling, is the fact that mutations in the same gene may cause autosomal dominant (AD) or autosomal recessive (AR) disease. This is the case with some AD and AR forms of EDMD, both of which have been associated with mutations in the *LMNA* gene [31].

Knowledge of the role of the different proteins in muscle pathology has been changing the original phenotype-based classification. Concomitantly, this has resulted in ill-defined boundaries both within the clinically-defined muscular dystrophies and between these and the myopathies or even neuropathies [32-34]. Although the disorders may still be grouped according to the classically distinguished main forms (EDMD, LGMD, etc.), they are now mostly referred to on the basis of the underlying protein defect (sarcoglycanopathy, laminopathy, etc.) or underlying pathogenic mechanism (e.g. glycosylation defect).

Figure 1.1.2.2 schematises the location of several proteins involved in three of the main forms of muscular dystrophy with overlapping phenotypes, namely the dystrophinopathies, limb-girdle and congenital forms, which are further detailed in Sections 1.1.3 to 1.1.5.

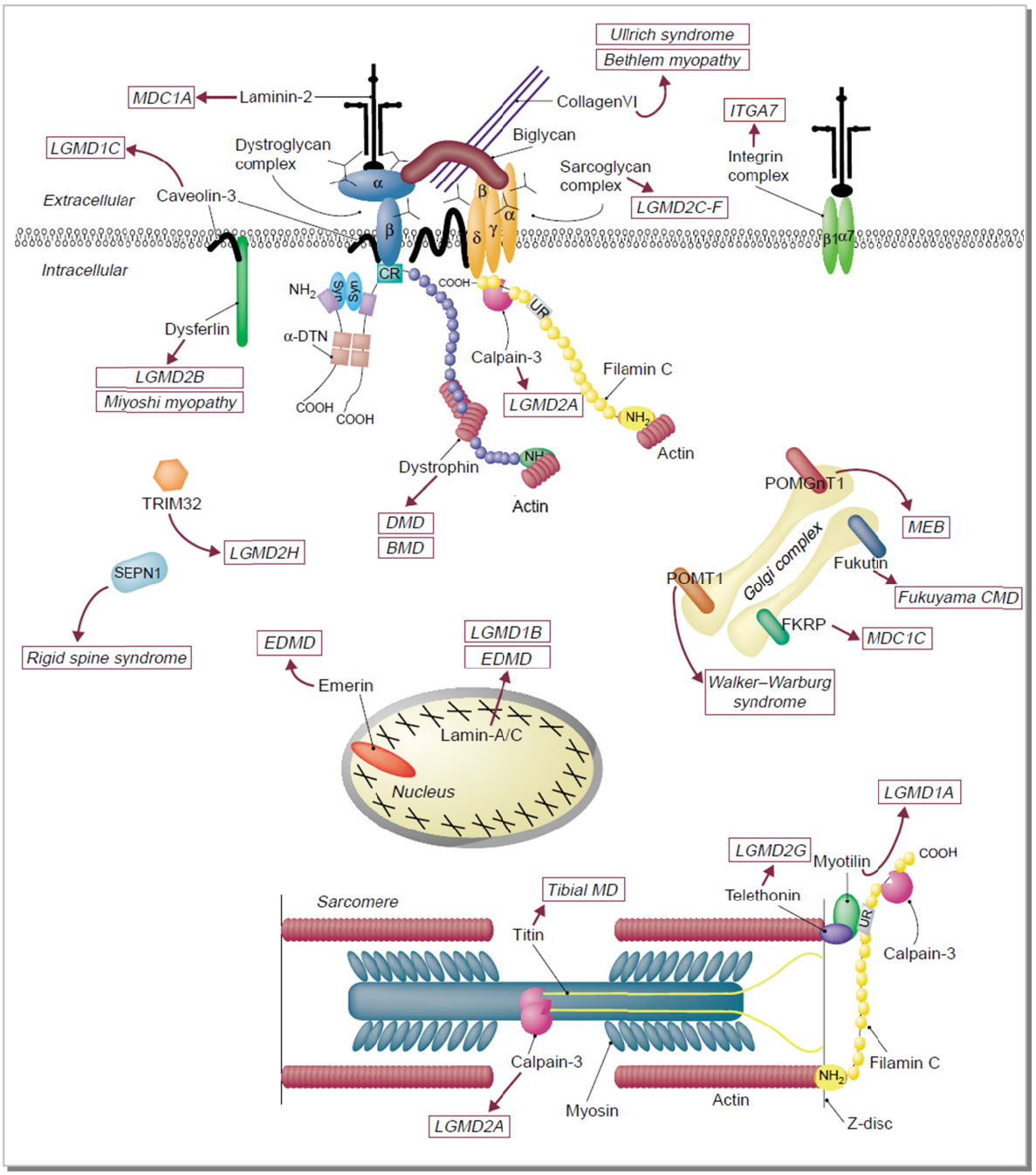

Figure 1.1.2.2

Schematic representation of several proteins associated with muscular dystrophies.

Locations of the various proteins are shown, and red boxes indicate some of the respective disorders with which they are associated. The DGC connects the extracellular matrix to the actin cytoskeleton. Further sarcomeric interactions are shown in the lower part of the figure. Calpain-3 (a muscle-specific Ca^{2+}-dependent cysteine protease involved in the wear and tear of muscle, through sarcomere remodeling and dysferlin-mediated membrane repair) is anchored to the giant structural/scaffold protein titin, when stable and inactive. Four of the proteins involved in the glycosylation process of α-dystroglycan, mediating its binding to the extracellular matrix, are shown in the Golgi complex. TRIM32 (Tripartite Motif-containing 32 – a ubiquitin ligase) and SEPN1 (selenoprotein N,1 – an EF-hand domain-containing protein) exemplify the cytosolic enzymes. Emerin and lamin-A/C, both of which may cause EDMD, exemplify the nuclear proteins.

α-DTN – dystrobrevins; Syn – syntrophins. (Adapted from [35]).

1.1.3. Dystrophinopathies

1.1.3.1 Clinical presentation

Duchenne muscular dystrophy (DMD; MIM# 310200) is a severe X-linked recessive, progressive muscle-wasting disease, affecting approximately 1 in 3500 live male births worldwide. Onset usually occurs before the age of five, with proximal lower limb weakness evidenced by calf hypertrophy, a waddling gait and a positive Gowers' sign. These patients subsequently present difficulties in running, jumping and climbing stairs. Still during the first decade of life, tendon retraction and lordosis sets in, and independent ambulation is eventually lost, with patients becoming confined to a wheelchair usually before the age of twelve. Muscle weakness then becomes more pronounced in the rest of the body, with development of contractures and scoliosis and deterioration of respiratory function. There is also worsening in the short term memory and learning ability of these patients [36]. The inexorable progression of the disease leads to death typically during the second decade of life, often due to respiratory insufficiency or cardiac failure.

In the allelic disorder known as Becker muscular dystrophy (BMD; MIM# 300376), the symptoms are similar to DMD but progression is slower and the severity of the disease is milder, to highly variable degrees. BMD may thus present as a mild form of DMD with later onset [37], a pseudometabolic form characterized by muscle cramps with myoglobinuria [38], a quadriceps myopathy [39] or a basically asymptomatic form showing only calf pseudohypertrophy and elevated serum creatine kinase (CK) levels (hyperCKemia) [37]. Additionally, patients may present an X-linked isolated dilated cardiomyopathy (XLCM / CMD-3B; MIM# 302045) with minimal skeletal muscle involvement [40]. All of these allelic forms are consequent to various degrees of deficiency in the subsarcolemmal protein designated *dystrophin,* and are thus collectively known as *dystrophinopathies*.

The serum CK levels remain 10-200 times higher than normal at least until the age of five, in both DMD and BMD patients, but these levels may decline with advancing age. While nerve conduction studies are normal, the electromyography shows myopathic changes [22].

The muscle biopsy, however, is what provides substantial clues towards a differential diagnosis. It is invariably dystrophic, and with disease progression there is increasing endomysial fibrosis and replacement of muscle by fatty tissue. Immunohistochemistry (IHC) shows abnormal staining for dystrophin, which is absent in DMD and reduced/irregular in BMD [24-28] (Figures 1.2.2.1 and 1.2.2.2). In the case of BMD, immunostaining for dystrophin is particularly useful, since the typical dystrophic changes

develop at a much more protracted pace. However, while positive staining for only certain domains of the protein is indicative of a BMD-type dystrophinopathy (due to a truncated or internally deleted protein), a general reduction in signal may indicate either BMD or may reflect a secondary reduction, consequent to a primary deficiency in other dystrophin-associated proteins [28].

Female carriers of a dystrophinopathy may be totally asymptomatic or present mild muscle weakness with an increased CK, calf hypertrophy, myalgia and cramps, and are particularly at risk of dilated cardiomyopathy [41]. Similarly, the muscle biopsy could be normal or pathological to variable degrees showing a mosaic immunostaining pattern of fibres either containing or lacking dystrophin (Figure 1.2.2.2). Occasionally, carrier females can present the full-blown clinical manifestations of DMD or BMD. These rare situations occur in carriers who simultaneously have Turner syndrome (45,X0 karyotype), carry an X-autosome translocation (where the normal X chromosome is preferentially inactivated), or for some reason present complete skewing from the random X-inactivation pattern [42].

1.1.3.2 The dystrophin gene

The *DMD* gene remains one of the largest genes described to date, spanning ~2,5 Mbp, which corresponds to ~0·1% of the total human genome [43-45]. It contains 86 exons and at least eight promoters, producing transcripts that encode various dystrophin isoforms composed from different segments of the basic sequence (Table 1.1.3.1 and Figure 1.1.3.1). The alternative mRNAs are generated by one of three processes: the use of (i) different, unique and often tissue-specific promoters, (ii) alternative splicing and/or (iii) different polyadenylation sites.

The full-length 14kb mRNA transcribed from *DMD*, driven by the muscle promoter (M), is the main gene product and is predominantly expressed in skeletal and cardiac muscle, with small amounts expressed in the brain [46-50]. The transcription of two other known full-length isoforms, with unique first exons spliced to the common set of 78 exons, is driven by the independent brain and Purkinje promoters - so named by virtue of this being their major, but not exclusive, sites of expression. The unique first exons of both transcripts are spliced directly to the second exon that is found in common with the muscle isoform. As a result, these three full-length dystrophin proteins are all encoded by 79 exons, differ only by a few N-terminal amino acids, and are proposed to be functionally equivalent.

An additional full-length lymphocyte isoform has also been described, but its transcription is negligible, even in peripheral blood lymphocytes, so that its biological significance is questionable [51-53].

The *DMD* gene also produces shorter proteins from at least four internal promoters that use unique first exons spliced into exons 30, 45, 56 and 63, to generate protein products of 260, 140, 116 and 71 kDa respectively [54-59]. The latter product, known as Dp71 (*dystrophin protein of 71 kDa*), is expressed in most adult tissues and is present in cardiac, but not skeletal muscle. Transcripts encoding this C-terminal protein are commonly alternatively spliced to generate multiple products of between 70 to 75 kDa [57]. Additionally, the same promoter gives rise to the much smaller product Dp40, which uses an alternative polyadenylation sequence in intron 70 [60]. Dp40, which thus lacks the normal C-terminus, is expressed in all adult and foetal tissues, in low abundance.

Exon exclusion and exon scrambling give rise to yet other transcripts [50, 67, 68], generating further protein diversity and accounting for the complex expression regulation of the tissue-specific dystrophin functions.

Table 1.1.3.1 Main dystrophin protein isoforms.

Promoter		Isoform			mRNA size (bp)	amino acids	Protein size (kDa)	Expression pattern	Ref.
Symbol	Site and unique 1st exon / 3'UTR	Symbol	Synonyms						
L	5' of B; spliced to exon 3 of M-dystrophin	Dp427l	L-dystrophin	lymphocyte dystrophin	13764	3562	427	Lymphoblastoid	51-53
B	5' of M	Dp427c	brain or C-dystrophin	cortical dystrophin	14069	3677	427	Cortical neurons (low in skeletal and cardiac muscle)	61
M	Between B and intron 1	Dp427m	M-dystrophin	muscle dystrophin	13993	3685	427	Skeletal and cardiac muscle; glial cells	20, 62-64
P	Between intron 1 and intron 2	Dp427p	P-dystrophin	Purkinje dystrophin	14000	3681	427	Purkinje cerebellar neurons (low in muscle)	65, 66
R	Intron 29	Dp260-1	R-dystrophin-1	retinal dystrophin	9916	2341	260	Retina	54
		Dp260-2	R-dystrophin-2		9773	2344			
B3	Intron 44	Dp140			7410	1225	140	CNS; kidney	55
S	Intron 55	Dp116	S-dystrophin	apo-dystrophin 2	5623	956	116	Schwann cells (adult)	56
G	Intron 62	Dp71	liver or G-dystrophin	apo-dystrophin 1	4623	617	71	Ubiquitous (except in skeletal muscle)	57, 59
	Intron 62; 3'UTR in intron 70	Dp40	-	apo-dystrophin 3	2200	340	40	Ubiquitous	60

LEGEND: 3'UTR – 3' untranslated region; CNS – central nervous system; Ref. – bibliographic references.

1.1.3.3 The dystrophin protein

Full-length dystrophin is a large rod-shaped protein associated with the sarcolemma by assembly into the DGC, which contributes to mechanical stability and plays a role in cell signaling [24-28].

Dystrophin's 3684 amino acids are organized into four regions based on sequence homologies and protein-binding capacities: the NH_2-terminal actin-binding domain, central rod domain, cysteine-rich domain and COOH-terminal domain (Figure 1.1.3.1).

The NH_2-terminal, or actin-binding domain (residues 14-240), contains a region that shows homology with α-actinin [69], which is a normal component of the actin filaments in smooth and skeletal muscle. It is believed to be involved in cross-linking F-actin, thereby connecting the filamentous elements of the cytoskeleton to the cell membrane.

The central rod domain (residues 253-3040) is formed by 24 spectrin-like triple-helical repeats of ~109 amino acids each, with homology to α-actinin and spectrin. These non-identical repeats are interrupted and flanked by four proline-rich hinges [70]. Recently, a further four small flexible junctions were identified [71]. These and the distinct surface properties of the repeats distinguish seven biologically relevant sub-domains, with connections to membrane phospholipids, microtubules and intermediate filaments (F-actin). This repetitive domain, initially held as redundant, is now thought to play a crucial role in sarcolemma scaffolding, through modulation of lipid-protein interactions [72].

The cysteine-rich domain (residues 3080-3360), with a density of 15 cysteines/280 amino acids and highly conserved among species, shows homology to the α-actinin carboxy terminus of the slime mold (*Dictyostelium discoideum*) [73]. It comprises three sub-domains: a WW domain with two conserved tryptophan residues [http://www.bork.embl-heidelberg.de/Modules/ww/], two putative EF-hand Ca^{2+}-binding sites and a ZZ domain. Besides the potential role for dystrophin in normal intracellular calcium metabolism, this domain is required for interaction with molecules that mediate attachment of dystrophin to the sarcolemma, by binding either directly to β-dystroglycan via the EF hands and the ZZ domain [74, 75], or indirectly to the dystrophin-associated glycoproteins.

Finally, the COOH-terminal domain (residues 3361-3685) has no known homology to known proteins, except to dystrophin-related proteins such as the dystrobrevins. An α-helical region mediates the interaction with syntrophins (cytoplasmic adapter proteins) [76]. This is followed by two sets of leucine zippers that interact with dystrobrevin [77]. The carboxy-terminal domain appears to be dispensable for normal muscle function [78].

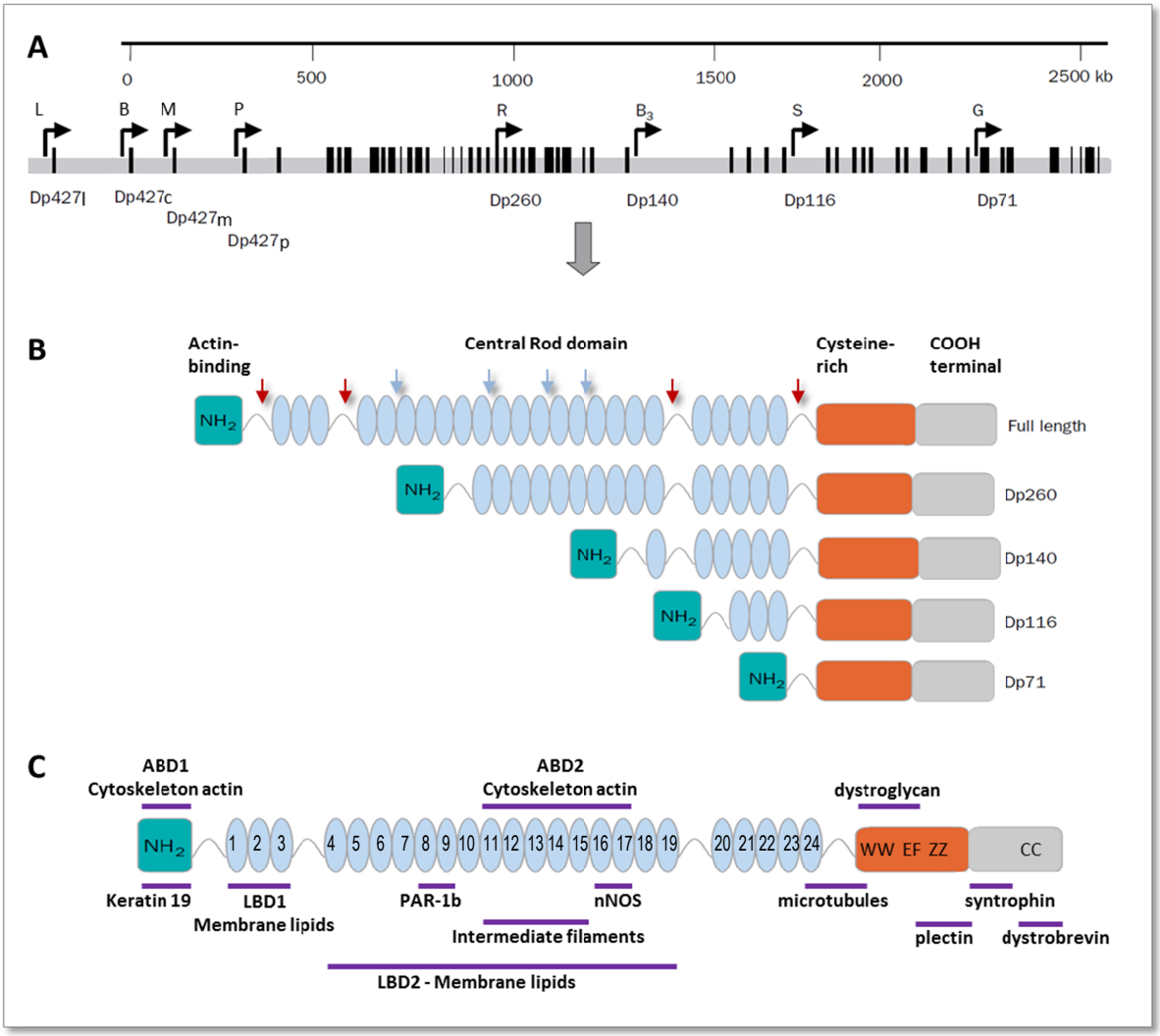

Figure 1.1.3.1 Schematic representation of the dystrophin gene and its main products.

A. Genomic organization of the *DMD* gene, with distribution of exons (black bars) and promoters (black arrows) and respective dystrophin proteins (Dp) below the gene. **B**. Domains of the various Dp. Proline-rich hinges and flexible junctions are indicated (red and blue arrowheads, respectively). **C.** Domain interaction sites. Purple lines represent partners, placed along the regions with which they interact. Spectrin-like repeats in the rod domain are numbered. Sub-domains in the cysteine-rich and COOH domains are identified: WW domain, EF hands, ZZ domain and paired coiled coils (CC).

ABD1, ABD2 - actin-binding domains 1 and 2; LBD1, LBD2 - lipid-binding domains 1 and 2; PAR-1b - polarity-regulating kinase-1b; nNOS - neuronal nitric oxide synthase. (Adapted from [71, 79]).

1.1.3.4 Mutations in the dystrophin gene

The vast majority of mutations described to date are those affecting the muscle isoform of dystrophin, resulting in the Duchenne or Becker phenotypes (DMD / BMD, or D/BMD). Mutations affecting the expression of other tissue-specific isoforms add phenotypic heterogeneity to these diseases; for example, altered expression of brain and/or retina isoforms have been associated with central nervous system (CNS) involvement sometimes found in D/BMD patients [80]. Rare mutations also account for the allelic X-

linked dilated cardiomyopathy, characterized by myocardial involvement without overt signs of skeletal myopathy [40]. Additionally, in rare instances some mutations usually associated with typical BMD can cause dilated cardiomyopathy without significant skeletal muscle involvement, especially in the earlier stages of the disease. The precise correlation between mutations in the *DMD* gene and cardiomyopathy is unclear, yet the study of such mutations may shed light on which functional domains are more relevant for one tissue or for the other.

The frequency of dystrophinopathies coupled with the high mutation rate ($1x10^{-4}$) in the *DMD* gene [81] has led to the description of hundreds of different mutations. According to studies in large cohorts, the most common changes are large genomic deletions or duplications (involving one or more exons), accounting for 43–85% and 7–11% of mutated alleles, respectively [82-84]. Most of the remaining ~25% are small insertions, deletions, exonic point mutations or splice site mutations, collectively termed “point mutations”.

The mutations most often seen in DMD are those that disrupt the reading frame, leading to complete or near complete absence of functional dystrophin in muscle. In contrast, BMD is usually associated with mutations that result in the preservation of an open reading frame, allowing production of either a reduced amount of normal dystrophin or an altered but partially functional protein, with an intact C-terminal and dystroglycan-binding region. This reading frame rule, relating genotype to phenotype [85], holds true even for extended in-frame exonic deletions that give rise to large internal truncations but a partially functional protein. The varying degree of severity seen in BMD patients is most likely associated with the residual functions of the mutated dystrophin.

In most cases, application of this reading frame rule to the known exon structure of the *DMD* gene makes it possible to predict whether a young male is likely to develop BMD or DMD [85, 86]. While this holds true for ~95% of the patients, there are some exceptions, most of which can be explained by changes that affect RNA splicing or alternative translation initiation [83, 87, 88]. A few rare cases have also been reported of patients with relatively benign BMD-type phenotypes but with complete absence of dystrophin in their muscle biopsies [89-91], supporting the belief that, under certain circumstances, other factors may assume a prominent role in determining disease severity. Exemplary evidence supporting such muscle function modulators relates to inflammatory changes in the dystrophinopathies, mediated by osteopontin; a polymorphism in the gene for transforming growth factor-β receptor-2 (*TGFBR2*) appears to exert a direct influence on osteopontin transcription, and is a strong genetic modifier of disease progression [92].

1.1.4. Limb-girdle muscular dystrophies

1.1.4.1 Overview

The limb-girdle muscular dystrophies (LGMD) may be inherited in an X-linked, autosomal dominant or autosomal recessive pattern. They constitute a clinically and genetically heterogeneous group of disorders characterized by progressive muscle weakness and wasting, predominantly of the scapular and pelvic girdle. Diagnostic criteria include facial muscle sparing, elevated CK levels and dystrophic features in the muscle biopsy. While most forms manifest initially in proximal muscles, some present with distal weakness. Age of onset, rate of progression and patterns of skeletal, respiratory and cardiac muscle involvement are variable and clinical manifestation ranges from mild impairment in the adult to childhood onset with severe disability [21, 22, 93].

Morbidity and mortality rates are equally variable. Much like the dystrophinopathies, early onset is often accompanied by rapid disease progression, with patients becoming wheelchair bound in their early teens and dying from respiratory or cardiac failure in their late teens, whereas patients with later onset usually show a slowly progressive course and may remain ambulant for more than three decades after disease manifestation. Moreover, the spectrum extends on either end, to congenital presentation on the one hand and subclinical forms on the other [21, 22, 93].

This group of muscle diseases was first proposed as a distinct nosological entity by Walton and Nattrass, in 1954 [18], essentially to distinguish it from the X-linked dystrophinopathies and from the autosomal dominant facio-scapulo-humeral muscular dystrophy (FSHD). Shortly afterwards, Chung and Morton studied a large group of patients, delineating and reinforcing the common features of LGMD [94].

As molecular genetic characterization of the entities within this group progressed over the years, it became apparent that the LGMDs cannot be defined on inheritance pattern or clinical grounds alone. Within a specific gene, some mutations may result in a severe disease progression while others may not even lead to limb-girdle weakness or a dystrophic muscle biopsy as a predominant feature. There is also no single underlying molecular mechanism in leading to LGMD, as the genes involved encode structural proteins of the sarcolemma, the sarcomere and the nuclear lamina, a variety of enzymes such as cysteine proteases, glycosyltransferases, ubiquitin ligases and enzymes involved in phosphatidylcholine biosynthesis, and even heterogeneous nuclear ribonucleoproteins (hnRNPs) [95-102].

In the 1995 consensus regarding the classification of the LGMDs [21], it was agreed that the different forms should not be defined according to clinical features alone, but essentially according to the molecular characteristics: type 1 LGMD defining AD and type 2 defining AR forms, with further division into sub-types using sequential alphabetical listing in the chronological order of the identification of the respective gene (or its locus). To date, at least 8 AD and 23 AR sub-types (or "forms") have been recognized [93]. Although both the dystrophinopathies and EDMD are held by some to be limb-girdle dystrophies, they are usually considered as individual clinical forms. EDMD was initially classified as a separate entity to distinguish it from the generally more severe Duchenne-type, which also followed an X-linked pattern of inheritance [22]. However, over the last couple of decades various underlying genetic defects were identified, and it was shown that the inheritance pattern of EDMD may be X-linked, AD or AR [29-31].

1.1.4.2 Epidemiology

In general, the LGMDs are considered rare diseases, and some sub-types have only been described in certain ethnic minorities or in a few families. Based on immunochemical and genetic testing, the overall frequency has been estimated to range from 1/14,500 to 1/123,000 in different countries, although prevalence and relative distribution varies widely between populations, depending on historic, geographic and cultural factors [103-107]. Some of the well documented geographic foci are found in Brazil (LGMD 2G), in the Canadian Manitoba Hutterites (LGMD 2H), in Denmark and England (LGMD 2I), in Finland (LGMD 2J) and in the French-Canadian (LGMD 2L) [93].

In Caucasians, the AR forms are found to account for ~90% of the known cases. From several worldwide reports, LGMD2A (or calpainopathy) appears to be the most common, accounting for 8-26% of all LGMDs, and in some populations it may be the only LGMD present (Reunion Island, Basque Country) with very high prevalence rates (1/14,300 to 1/20,000). LGMD2B (or dysferlinopathy) is also relatively common, accounting for 3-19% of all LGMDs. As a group, the sarcoglycanopathies (SGs – LGMD sub-types 2C to 2F) are a common cause of LGMD, accounting for 3-18% of cases [93]. These too can be over- or underrepresented in different populations, with some populations having only one mutation type, which is probably related to founder effects and consanguinity.

The AD forms of LGMD account for the remaining ~10% of known cases, and most of the loci have been described in single extended pedigrees [93].

Table 1.1.4.1 Overview of LGMD forms. Compiled from [30, 93] and the following databases: http://www.ncbi.nlm.nih.gov/omim; http://neuromuscular.wustl.edu/; http://emedicine.medscape.com/; http://www.musclegenetable.org (last accessed March 2015).

LGMD form (OMIM #)	Gene (gene product)	Clinical features				Muscle biopsy (Histology / EM / IHC / WB)	Allelic Disorders and other findings
		Onset	Presentation and progression	WCB (age)	Distinguishing features		
LGMD type 1 (autosomal dominant)							
LGMD 1A (159000)	*MYOT* (Myotilin/Titin immunoglobulin domain protein; TTID)	Young adulthood to mid 70s (mean 27 yr)	Hip girdle weakness precedes and often greater than that of the shoulder girdle. Distal muscle weakness occurs later, often causing foot drop. Can also present with distal and proximal weakness. Neuropathy in > 50% (may account for distal weakness). Slowly progressive. Cardiomyopathy / arrhythmia in 50%. Dysarthria, hyporeflexia, absent ankle jerks are common. Respiratory insufficiency is rare.	Late loss of ambulation; often 10 yr after onset.	Nasal and dysarthric speech in some patients. Swallowing difficulty. Distal weakness. Achilles tendon contractures.	Fibre degeneration, size variation, splitting, centralized nuclei, rimmed vacuoles, spheroid bodies. Inclusions with multiple proteins: MYOT, DYS, NCAM, DES, PLEC1, ubiquitin, gelsolin and prion protein. EM: Z-disk streaming and sarcomeric disruption.	Allelic to Spheroid body myopathy, Myofibrillar myopathy type 3 (MFM3).
LGMD 1B (159001)	*LMNA* (Lamin A/C)	< 10 yr to the mid 30s.	Presenting symptom usually hip girdle weakness, then shoulder girdle weakness. Distal weakness occurs later and possibly facial weakness. Slow progression. Respiratory complications and contractures are common. Cardiac disease (2/3 patients) begins by the 30s-50s: atrioventricular conduction disturbances, bradycardia, dilated cardiomyopathy, sudden cardiac death.	?	Muscle symptoms precede cardiac symptoms. Cardiomyopathy and arrhythmia. There may be family history of sudden cardiac death. Mild joint contractures with sparing of the elbows.	Muscle biopsy shows mild dystrophic changes. Lamin A may aggregate in the nucleus and be present in the cytoplasm.	Allelic to AD- and AR-EDMD, Lamin-related CMD (L-CMD), AD dilated cardiomyopathy with AV block, Familial lipodystrophy, CMT type 2A, Mandibuloacral dysplasia, Quadriceps myopathy, Hutchinson-Gilford progeria, Werner syndrome.
LGMD 1C (607801)	*CAV3* (Caveolin-3)	First or second decade, but also early adulthood.	Presentation usually with proximal weakness but can be distal. May present without muscle weakness but with hyperCKemia, myalgia, exercise-induced cramps or rippling muscles, hypertrophic cardiomyopathy and rhabdomyolysis. Progression is slow to moderate.	Adults usually remain ambulant.	Presentation with myalgias, rippling muscles or asymptomatic hyperCKemia. Calf hypertrophy and toe walking may be prominent.	Dystrophic changes. Reduced or absent IHC staining for caveolin-3 and often also dysferlin,. EM shows reduced number of caveolae.	Allelic to Rippling-muscle disease, HyperCKemia, Distal myopathy, Hypertrophic cardiomyopathy. Both AD and AR inheritance has been described.
LGMD 1D (601419)	*DES* (Desmin)	Early adulthood	Presentation with proximal weakness. Progression is slow. Cardiac arrhythmia (earliest cardiac feature) and cardiomyopathy are noted in all patients beginning 1-2 decades after weakness and may lead to sudden death.	All remain ambulatory	-	EM: Z-disk streaming. IHC: Intrasarcoplasmic aggregates; >50% fibres: DES, MYOT, DYS, αβ-crystallin, β-amyloid precursor protein, cell division cycle kinase 2, actin, NCAM, PLEC1, prion protein.	Allelic to myofibrillar myopathy type 1 (desmin-related myofibrillar myopathy). Designated LGMD1D in OMIM and LGMD1E in HUGO. One large family described.

LGMD 1E (603511)	*DNAJB6* (DNAJ/HSP40 Homolog, subfamily B, member 6)	Early 20s-60s. (mean 38 yr)	Onset mostly with proximal weakness, although distal weakness can predominate. Legs are usually affected more than arms, particularly the soleus, adductor magnus, semimembranosus and biceps femoris. Progression is slow. Dysphagia may be preset (~20%). Serum CK is elevated. No cardiomyopathy is noted.	20-30 yr after onset. Most remain ambulant into late life.	-	Small rounded and angulated fibres that express neonatal and fetal myosin. Rimmed vacuoles. Aggregates contain SMI-31, TDP-43 and DNAJB6.	(Nomenclature confusing: designated LGMD 1E in OMIM and LGMD 1D in HUGO).
LGMD 1F (608423)	*TNPO3* (Transportin 3)	10-20 yr (birth to mid 50s).	Presentation with proximal leg weakness, later also distal. Early onset correlates with rapid progression and early loss of ambulation. Prominent atrophy of lower limb muscles.	Second or third decade, in younger patients.	May have facial and respiratory weakness and/or spinal deformity.	Variable fibre size and occasional degeneration; rimmed vacuoles. Abnormal nuclear morphology. Increased DES expression.	One large family described.
LGMD 1G (609115)	*HNRPDL* (Heterogeneous nuclear ribonucleoprotein D-like protein)	15-53 yr (mean 37 yr)	Mild form. Presents with proximal leg weakness. Some present with upper limb weakness or muscle cramps. Progression to upper limb weakness and permanent restriction of finger and toe flexion, with reduced range of movement in the interphalangeal joints. Normal strength retained in the intrinsic hand muscles.	50% lose ambulation 10 yr after onset	Cataracts in 50% patients. Limited finger and toe flexion.	Type 2 fibre predominance. Necrotic fibres. Discrete perimysial fibrosis. Irregular nuclei. Rimmed vacuoles. Milder patients may be normal.	Two families described.
LGMD 1H (613530)	(3p25.1–p23) (?)	16-50 yr	Proximal upper and lower limb weakness. Calf hypertrophy. Proximal atrophy. Reduced tendon reflexes. Slow progression: More severe with younger onset.	?	Southern Italian family. Incomplete penetrance. (Multiple mtDNA deletions)	Fibre size variation. Endomysial fibrosis.	Multiple deletions in mtDNA. One southern Italian family.
LGMD type 2 (autosomal recessive)							
LGMD 2A (253600)	*CAPN3* (Calpain-3)	8-15 yr (range 2-40 yr)	Typically presents with scapular winging, severe weakness of hip adductors and elbow flexors, contractures. Normal respiratory function. Presentation may be with toe walking. ~10% have asymptomatic hyperCKemia. No facial and cardiac involvement.	11-28 yr after onset (mean late 2nd decade).	Similar to FSHD dystrophy, but without facial weakness.	Correlation between degree of deficiency and clinical phenotype can be total, partial, or nonexistent. DYS and SGs normal. DYSF can be reduced.	LGMD2I may have similar phenotype. Presence of contractures requires differentiation from LGMD1B, EDMD, laminin-α2 deficiency and Bethlem myopathy.
LGMD 2B (253601)	*DYSF* (Dysferlin)	10-39 yr (range 10-70 yr)	LGMD or Miyoshi myopathy (MMD1) presentations. LGMD: pelvic and femoral muscles affected first; later proximal portions of the arms. MMD1: gastrocnemius weakness and difficulty with toe walking. With disease progression, these become indistinguishable. ~35% of patients have combined proximal and distal weakness.	10-30 yr after onset	Early weakness and/or atrophy of gastrocnemius, inability to walk on toes, waddling gait, atrophic distal biceps, spared periscapular and deltoid muscles. Often sudden onset in the late teens or early 20s.	DYSF reduced or absent. Absent staining more specific for LGMD2B, while reduced staining can be seen in other diseases (e.g. LGMD2A). DYS and SGs: normal. On WB, DYSF is reduced 0-15% of normal.	Allelic to MMD1. A few families also reported with an anterior tibial myopathy and with paraspinal (axial) myopathy. Congenital to 8th decade onset has been reported. Carriers may be symptomatic.

LGMD 2C (253700)	*SGCG* (γ-Sarcoglycan)	1-12 yr (mean 5-6 yr)	Often presents DMD-like course, but may have mild BMD-like phenotype or intermediate. Positive Gowers sign. Lumbar hyperlordosis and scoliosis; scapular winging. Calf and tongue hypertrophy and Cardiac involvement may appear late in disease course.	Loss of ambulation: 10 to 37 years; Mean 16 years	Muscle hypertrophy especially of the calf and tongue. Mental development is normal. Neurosensory hearing loss.in some patients.	Dystrophic pattern of necrosis and regeneration (similar to DMD). IHC: γ-SG absent or reduced. Other SGs: β mostly absent or reduced, α and δ normal or reduced. DYS normal or reduced.	Formerly known as Severe Childhood Autosomal Recessive Muscular Dystrophy (SCARMD) or Adhalin deficiency.
LGMD 2D (608099)	*SGCA* (α-Sarcoglycan)	2-15 yr	Generally milder than LGMD 2C. Limb-girdle weakness (first proximal, then also distal), muscle atrophy, scoliosis, scapular winging, calf pseudohypertrophy and contractures. Cardiomyopathy is rare.	Loss of ambulation in teens (severe cases).	-	Necrosis and degeneration of muscle fibres. IHC: α-SG absent. Other SGs reduced or absent.	Formerly known as Primary Adhalinopathy. Intrafamilial variability.
LGMD 2E (604286)	*SGCB* (β-Sarcoglycan)	<3-12 yr	Severe, similar to outlier DMD. Rare cases with mild phenotype. Proximal weakness. Prominent tongue hypertrophy. Scapular winging and muscle wasting of shoulders.	10-15 yr	-	Necrosis and degeneration of muscle fibres. IHC: all SGs absent or markedly reduced; DYS reduced, not absent.	Some intrafamilial variability.
LGMD 2F (601287)	*SGCD* (δ-Sarcoglycan)	2-12 yr	Severe phenotype with proximal weakness. Calf hypertrophy. Cramps. Cardiac function usually normal but dilated cardiomyopathy has been described.	9-16 yr	Early onset is severe with death in the second decade. Mental development is normal.	Degeneration and regeneration. IHC: δ-SG absent; α and β absent; γ reduced or absent; DYS present but reduced.	-
LGMD 2G (601954)	*TCAP* (Telethonin)	2-15 yr	Weakness predominantly proximal, but ~50% present with foot drop and anterior compartment atrophy. All eventually develop distal leg weakness. Gluteal and thigh atrophy may be prominent. Calf hypertrophy in ~50%. Calf atrophy in ~50% (resemble MMD1 or LGMD2B). Joint laxity in distal arms. Cardiomyopathy in ~55%.	3rd to 4th decade	Patients may have initial anterior tibial weakness causing foot drop or a typical LGMD phenotype. Difficulty walking on heels occurs prior to difficulty walking on toes Areflexia in lower limbs.	Degeneration and regeneration. Increased variation in fibre size. Some show lobulated fibres. Centralized myonuclei. Rimmed vacuoles. IHC: telethonin absent; α-DG normal.	Allelic to Dilated Cardiomyopathy type 1N (DCM 1N) and Congenital Muscular Dystrophy (presentation in infancy).
LGMD 2H (254110)	*TRIM32* (Tripartite motif–containing gene 32)	8-27 yr (range – into 30's)	Onset with proximal weakness of quadriceps and pelvic girdle. Back pain and fatigue are common. Neck flexor and facial weakness, scapular winging, respiratory insufficiency, ankle contractures and cramps. Slow progression. May have only asymptomatic hyperCKemia.	Ambulatory to >50 yr	Flat smile due to facial weakness. Waddling gait and exercise-induced myalgia. No cardiac symptoms, but mild ECG changes may be noted.	Mild dystrophic changes with fibre size variation, fibre splitting and internal nuclei. No protein accumulations or inclusions have been identified.	Almost exclusively in the Hutterite population of Manitoba. Allelic to Sarcotubular myopathy and Bardet-Biedl syndrome 11.

LGMD 2I (607155)	*FKRP* (Fukutin-related protein)	0.5-27 yr (61% <5 yr)	Varies from severe CMD to mild, late-onset LGMD. In LGMD, patients can have severe DMD-like presentation with restrictive respiratory failure, or more commonly, a BMD-like onset. Myalgia or cramps in ~60%. Cardiac involvement in ~60%. Isolated hyperCKemia in some cases. Mild or no cognitive impairment in the LGMD phenotype (as opposed congenital forms).	Highly variable: ≤ 5 – late 40s.	Prominent muscle hypertrophy and cardiomyopathy (DMD-like). May have prominent thigh and tongue hypertrophy and severe weakness and wasting of upper arms, neck flexors and axial muscles; these features can help in distinguishing from DMD.	Necrosis and variation in fibre size. IHC: reduced staining for the glycosylated portion of α-DG; laminin-α2 may also be reduced; DYS, SGs and β-DG are normal. WB: α-DG shows decreased mass (decreased glycosylation); laminin-α2 reduced or absent.	Also known as Muscular dystrophy-dystroglycanopathy type C5 (MDDGC5). Allelic with Congenital muscular dystrophy with muscle hypertrophy and normal CNS (MDC1C), with Myopathy with abnormal merosin and with Walker-Warburg Syndrome (WWS).
LGMD 2J (608807)	*TTN* (Titin)	10-30 yr	Onset with proximal weakness, upper limb involvement, generalized weakness in childhood, persistent focal/asymmetric weakness and mild bulbar/facial weakness. Tibialis anterior weakness may develop. Allelic Finnish distal myopathy (TMD) usually presents in the 4th decade, with tibialis anterior weakness, which may be asymmetric. Proximal weakness may develop later. LGMD phenotype less common than TMD.	Within 20 yrs of onset (some ambulant past age 60 yr)	This is a severe LGMD described in the Finnish population. Distal muscles are affected as the disease progresses. No cardiomyopathy noted.	Myopathic. No vacuoles. WB: Severely reduced C-terminal titin fragments (important for calpain-3 binding and cell signaling pathways); secondary deficiency of calpain-3. IHC: mislocalization of obscurin.	Allelic with TMD, Myopathy with Early Respiratory failure, Dilated cardiomyopathy (CMD1G), Familial hypertrophic cardio-myopathy (CMH 9), Cytoplasmic body myopathy, Multicore disease (CM + Fatal dilated cardiomyopathy) and Centronuclear myopathy.
LGMD 2K (609308)	*POMT1* (O-mannosyl-transferase 1)	1-6 yr	Severe proximal muscle weakness with slow progression. Mild pseudohypertrophy. Joint contractures (especially ankles) in ~50%. Rigid spine and lumbar lordosis. Facial dysmorphic features and mental retardation may occur (IQ 50 to 76), though brain MRI is normal.	-	Global delay, mental retardation and microcephaly may be present.	Dystrophic changes. WB: α-DG shows decreased mass (decreased glycosylation).	Also known as Muscular dystrophy-dystroglycanopathy type C1 (MDDGC1). Allelic with WWS.
LGMD 2L (611307)	*ANO5* (Anoctamin 5)	11-51 yr (mean 33 yr)	Initially difficulty walking or standing on toes. Frequently presents with proximal shoulder and pelvic girdle weakness. Asymmetric muscle involvement. Myalgia is common. Scapular winging in ~33%. Slow progression. May present as distal myopathy (MMD3) with calf weakness. Proximal weakness may develop later. With progression, phenotypes overlap and merge.	~12yr after onset.	Often asymmetric quadriceps, hamstring, biceps and brachioradialis.	Dystrophic changes with fibre splitting. Sarcolemmal membrane lesions (defective membrane repair, as in LGMD2B). IHC: DYS reduced; DYSF normal. WB: Calpain-3 reduced.	Allelic with Miyoshi Muscular Dystrophy type 3 (MMD3). There is similarity between LGMD2L and LGMD2B, both presenting with either a LGMD phenotype or a distal myopathy phenotype.
LGMD 2M (611588)	*FKTN* (Fukutin)	<0.5-10 yr	Usually presents with hypotonia or delayed motor milestones before age 2 yr. Moderate progression with proximal greater than distal weakness affecting legs more than arms. Spinal rigidity, joint contractures and calf hypertrophy may be present.	Acquire ambulation but lose it during infancy	Rare cases. MRI may show cortical and posterior fossa pathology.	Dystrophic with fibre necrosis. IHC: decreased staining for α-DG. WB: glycosylated α-DG nearly absent; laminin-α2, -β1 and -γ1 slightly reduced.	Also known as Muscular dystrophy-dystroglycanopathy type C4 (MDDGC4). Allelic with FCMD and with Familial Dilated Cardiomyopathy (CMD1X).

LGMD 2N (613158)	*POMT2* (O-mannosyl-transferase 2)	Onset usually in the first decade.	Hypotonia and delayed motor milestones, but walking is achieved. Calf hypertrophy and scapular winging. No brain or eye abnormalities are present. Intellect is normal.	-	Rare cases described with LGMD phenotype. May present with global delay. Very high serum CK.	Muscle biopsy may show inflammatory changes. IHC: decreased staining for α-DG.	Also known as Muscular dystrophy-dystroglycanopathy type C2 (MDDGC2). Allelic with WWS.
LGMD 2O (613157)	*POMGNT1* (O-mannose β-1,2-N-acetyl-glucosaminyl-transferase)	Infancy or early childhood	Proximal weakness and wasting of proximal muscles. Muscle hypertrophy. Positive Gowers sign. Difficulty climbing stairs. Fatigue.	-	Rare cases described with LGMD phenotype.	Dystrophic changes. IHC: decreased staining for α-DG.	Also known as Muscular dystrophy-dystroglycanopathy type C3 (MDDGC3). Allelic with Muscle-Eye-Brain disease (MEB).
LGMD 2P (?)	*DAG1* (Dystrophin-associated glycoprotein 1)	First decade	Delayed walking and fatigue, proximal more than distal weakness. Calf or thigh hypertrophy. Achilles joint contracture, hyperCKemia and moderate-to-severe MR (Brain MRI normal). Slow progression.	Patients remain ambulant.	Rare cases described with LGMD phenotype. Mild to severe MR. Slight microcephalus.	Dystrophic changes. IHC: decreased staining for α-DG	Also known as Muscular dystrophy-dystroglycanopathy type C7 (MDDGC7) or LGMD with Mental Retardation.
LGMD 2Q (613723)	*PLEC1* (Plectin)	Early childhood	Delayed motor development. Slow progression with proximal more that distal weakness. Late-onset contractures.	Adults may lose ability to walk	Rare families have been reported with LGMD but without skin manifestations.	Dystrophic changes. Plectin expression reduced and almost no expression of plectin 1f mRNA.	This disease is allelic with Epidermolysis Bullosa Simplex with Muscular Dystrophy (EBS-MD, MIM#226670).
LGMD 2R (615325)	*DES* (Desmin)	15-27 yr	Features include onset in the second or third decade with slowly progressive proximal and facial weakness. Scapular winging or respiratory involvement may be present. Scoliosis or scapular winging has been reported. Cardiac symptoms are common.	Loss of ambulation in adulthood	One Turkish family reported with a limb girdle phenotype.	Dystrophic changes with type 2 fibre predominance. No myofibrillar abnormalities or protein aggregation.	Allelic with AD Dilated Cardiomyopathy type 1I (CMD1I), MFM1 and with Neurogenic Scapuloperoneal syndrome, Kaeser type (SCPNK).
LGMD 2S (615356)	*TRAPPC11* (Transport prot. particle complex, subunit 11)	Early childhood	Ranges from LGMD phenotype with childhood onset to myopathy with neurological involvement. Facial weakness, scapular winging, myalgias. Mild-to-moderate intellectual disability. Seizures, ataxia, and ocular abnormalities can occur.	-	Rare families reported. Short stature, microcephaly and ocular abnormalities are distinguishing features.	Dystrophic changes. Increased glycosylation of LAMP1 and LAMP2	Also known as LGMD with Movement Disorder and Intellectual Disability.
LGMD2T	*GMPPB* (GDP-mannose pyrophosphoryl-ase B)	Birth or early infancy.	Mild limb-girdle weakness. Mild intellectual disability or normal cognitive function. May present with microcephaly. All have hyperCKemia. May present with exercise intolerance.	-	Reported in a few unrelated patients.	Dystrophic findings on muscle biopsy. Hypoglycosylation of α-DG.	Allelic with Muscular dystrophy-dystroglycanopathy type A14 (MEB-like, MDDGA14, MIM#615350) and type B14 (with MR, MDDB14, MIM#615351).

LGMD2U	*ISPD* (Isoprenoid synthase domain containing)	Early / late	DMD-like progression. Muscle pseudohypertrophy, including the tongue. Respiratory and cardiac functions also decline, resembling other dystroglycanopathies.	Loss of ambulation in early teens (<12 yr).	Usually no brain involvement	Muscle fibre necrosis & regeneration. Increased endomysial connective tissue. IHC: α-dystroglycan absent or severely reduced, laminin-α2 reduced.	Allelic with Muscular dystrophy-dystroglycanopathy type A7 (MEB-like, MDDGA7, MIM#614643).
LGMD2V	*GAA* (Alpha-1,4-glucosidase)	2nd to 7th decade	Mild, late-onset form of Pompe disease (massive accumulation of glycogen in muscle, heart and liver). Progressive proximal muscle weakness primarily affecting the lower limbs. Disproportionate axial and respiratory muscle involvement in comparison with limb muscle involvement. Outcome depends on respiratory muscle failure.	-	May be distinguished by muscle pathology.	Mild non-dystrophic, myopathic features on muscle biopsy (glycogen accumulation).	Allelic with Glycogen storage disease type II (MIM#232300).
LGMD2W	*LIMS2/PINCH2* (Lim and senescent cell antigen-like domains 2)	Childhood	Patients show a childhood onset LGMD with macroglossia and calf enlargement. Severe quadriparesis and relative sparing of the face. Develop biventricular cardiac dysfunction in the 3rd decade.	-	Characteristic broad-based triangular tongue.	IHC: irregular staining for LIMS2.	-

LEGEND: AD – autosomal dominant; AR – autosomal recessive; CK – creatine kinase; CMT – Charcot-Marie-Tooth Disease; CNS – central nervous system; DES – desmin; DYS – dystrophin; DYSF – dysferlin; ECG – echo-cardiogram; EDMD – Emery-Dreifuss Muscular Dystrophy; EM – electron microscopy; FCMD – Fukuyama Congenital Muscular Dystrophy; HUGO – Human Genome Organization; IHC – immunohistochemistry; IQ – intelligence quotient; LAMP1 – lysosome-associated membrane protein 1; LAMP2 – lysosome-associated membrane protein 2; MEB – Muscle-Eye-Brain disease; MIM - Mendelian Inheritance in Man; MR – mental retardation; MRI – magnetic resonance imaging; mtDNA – mitochondrial DNA; MYOT – myotilin; NCAM – neuronal cell adhesion molecule; OMIM – Online Mendelian Inheritance in Man; PLEC1 – plectin; WB – western blot; WCB – wheelchair bound; WWS – Walker-Warburg Syndrome; yr – year(s).

1.1.5. Congenital muscular dystrophies

1.1.5.1 Overview

The Congenital Muscular Dystrophies (CMDs) are a common cause of autosomal recessive neuromuscular disorders. In general, they result in severe diffuse proximal weakness at birth or within the first months of life, with a clinical course that is either slowly progressive or non-progressive. Contractures are common as are central nervous system abnormalities, which range from sub-clinical to severe, with deficiencies of myelin or neuronal migration [108, 109].

Muscle biopsy shows signs of dystrophy, including a marked increase in endomysial and perimysial connective tissue. There is variability in fibre size with small, round fibres, as well as immature muscle fibres, but degeneration and necrosis is uncommon. However, with the exception of partial or total absence of laminin-α2 (or merosin) and/or hypoglycosylation abnormalities in α-dystroglycan (α-DG), there are few or no particular distinguishing features differentiating these disorders from some LGMDs and from some congenital myopathies [110].

There are several hallmarks in the description of CMD forms, after the first “infantile myopathy” reported by Frederick Batten [16]. In 1930, Otto Ullrich described a peculiar form of CMD characterized by significant distal hyperlaxity and proximal contractures [111], now known as Ullrich Congenital Muscular Dystrophy (UCMD). Thirty years later, Fukuyama and colleagues described the first form with severe mental retardation and structural brain involvement (FCMD) [112]. Other diseases involving the muscle, eye and brain were subsequently described and are now grouped as Muscle-Eye-Brain disease (MEB) or Walker-Warburg Syndrome (WWS), distinguished essentially according to clinical severity [108, 109]. The “classical” (merosin-deficient) form of CMD was said to have normal intellect and no clinical involvement of the central nervous system; however, although the brain is structurally normal, some patients have been identified with white matter changes [110].

The discovery of causative mutations in multiple genes made the concept of CMD evolve from a somewhat narrowly defined clinical entity to a more inclusive group of sub-types. Moreover, with the recent identification of several new genes expanding the spectrum of the known forms, classification of the CMDs has suffered significant changes and has created some confusion between the HUGO (Human Genome Organization) and the OMIM (Online Mendelian Inheritance in Man) nomenclatures. As in the case of the LGMDs, the current tendency is to subdivide the CMD forms according to the combined

clinical and underlying patho-genetic mechanism, namely defects of structural proteins of the extracellular matrix or basal lamina, of the glycosylation process, of the endoplasmic reticulum, of the nuclear envelope and of the mitochondrial membrane [108, 109].

The large group that has come to be known as the dystroglycanopathies have in common the hypoglycosylation of α-DG, with specific O-glycosylation and N-glycosylation defects caused by mutations in known or putative glycosyltransferases, as well as in the dystroglycan (*DAG1*) gene itself [113-117]. This group of muscular dystrophy-dystroglycanopathy disorders includes a range of clinical phenotypes [118-127]. WWS, MEB and FCMD represent the most severe end of the spectrum, whereas the mildest variant of the dystroglycanopathies is an adult-onset limb-girdle muscular dystrophy (LGMD2I) associated with mutations in the fukutin-related protein (*FKRP*) gene [118].

Cognitive impairment ranging from intellectual disability to mild cognitive delay, structural brain and/or eye abnormalities, and seizures are found almost exclusively in the dystroglycanopathies (particularly the more severe forms) while white matter abnormalities without major cognitive involvement tend to be limited to the laminin-α2-deficient sub-type. However, as may be seen in Table 1.1.5.1, there is often phenotypic overlap amongst the CMD forms, and there is also allelism between these, the limb-girdle muscular dystrophies and the congenital myopathies.

Several less known CMD subtypes have been reported in a limited number of individuals. Table 1.1.5.1 describes a listing of 27 CMD forms, as proposed by different authors [108, 109, 128] and also resorting to several disease databases.

1.1.5.2 Epidemiology

Information on CMD prevalence and incidence is scarce. There appear to be some regional discrepancies in terms of prevalent sub-types, and reported estimates of the incidence range between 1 and 6 per 100,000 live births [110, 129, 130]. Classical, merosin-deficient CMD was originally reported to be the most common form amongst European populations [131, 132] whereas FCMD has a high prevalence in Japan and is extremely uncommon elsewhere [133-135].

Although CMDs as a group are considered relatively common neuromuscular disorders, studies in large cohorts of CMD patients identify mutations in only 25-50% of the cases [134, 135], implying that there are yet to be identified many more causative genes than are currently known.

Table 1.1.5.1 Overview of CMD forms. Compiled from [108] and the following databases: http://www.ncbi.nlm.nih.gov/omim; http://neuromuscular.wustl.edu/; http://emedicine.medscape.com/; http://www.musclegenetable.org (last accessed March 2015).

CMD form (OMIM #)	Gene (gene product)	Clinical features			MRI / Muscle biopsy	Allelic Disorders and other findings
		Onset	Presentation and progression	CNS involvement		
Defects of structural proteins						
MDC1A (607855)	*LAMA2* (Laminin-α2)	Birth to early infancy.	Present with severe congenital hypotonia, severe axial and proximal muscle weakness, feeding difficulty, and respiratory insufficiency. Reduced fetal movements may be noted in utero. Delayed motor development. Most infants eventually sit unsupported, but standing and walking with support is achieved in only about 25%. Kyphoscoliosis and contractures are common. External ophthalmoplegia may occur late but is rare. Weakness is static or minimally progressive. Complications are related to respiratory compromise, feeding difficulty, scoliosis, and (in ~33%) cardiac abnormalities. Death often occurs after 10-30 yrs due to respiratory failure.	Mild mental retardation or perceptual-motor difficulties are observed in a few cases. Seizures occur in up to 30% of patients.	MRI: White matter hypomyelination and hypodensities invariably present by age 6 months, even in cases with normal intelligence. Structural brain changes include abnormal cortical gyration, focal cortical dysplasia and hypoplasia of the pons and/or cerebellum. Muscle biopsy: dystrophic changes. IHC: absence of laminin-α2 chain.	Most common form in some countries (~40% of all cases). May resemble Rigid Spine muscular dystrophy (RSMD) or EDMD. Allelic to MDC1B. Clues to laminin-α2 deficiency include MRI abnormalities, seizures, and demyelinating neuropathy.
MDC1B (604801)	(1q42) (?)	Early childhood	Presents with generalized hypotonia. Delayed early motor milestones. Weakness as in LGMD phenotype. Facial weakness, contractures of the Achilles tendon, diaphragm involvement, spinal rigidity and early respiratory failure. Acquire ambulation and remain ambulant for 20-30 yr.	Normal intelligence	Dystrophic changes on biopsy. . IHC: reduced staining for laminin-α2 chain (secondary deficiency).	Also known as Congenital Muscular Dystrophy with Respiratory Failure and Muscle Hypertrophy.
Integrin α7-deficient CMD (613204)	*ITGA7* (Integrin α7)	Onset in infancy	Present with hypotonia in infancy. Walk late (age 2-3 yr). May have contractures and respiratory failure. May require noninvasive ventilation in the first decade and become wheelchair bound in the second decade.	Delayed motor milestones. May have mental retardation.	Mild dystrophic changes. Lack of integrin-α7 staining.	Very rare disorder.
CMD with epidermolysis bullosa (226670)	*PLEC1* (Plectin)	Infancy to young adult	Progressive proximal weakness often leading to wheelchair use by the second decade. Epidermolysis bullosa can be severe, even resulting in death and presents with severe blistering. Other skin findings can include nail dystrophy and scalp alopecia. Systemic features include growth retardation, anemia, laryngeal webs, tooth decay, pyloric atresia, infantile respiratory distress and cardiomyopathy. Presentation as a late onset (20-40 yr) muscular dystrophy has mild skin manifestations that may not cause significant disability.	-	Degenerative changes with increased connective tissue.	Allelic to LGMD 2Q (MIM#613723) without epidermolysis bullosa.

UCMD1 (120220)	*COL6A1* (Collagen VI α-1 polypeptide)	Neonatal to second decade	Ullrich Congenital Muscular Dystrophy (UCMD) often presents neonatally with hypotonia, rigidity and kyphosis of the spine, proximal joint contractures, torticollis, hip dislocation, distal joint hyperlaxity and protruding calcaneus. Weakness more distal than proximal. Facial dysmorphism includes micrognathia, round face with drooping lower lids and prominent ears. Skin changes include follicular hyperkeratosis and keloids. Normal cardiac function. May never walk or walk for a short time with loss of ambulation after 3-10 yrs. Respiratory insufficiency develops in the first or second decade. Bethlem Myopathy (BM) is milder with a slower progression. Often AD with onset in the first or second decade, but may be as late as the sixth decade. Proximal muscle weakness and wasting, including respiratory muscles. Flexion contractures of fingers, wrists, elbows and ankles. Proximal contractures of knees, hips and shoulders, often preceded by joint laxity. Skin changes similar to UCMD. Adult forms may require a wheelchair after 40-50 yrs.	Delayed motor milestones. Normal intelligence.	(UCMD): Brain MRIs are normal. Muscle biopsy: fibre necrosis and Regeneration; increased variation in fibre size; type 1 fibre predominance. IHC: collagen VI absent; laminin-α2 normal. (BM) Muscle biopsy shows nonspecific myopathic changes.	All three collagen VI α-chain genes can originate UCMD (MIM#254090) or BM (MIM #158810) and both forms can be either AD or AR. Allelic to Ossification of the Posterior Longitudinal Ligament of the Spine (OPLL; MIM#602475), to Congenital Myosclerosis (MIM#255600) [7] and to an AD LGMD phenotype with proximal weakness and no significant contractures.
UCMD2 (120240)	*COL6A2* (Collagen VI α-2 polypeptide)					
UCMD3 (120250)	*COL6A3* (Collagen VI α-3 polypeptide)					
Defects of glycosylation						
MDDGA1 (236670)	*POMT1* (Prot. O-mannosyltransferase 1)	Onset prenatally or at birth	Most severe glycosylation disorders. Present as Walker-Warburg (WWS) or Muscle-Eye-Brain (MEB) Syndromes. Common features include severe hypotonia, congenital contractures, microcephaly, retinal atrophy, cataract, glaucoma, corneal clouding and myopia. WWS is the most severe, with virtually no active movement at birth. Additional features include blindness, absent auditory canals, cleft lip and palate, hypoplastic genitalia and renal dysplasia. Most die within the first year of life. Patients with MEB have less severe features. May present muscle hypertrophy, rigid spine, scoliosis, macroglossia, midface hypoplasia and mild micrognathia. They may acquire ability to walk and a few words. Survival is usually longer.	Seizures WWS: lack of motor development; profound MR. MEB: severe MR.	Hydrocephalus, disorganized brain cytoarchitecture, ventricular dilatation, white matter changes, cerebellar hypoplasia and dysplasia, brainstem hypoplasia, flattening of the pons. WWS: Type II cobblestone lissencephaly, thin cortical mantle, aplasia of the corpus callosum. MEB: Cerebellar cysts, pachygyria with preferential frontoparietal involvement, polymicrogyria, diffuse white matter changes. Decreased glycosylation of α-DG.	Most MDDGA1 present as WWS. Allelic with Muscular dystrophy-dystroglycanopathy type C1 (MDDGC1; LGMD 2K).
MDDGA2 (613150)	*POMT2* (Prot. O-mannosyltransferase 2)					Most MDDGA2 present as MEB. Allelic with CMD-dystroglycanopathy with MR type B2 (MDDGB2) and with LGMD-dystroglycanopathy C2 (MDDGC2; LGMD 2N).
MDDGA3 (253280)	*POMGNT1* (Prot. O-mannose β1,2-N-acetylglucosaminyltransferase 1)					Most MDDGA3 present as MEB. Also known as Santavuori congenital muscular dystrophy. Allelic with LGMD-dystroglycanopathy C3 (MDDGC3; LGMD 2O).

MDDGA4 (253800)	*FKTN* (fukutin)		Presents with weak sucking, lack of head control and a weak mouth in the neonatal period. Some show poor fetal movements in utero. Severe cases may only be able to sit with support; less severe forms can stand and walk at age 2-8 years. In most, cardiac disease develops after age 10 yr, resulting in dilated cardiomyopathy and congestive heart failure. Progressive weakness and respiratory failure leads to death (2 yr to mid-teens). Mild cases have abnormal eye movements, poor pursuits, and strabismus. Severe cases may cause retinal detachment, microphthalmos, cataracts or severe myopia.	In severe cases, progressive hydrocephalus, seizures (50%) and severe mental retardation.	Type II cobblestone lissencephaly ranging from cobblestone polymicrogyria and/or pachygyria to complete agyria due to neuronal migration abnormalities. May show dysplasia of pyramidal tracts and mild ventricular dilation. Cerebellar cysts. Decreased glycosylation of α-DG.	Known as Fukuyama Congenital Muscular Dystrophy (FCMD). Allelic with LGMD 2M (MDDGC4), Congenital muscular dystrophy (MDDGB4) and Familial dilated cardiomyopathy (CMD 1X).
MDDGA5 (613153)	*FKRP* (fukutin-related protein)	Onset at birth	Present with severe hypotonia, reduced movement and activity. May present microcephaly. Diffuse limb, neck, trunk and facial weakness. Wasting of shoulder and other muscles. Calf hypertrophy in most patients. Unable to walk; may sit unassisted. Contractures of knees, ankles, elbows and spine in some patients. Respiratory insufficiency.	Seizures are common. Moderate-to-severe mental retardation, delayed psychomotor development, absent speech.	Hydrocephalus; cobblestone lissencephaly; agyria; cerebellar cysts. Decreased glycosylation of α-DG.	Also known as Congenital Muscular Dystrophy with Muscle hypertrophy (MDC1C; MDDGB5). Allelic with LGMD 2I (MDDGC5).
MDDGA6 (613154)	*LARGE* (acetylglucosaminyltransferase-like protein)	Onset prenatally or at birth	Presents with severe hypotonia, moderate muscle hypertrophy and weakness, mild contractures, retinal dysplasia, optic atrophy, cataracts.	Global developmental delay. Profound mental retardation.	MRI: White matter changes: periventricular to arcuate fibres. Structural abnormalities: abnormal neuronal migration; small brainstem. Decreased glycosylation of α-DG.	Also known as MDC1D. Allelic with Congenital Muscular Dystrophy (MDDGB6).
MDDGA7 (614643)	*ISPD* (isoprenoid synthase domain-containing protein)	Onset prenatally or at birth	Presents as WWS. More specific features include small low-set ears, visceral malformations; limb deformations and thumb abduction. Death occurs within the first yr of life in 80% of patients. Often show decreased fetal movements. Rarely, presents with less severe phenotype: proximal weakness with no CNS or eye involvement, although patients are non-ambulant.	Areflexia. Profound mental retardation.	Dystrophic changes with disruption of the basal lamina. Decreased glycosylation of α-DG.	2nd most common form of WWS. Milder form with no CNS or eye involvement (MDDGB7). Allelic to Limb-Girdle Muscular Dystrophy (MDDGC7).
MDDGA8 (614830)	*GTDC2* (glycosyltransferase-like domain-containing protein 2)	Onset prenatally or at birth	Presents as WWS, with lack of psychomotor development. Other features include retinal dysplasia, microphthalmia and macrophthalmia. Death occurs within the first yr of life.	Lack of psychomotor development.	Cobblestone lissencephaly, severe hydrocephalus, cerebellar hypoplasia.	Found in consanguineous families. Gene alias: *POMGNT2*.
MDDGA10 (615041)	*TMEM5* (transmembrane protein 5)	Onset prenatally or at birth	Presents as severe WWS or MEB. Specific features include retinal dysplasia, visceral malformations, and gonadal dysgenesis. Limb deformation seen in some cases. Death in utero or within first yr of life.	-	Cobblestone lissencephaly, neural tube defects, cerebellar dysplasia. Decreased glycosylation of α-DG.	-

MDDGA11 (615181)	*G3GALNT2* (beta-1,3-N-acetylgalactosaminyltransferase 2)	Onset at birth or in infancy	Presents as WWS or MEB. Patients with WWS phenotype show epilepsy and no motor milestones or cognitive development. Eye features include optic nerve hypoplasia, cataracts and microphthalmia. Patients with MEB show motor and cognitive delay. Eyes may be normal or show optic nerve hypoplasia.	-	WWS: hydrocephalus, cobblestone lissencephaly. MEB: polymicrogyria, leukoencephalopathy, cerebellar cysts or dysplasia. Muscle fibre size variation, regeneration, internal nuclei. Decreased glycosylation of α-DG.	-
MDDGA12	*SGK196* (Protein kinase-like protein SGK196)		Presents as severe WWS. Eye features include hyperplastic primary vitreous, myopia and microphthalmia. Death at age 3.	-	Hydrocephalus, agyria, compressed cerebellar hemispheres and brainstem. Normal staining for DYS, SGs and laminin-α2.	Reported in 1 family.
MDDGA13 (615287)	*B3GNT1* (beta-1,3-N-acetylglucosaminyltransferase)	Prenatal onset	Presents as severe WWS, with intrauterine hydrocephalus. Severe hypotonia.	Seizures. Lack of psychomotor development.	Ventricular enlargement; diffuse widening of gyri; disorganization of cortical sulci with cobblestone lissencephaly. Decreased glycosylation of α-DG.	Reported in 1 family.
MDDGA14 (615350)	*GMPPB* (GDP-mannose pyrophosphorylase B)	Onset at birth	Presents as MEB. Intrauterine oligohydramnios. Increased muscle tone at birth. Microcephaly, sensorineural hearing loss, retinal dysfunction, cleft palate. Muscle weakness, ataxia, feeding difficulties and inability to walk unsupported.	Delayed psychomotor development; Severe MR; absent speech.	Pontine hypoplasia and cerebellar hypoplasia. Decreased glycosylation of α-DG.	Reported in 1 family.
CDG1U (615042)	*DPM2*	-	Distinctive phenotype characterized by muscular dystrophy, Severe MR, microcephaly, myoclonic epilepsy, and cerebellar hypoplasia on brain MRI	MR and severe epilepsy	Cerebellar hypoplasia seen on brain MRI.	Known as Congenital Disorder of Glycosylation Type Iu.
CDG10 (612937)	*DMP3*	-	Presents with mild muscular dystrophy, dilated cardiomyopathy and stroke-like episodes. Show abnormal profile of transferrin isoelectric focusing, suggesting a congenital disorder of glycosylation type I pattern.	MR and severe epilepsy	-	Known as Congenital Disorder of Glycosylation Type Io.
	DAG1	-	Primary dystroglycanopathy. Phenotype like early onset LGMD with mental retardation. Normal brain MRI.	-	-	-

Defects in Proteins of the endoplasmic reticulum and nucleus						
RSMD1 (602771)	*SEPN1* (Selenoprotein N, 1)	Onset in infancy	Non-progressive or slowly progressive. Present with variable degrees of proximal weakness and hypotonia. Onset of clinical signs usually occurs during the first year, with delayed milestones. Characterized by early spine rigidity (onset at 3-7 yr) and scoliosis (onset at 4-12 yr). Limited flexion of neck. Contractures of the face, proximal limbs and finger extensors may also be present. Characteristic nasal, high-pitched voice. Severe cases have poor head control and never acquire ability to walk. Progressive respiratory failure often leads to death before adulthood.	Delayed motor development. Early spinal rigidity. Intelligence normal.	Normal brain MRIs. Occasional fibre necrosis. Minicores may be present: showing sarcomere disorganization and mitochondria depletion. Cores stain positive for αB-crystalline. filamin C and DES. Often type I fibre predominance.	Also known as SEPN1- related Rigid Spine Muscular Dystrophy. Allelic to Congenital Myopathy with Fibre-type Disproportion (CFTD), Multiminicore Disease (MmD) with external Ophthalmoplegia (MIM# 255320), antenatal MmD with Arthrogryposis (MIM# 607552) as well as desmin-related myopathy with Mallory body-like inclusions (MB-DRM).
SEPN1-related CMD	*SECISBP2 / SBP2* (Selenocysteine insertion sequence-binding prot. 2)	2 yr to adulthood	Hypotonia, axial muscle weakness, and spinal rigidity, as well as mild facial, proximal, and respiratory weakness. Other features include azoospermia, cutaneous photosensitivity, impaired T-cell proliferation, increased fat mass with enhanced insulin sensitivity, and hearing loss.	Severe cases may present delayed motor and speech developmental milestones and mild bilateral high-frequency hearing loss. Predominant selective involvement of sartorius muscle, seen on MRI, can help distinguish from other rigid spine myopathies.	Marked predominance of type 1 fibres with occasional areas of minicores. WB (fibroblasts): markedly reduced levels of selenoprotein N.	Previously known as Rigid Spine Syndrome. Rare multisystemic disorder.
L-CMD (613205)	*LMNA* (Laminin A/C)	Prenatal onset or onset in infancy	Present with severe hypotonia, severe axial, proximal and distal muscle weakness. Head drop and respiratory insufficiency due to muscle weakness. Rigid spine and joint contractures. Failure to thrive. May show decreased fetal movements.	Delayed motor development	Dystrophic features and atrophic fibers seen on muscle biopsy Variability in fiber size	Known as (AD) LMNA-related Congenital Muscular Dystrophy.
(608441)	*SYNE1* (nesprin 1)		A form of CMD with adducted thumbs, MR and ophthalmoplegia. Mild cerebellar hypoplasia.	CMD with adducted thumbs	-	Reported only in two siblings.

Defects in Mitochondrial membrane protein						
MDCMC (602541)	*CHKB* (Choline kinase beta)	Onset at birth	Presents with generalized hypotonia and proximal weakness, but patients achieve walking (at 15–42 months). Muscle wasting. Slowly progressive. MR and autistic features are constant. Dilated cardiomyopathy and ichthyosiform skin changes are also frequent.	MR. May present microcephaly. May be distinguished by abnormal mitochondria in muscle and by ichthyosiform skin changes.	Fibre size variation. Necrotic and regenerating fibres. Mitochondria appear depleted in the centre of the muscle fibre, and accumulate at the periphery. EM: Mitochondrial enlargement.	Known as Megaconial type Congenital Muscular Dystrophy or as Late walking CMD with mitochondrial structural abnormalities.

LEGEND: α-DG – α-dystroglycan; AD – autosomal dominant; AR – autosomal recessive; BM - Bethlem Myopathy; CFTD - Congenital Myopathy with Fibre-type Disproportion; CNS – central nervous system; DES – desmin; EDMD – Emery-Dreifuss Muscular Dystrophy; EM – electron microscopy; FCMD – Fukuyama Congenital Muscular Dystrophy; HUGO – Human Genome Organization; IHC – immunohistochemistry; MEB – Muscle-Eye-Brain disease; MIM - Mendelian Inheritance in Man; MmD - Multiminicore Disease; MR – mental retardation; MRI – magnetic resonance imaging; OMIM – Online Mendelian Inheritance in Man; POMGNT2 - Protein O-mannose β1,2-N-acetylglu-cosaminyltransferase 2; RSMD – Rigid Spine Muscular Dystrophy; UCMD - Ullrich Congenital Muscular Dystrophy; WB – western blot; WWS – Walker-Warburg Syndrome; yr – year(s).

1.2 DIFFERENTIAL DIAGNOSIS

Identifying the gene involved and the causal mutation in each patient has become the gold standard in diagnosis. This is important for the provision of adequate medical management and genetic counseling. For instance, patients who have a disorder that confers increased risk of developing cardiomyopathy or respiratory failure, require closer monitoring of these functions. Proper genetic counseling and prenatal diagnosis can only be offered if the underlying molecular defect is known. Moreover, many novel therapies currently under development are mutation-specific, implying that enrollment in the respective clinical trials will be based on the specific molecular diagnosis.

However, the ever-increasing numbers of disease genes that can be tested for, together with the expansion of phenotype-genotype correlates, pose major difficulties in molecular genetic testing. Although the common primary symptom of muscular dystrophies is muscle weakness, some of the forms have distinctive features or a combination thereof, and clinical information remains of paramount importance in the diagnostic process. Nonetheless, more often than not, the underlying genetic cause cannot be predicted on the basis of clinical information alone; besides a comprehensive clinical history and thorough physical examination, the diagnostic approach may include biochemical data, electrophysiological investigations, imaging and, most importantly, muscle biopsy analysis, ultimately leading to genetic confirmation of the diagnosis.

1.2.1. Clinical assessment

1.2.1.1 Family history

It is important to enquire as to the existence of other family members with a similar disorder, as this can elucidate if the pattern of inheritance is more likely to be X-linked, autosomal recessive or autosomal dominant. Consanguinity among the parents should be ascertained since it is a common occurrence in recessive forms. Also, in some recessive disorders heterozygous carriers may manifest particular sub-clinical signs, such as mild or moderately elevated levels of serum creatine kinase (Section 1.2.2.1), fatigue, myalgias, muscle weakness or abnormal cardiac function. Finally, knowledge of geographic origin and ethnicity may be useful to guide molecular testing because of private mutations and founder effects that are seen among specific populations.

1.2.1.2 Age at onset

Age at onset may range from perinatal to late adulthood. Although a wide range may be found, some forms have a typical onset. For instance, among the LGMDs, those conditions which are most likely to present in childhood are LGMD1B (laminopathy), LGMD1C (caveolinopathy), LGMD2A (calpainopathy), LGMD2C-F (sarcoglycanopathies) and LGMD2I (fukutin-related proteinopathy); among these, onset is best defined in LGMD2A and 2E (early teens) as well as in LGMD2B (around 20 years of age).

1.2.1.3 Pattern of muscle weakness

With the exception of the generalised hypotonia at birth that is seen in some CMDs, the distribution of muscle weakness, especially at onset, helps distinguish between the main forms of muscular dystrophy (Figure 1.1.1.4). Additionally, some subtypes show particular signs, as is the case with scapular winging, which is almost pathognomonic for the LGMD sub-type 2A (calpainopathy). Specific patterns and combinations of muscle atrophy and hypertrophy, including macroglossia, are found more readily in certain sub-types. An example is initial hypertrophy of the calves, which is common in dystrophinopathy, caveolinopathy, calpainopathy, the sarcoglycanopathies, anoctaminopathy and fukutin-related proteinopathy.

1.2.1.4 Muscle strength and function

Motor function assessment should involve graded and timed tests. Muscle strength is often measured manually and graded from 0 (no contraction) to 5 (normal power), according to the Medical Research Council (MRC) scale, which evaluates the ability of patients to move the various groups of muscles against gravity and against the clinician's resistance [136]. The Walton and Gardner-Medwin (WGM) scale grades global motor disability, ranging from 0 (pre-clinical - able to run, walk, sit and stand up unaided) to 10 (bed-ridden) [137-140]. In ambulant patients, functional endurance can be assessed using the 6-Minute Walk Test (6MWT) that measures the distance walked over a total of six minutes on a hard, flat surface [141]. The Motor Function Measure (MFM) scale is a global scale validated for different neuromuscular disorders, offering a continuous assessment regardless of disease severity and ambulatory status [142, 143].

Tests have also been designed to specifically assess lower limb, upper limb or hand muscle weakness. Lower limb muscle weakness can be quantified using the Gait-Stairs-

Gowers-Chair (GSGC) scores that evaluate four main motor performances, by measuring the time the patient takes to walk 10 metres, to climb 4 steps up a staircase, to perform Gowers' manoeuvre and to rise from a sitting position [144, 145]. The arm function test (AFT) grades the ability of the patient to raise the upper arms above the head, ranging from 0 (full arm abduction) to 6 (unable to raise the hands to the mouth) [146]. The Jebsen Hand Function Test (JHFT) evaluates fine and gross motor hand function using simulated activities of daily living, and is scored according to the time taken to complete each task [147, 148].

Finally, tests that combine different measurements have also been validated for specific disorders, such as facio-scapulo-humeral dystrophy (FSHD), DMD and CMD [149, 150].These are particularly useful to ascertain disease progression.

1.2.1.5 Joint contractures

The distribution of joint contractures and time of their development during the course of the disease is typical in some disorders, as is spinal rigidity. Even within the same group of disorders, the different sub-types may sometimes be distinguished according to onset and pattern of joint stiffness. Among the CMDs, for example, while Ullrich Congenital Muscular Dystrophy (UCMD) usually presents as neonatal hypotonia followed by knee and elbow contractures, there are other CMD forms where congenital weakness is followed by early spine rigidity and limited flexion of the neck, with or without contractures of the face (Table 1.1.5.1). In non-congenital forms, this key diagnostic feature is exemplified with the distinction of EDMD from the LGMDs; a child with EDMD typically begins toe-walking and is unable to fully extend the elbows, and progressive contractures soon lead to a rigid spine [151], whereas in LGMD contractures develop later in the clinical course.

1.2.1.6 Respiratory function

Respiratory involvement is common among the muscular dystrophies. This may be evident from onset or may develop in the more advanced stages, providing clues as to the diagnosis of the disease. The probability of having episodes of Respiratory Failure is also correlated with the underlying disease, and may be considered inevitable (DMD), frequent (UCMD and LGMD types 2C, 2D 2F and 2I), occasional (BMD and EDMD) or uncommon (FSHD, LGMD types 2A, 2B, 2G and 2H) [152].

Nocturnal hypoventilation is suspected when patients complain of sleep disturbances, early morning headaches and/or daytime drowsiness, and this can be confirmed by several methods, including overnight pulse oximetry [152]. Measurements of forced vital capacity (FVC), in both the sitting and the supine position, determine whether or not there is reduced respiratory function. The maximum FVC recorded and its rate of decline may predict survival time. Moreover, a characteristic pattern of FVC has been documented for patients with DMD, where there is a rising phase, a plateau around 10-12 years of age, followed by a rapid decline inevitably leading to RF [153].

1.2.1.7 Cardiac involvement

Cardiac involvement is also seen more frequently in some forms of muscular dystrophy as compared to others. This may even be one of the main clinical features, manifesting as a conduction defect and/or dilated cardiomyopathy or, less frequently, as hypertrophic cardiomyopathy. Patients with a laminopathy inevitably develop atrioventricular block, atrial paralysis or atrial fibrillation by the age of 30, requiring a pacemaker or an implantable defibrillator. Indeed, in EDMD patients where such lamin A/C deficiency is the underlying cause, cardiac disease is part of the cardinal triad of distinguishing features, (with arrhythmias, conduction block and cardiomyopathy), and is the major risk for mortality [151, 154].

1.2.2 Laboratory investigations

1.2.2.1 Serum creatine kinase

The serum level of CK is a sensitive parameter of muscle damage, since it is readily released in cellular injury. Consequently, the degree of persistent CK elevation (hyperCKemia) in part reflects the underlying disease process, varying with the different forms and thereby providing an approximate indication of the type of muscle disease involved [155-157].

Highest CK levels are seen with conditions causing muscle fibre necrosis, as in the dystrophinopathies, where CK values are elevated 10- to 200-fold above the upper limit. These high levels are detected from birth, and may be the first sign of the disorder as an incidental finding in blood tests during investigation of other symptoms. They are also mild or moderately elevated in about two thirds of female carriers [158]. Other muscular

dystrophies often exhibiting hyperCKemia include several LGMD and some CMD sub-types, such as calpainopathy, dysferlinopathy, the sarcoglycanopathies, anoctaminopathy and fukutin-related proteinopathies [30].

Lesser degrees of CK elevation are seen in more indolent myopathies, such as facio-scapulo-humeral dystrophy, myotonic dystrophy and inclusion body myositis [155]. Disorders such as mitochondrial or other metabolic myopathies, that cause muscle atrophy without cell membrane injury, often have normal CK levels [155, 156].

1.2.2.2 Electromyography

This method is important for establishment of the myopathic nature of the disease and for exclusion of neurogenic processes that cause weakness, such as myasthenic syndromes, myotonias and other peripheral nerve disorders. However, EMG is becoming less favoured because, besides being an invasive technique, the findings are non-specific and do not contribute towards the differentiation of the muscular dystrophies [159].

1.2.2.3 Imaging

The pattern and distribution of structural muscle changes can be detected by imaging techniques such as ultrasound, computational axial tomography (CAT) scan and magnetic resonance imaging (MRI). Several studies have shown that specific distributions of muscle abnormalities are specific for some muscular dystrophies. Although CAT scan pioneered the field of identifying specific muscle changes associated with different diseases [160], this is now almost completely replaced by muscle MRI, which simultaneously avoids ionizing radiation and enables multiplanar scanning [161]. These imaging techniques may be a useful adjunct to differential diagnosis of myotonic dystrophy, EDMD, the CMDs and particularly of certain LGMD sub-types [152, 161-164].

Muscle MRI can also assist in choosing an appropriate muscle to biopsy, so that muscles that are affected by the disease process but not completely atrophied are targeted [165].

Cerebral MRI may also be useful in determining the complete clinical phenotype associated with some forms of muscular dystrophy. This helps distinguish the LGMDs from the CMDs as well as between the different forms of CMD, where leukodystrophic changes are often associated with the classical merosin-deficient forms and structural brain abnormalities with the ‘non-classical’ forms, such FCMD [166].

1.2.2.4 Muscle biopsy

Histological and immunohistochemical analysis of the muscle biopsy plays a key role in the assessment of patients, providing useful diagnostic information to guide genetic analysis. At the start, standard muscle histopathology provides information about muscle architecture and is useful to exclude most congenital myopathies with specific ultrastructural changes (such as nemaline rods or central cores), or to exclude a primary neuropathic disorder. The histological findings that define muscular dystrophies include variation in fibre size, including congenital fibre type disproportion (CFTD), the presence of degenerating and regenerating fibres, invasion by macrophages and, ultimately, replacement by adipose and connective tissue (Figure 1.2.2.1).

The relative prominence of different dystrophic features may vary depending on the type of disorder, the age of the patient and the muscle biopsied (since the degree of involvement varies among different muscle groups in the different forms). Whereas DMD and other severe forms usually present the full dystrophic picture, in FSHD, for instance, inflammatory changes are often the main feature; in oculo-pharyngeal muscular dystrophy (OPMD), rimmed vacuoles and nuclear inclusion bodies are typical; rimmed vacuoles are also prominent in titinopathy (LGMD2J) and in telethonin deficiency (LGMD2G); increased variation in fibre size may be the key finding in EDMD [167-169]. Neonates with UCMD (collagen VI deficiency) may only show non-specific myopathic changes or CFTD [170].

Necrosis may not be evident in the CMDs, but the presence of fibrosis and adipose tissue reflect the loss of muscle fibres. The pattern of muscle histology in the LGMDs is typically dystrophic, but the extension of pathology does not always correlate with disease severity (as seen in Tables 1.1.4.1 and 1.1.5.1).

The histological features alone are therefore often insufficient to distinguish many forms of muscular dystrophy, and even less so to distinguish the different sub-types within each group. Muscle biopsy analysis by means of immunostaining/immunofluorescence or Western blot (immunoblot), with labeled antibodies that react specifically with individual muscle proteins, may help separate the disease entities. There are currently over forty commercially available antibodies to proteins involved in the muscular dystrophies that can be used for immunohistochemical (IHC) and/or immunoblot analysis [171].

IHC studies using antibodies to dystrophin will reveal complete absence in DMD and a decrease in quantity in BMD. In DMD carriers, the muscle biopsy may show a mosaic immunostaining pattern of fibres either containing or lacking dystrophin. Further analyses performed by Western blot techniques will enable quantitation and the identification of dystrophin with abnormal molecular weight (Figure 1.2.2.2).

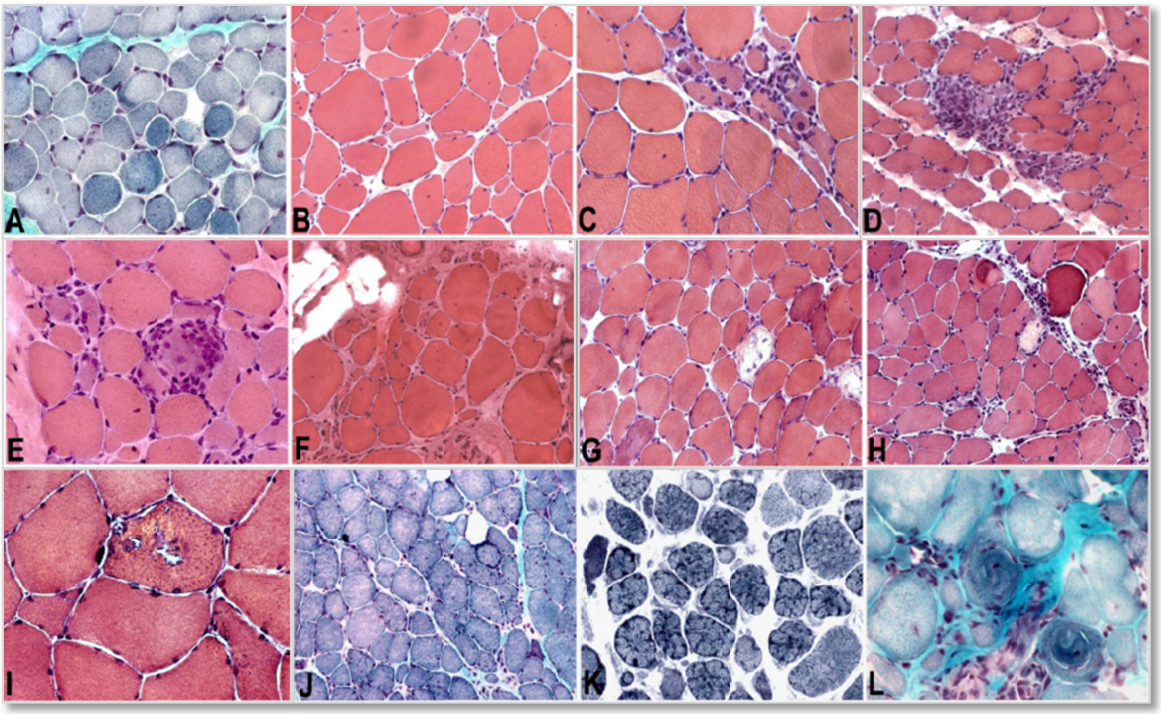

Figure 1.2.2.1

Features of dystrophic muscle: increased fibre size variability (A-F), increased central nuclei (B,C,F), endomysial fibrosis and fatty replacement (F), regenerating fibres (C,D), necrotic changes such as phagocytosis (E) and jaline fibres (G,H) and inflammatory changes (D). Additional changes include rimmed vacuoles (I), lobulated fibres (J,K) and whorled/ coiled fibres (L). (Adapted from [152]).

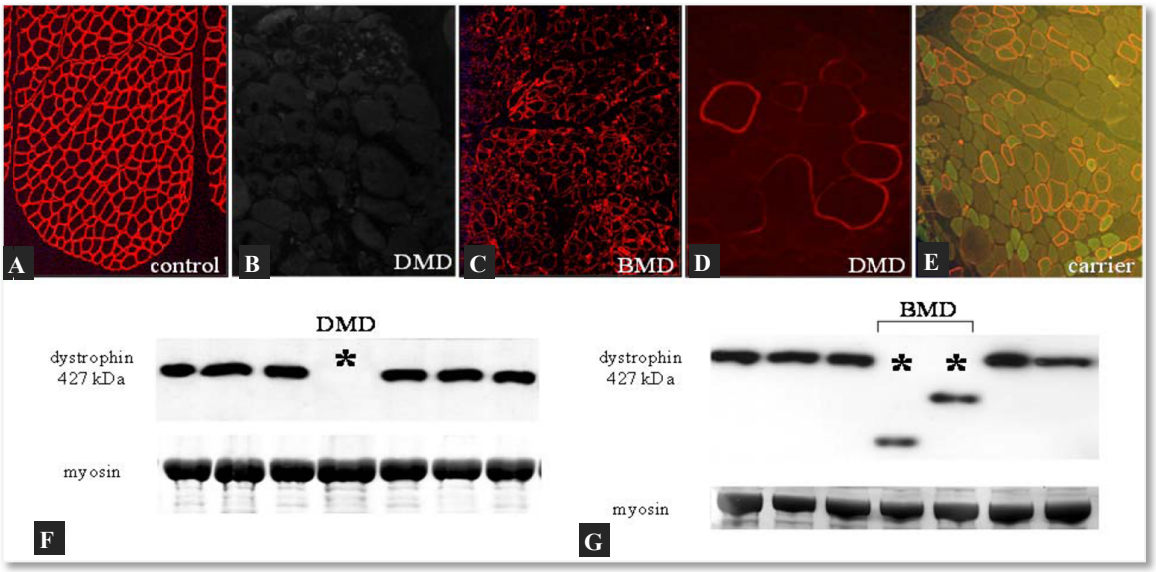

Figure 1.2.2.2

Immunostaining for dystrophin. **A-E:** immunofluorescence in cross sections from skeletal muscle biopsy, showing continuous labeling in all myofibres of control (A), complete absence in a DMD patient (B), reduced and patchy staining in a BMD patient (C), scattered dystrophin-positive (revertant) fibres, sometimes observed in older DMD patients (D), and a mosaic pattern of dystrophin-positive and -negative fibres in a DMD carrier (E). **F-G:** Western blot analysis showing complete absence in DMD (F) and dystrophin of reduced weight and amount in BMD (G). Myosin heavy chain is used for normalizing protein amounts. (Adapted from [152]).

Provided that protein degradation is excluded, absence of labeling for one protein should indicate a primary defect in the respective gene; this is true for instance in the case of emerin and collagen VI (Figure 1.2.2.3).

Mutations in the *EMD* gene result in absence of emerin IHC staining in the nuclear envelope of skeletal muscle, and this is specific for X-linked EDMD [172]; however, EDMD may also result from mutations in other genes, such as *FHL1* (X-linked EDMD) or *LMNA* (AR- and AD-EDMD), for which there are no robust protein assays [173, 174]. Indeed, lamin A/C staining usually appears normal by IHC, when mutations in the *LMNA* gene are identified as causal in some of the laminopathies, such as LGMD1B [173].

Reduced or absent staining for collagen VI is also disease-specific, indicating a primary collagenopathy (UCMD or Bethlem myopathy) [175, 176], although in Bethlem myopathy (the more benign form) these alterations may be very subtle [177]. In either case, diagnostic confirmation relies on genetic testing which is required to identify the causal mutation among one of the three collagen VI α-chains.

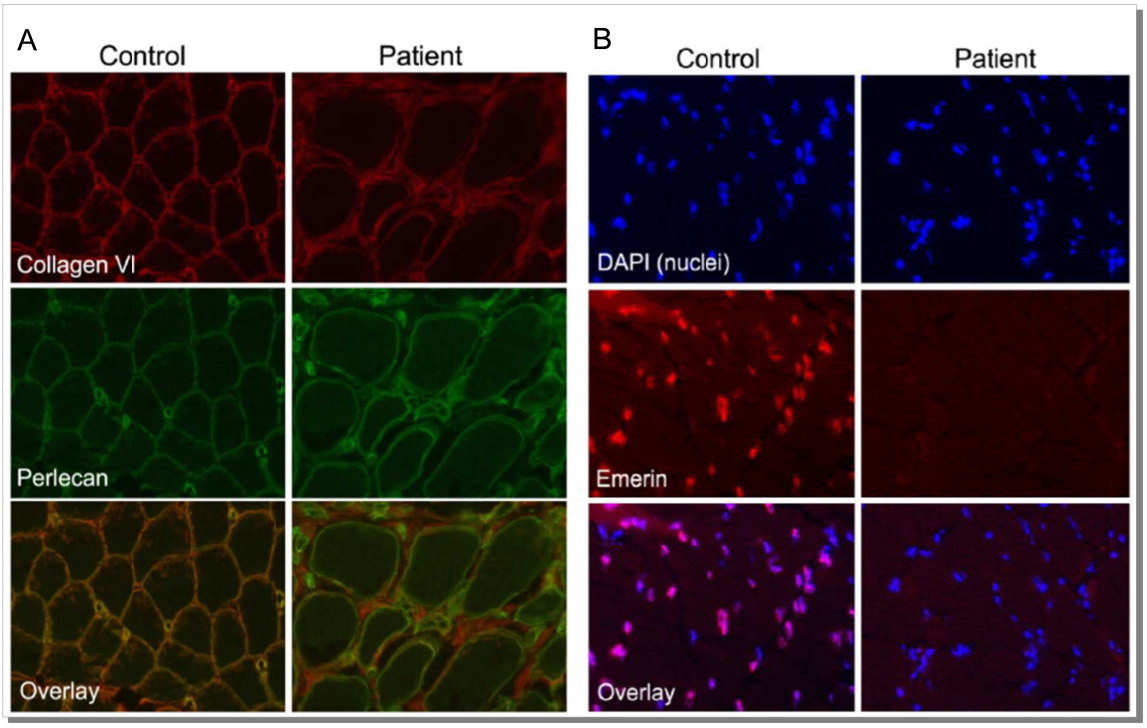

Figure 1.2.2.3
Immunostaining for collagen VI and emerin. **A:** Patient with UCMD. Collagen VI is reduced or lost at the sarcolemmal membrane but may be present in interstitial connective tissue. Stain intensity equalizing with perlecan, in control muscle, followed by image overlay, reveals a dominance of perlecan (green labeling), indicating a relative deficiency of collagen VI (red labeling). **B:** Patient with X-linked EDMD, showing DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) staining of nuclei but absence of staining for emerin in the nuclear envelope. (Adapted from [174]).

Due to structural and/or functional association between many of the muscle proteins, abnormal staining patterns may reflect secondary effects. In particular, the proteins of the DGC appear to be strictly interrelated in terms of presence and correct localization: dystrophin deficiency in DMD, BMD and manifesting carriers is accompanied by a secondary reduction of sarcoglycans and dystroglycans [178-181]; in turn, deficiency of a particular sarcoglycan is often associated with the secondary reduction of other sarcoglycans [182, 183].

Secondary deficiencies of calpain-3 have been described in several forms of muscular dystrophy, including dysferlinopathy and titinopathy; however, in some other forms this deficiency may reflect rapid enzyme degradation and/or inadequate biopsy processing conditions. Similarly, non-specific secondary abnormalities in dysferlin may be seen in calpainopathy and in caveolinopathy [174, 184-187]. Western blot analysis, however, is specific for primary deficiencies in calpain-3 and in dysferlin (Figure 1.2.2.4). Secondary reduction of α-dystroglycan and/or laminin-α2 is usually seen and in LGMD sub-types with glycosylation defects as the underlying pathological mechanism (as seen in Tables 1.1.4.1 and 1.1.5.1). For this reason, biopsies should be tested for several proteins concurrently in immunohistochemical studies. Ideally they should also be analyzed by Western blot, and the results interpreted in the context of the clinical phenotype.

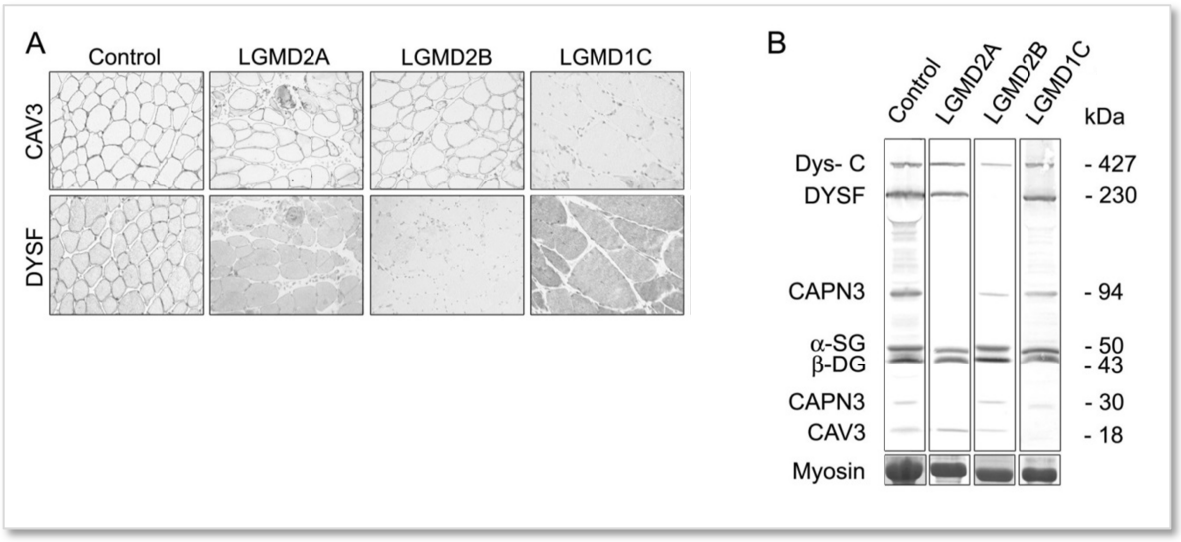

Figure 1.2.2.4

Comparative expression of dysferlin, caveolin-3 and calpain-3. **A:** Immunohistochemical analysis revealing deficiency of caveolin-3 and/or dysferlin in LGMD2A, LGMD2B and LGMD1C. **B:** Immunoblotting is more reliable for diagnosis of primary calpain-3 deficiency (LGMD2A) and primary dysferlin deficiency (LGMD2B). CAV3 - caveolin-3; DYSF - dysferlin; CAPN3 - calpain-3; Dys-C - dystrophin C-terminal; α-SG - α-sarcoglycan; β-DG - β-dystroglycan. (Adapted from [171]).

1.2.2.5 Genetic analysis

The approach in molecular diagnosis of a disease is highly dependent on the mutation type and spectrum of the candidate gene. For example, intragenic deletions and duplications make up 75-90% of the mutations detected in the dystrophin gene [83, 84], whereas in most of the LGMD and CMD genes similar high frequencies are comprised by point mutations. Deletion and duplication screening should therefore be the starting point in patients with a clinically suspected dystrophinopathy, prior to sequencing, while clinical suspicion of other disorders may warrant the inverse approach.

In myotonic dystrophy type 1 (DM1), the underlying genetic basis is the expansion of a (CTG)n triplet repeat in the 3'-untranslated region of the dystrophia myotonica protein kinase (*DMPK*) gene [188]. The detection of these trinucleotide repeats, which are pathogenic above 50 but can reach ~5000 copies [189], requires analysis by triplet-primed polymerase chain reaction (TP-PCR) and/or Southern blotting (SB) – the latter enabling sizing of the expansions. Essentially all individuals with DM1 have an expanded (CTG)n allele.

A trinucleotide repeat expansion is also the underlying cause of OPMD. Here, the (GCN)n triplet in the first exon of the polyadenylation-binding protein nuclear 1 gene (*PABPN1*), becomes pathogenic when expanded to 12-17 repeats (under 10 repeats is normal) [190]. The comparatively much smaller size of this expansion in relation to that involved in DM1 makes it amenable to genetic testing by fragment size analysis.

FSHD is caused by yet another type of mutation: a reduction in the number of 3.3 kb tandem repeats (termed D4Z4), found in a non-protein-coding region of chromosome 4q. Disease develops in individuals with repeat sizes smaller than 35 kb (8 or fewer D4Z4 repeats) [191].

DM1, OPMD and FSHD are readily diagnosed clinically with a high degree of certainty and without the need of a muscle biopsy. Moreover, molecular diagnostic confirmation in these disorders is highly specific - not only gene-directed but also mutation-directed.

However, other forms of muscular dystrophy, LGMDs and CMDs in particular, present a high level of clinical and genetic heterogeneity, with overlapping phenotypes and a large number of candidate genes for each disease entity. The majority of these genes have a high frequency of diverse point mutations, requiring thorough sequence analysis. Furthermore, since many are amongst the largest human genes known to date (e.g. *DMD* with 79 exons, *SYNE1* with 147 exons and *TTN* with 363 exons: see Appendix I), compounded by the feature of generating multiple splice isoform mRNAs, analysis of more than one gene can be extremely laborious and costly.

One way to deal with such high level of heterogeneity is to perform co-segregation analysis in the families using polymorphic markers linked to the various candidate genes (see Appendix II). In the least, this may enable exclusion of some candidates, thereby narrowing down the number of genes that need to be sequenced. In cases where no linked markers are known or no key family members are available for study, simultaneous screening of mutation hotspots in the larger genes or the use of sequencing panels directed at a combination of mutations found to be most frequent in certain populations, may reduce time and cost of molecular diagnosis.

Several diagnostic algorithms have also been proposed to guide genetic testing, based on clinical and pathological phenotype in the patient, the pattern of inheritance and the frequency with which mutations are identified in each of the disease-associated genes [30, 93, 105, 108, 110, 165, 173, 174, 177, 187, 192].

A major part of the work in molecular diagnosis involves determining whether a genetic variant identified in a patient is in fact the disease-causing mutation or simply a non-pathogenic polymorphism. If the variant has been previously documented as causal, a firm diagnosis can be made. Databases that can be consulted include the *1000 Genomes* browser (http://browser.1000genomes.org), the single nucleotide polymorphism database (dbSNP - http://www.ncbi.nlm.nih.gov/SNP.index.html) and the Exome Variant Server at the University of Washington (http://snp.gs.washington.edu/EVS/). Locus specific databases (LSDBs) for single disease genes are an exceptionally valuable resource since they collate information on both known pathogenic variants and known polymorphisms. In particular, a group of LSBDs for multiple neuromuscular disease genes is found in the Leiden Muscular Dystrophy pages© (http://www.dmd.nl). Genetic variants that have not been published or listed in databases require further studies to determine their nature; these include *in silico* analysis aided by in-house or commercially available applications, expression analysis (mostly mRNA studies) and population screening.

Even in highly specialized diagnostic laboratories, the underlying genetic cause is only established in 50-80% of the patients, depending on the inclusion criteria of the cohorts [104, 106, 107, 152]. The most likely explanation is that many causal genes remain unidentified. Approaches that capitalize on the recent next generation sequencing (NGS) technologies will enable the simultaneous screening of all candidate genes (gene panels) and facilitate the identification of new genes (whole exome or whole genome sequencing) [193, 194]. As these technologies move into the diagnostic setting, medical evaluation of patients with muscular dystrophy will undergo a shift in paradigm, moving towards a “genetics-first” strategy.

2. AIMS

The determination of the gene and the genotype involved in each patient with muscular dystrophy is of pivotal importance for patient management and follow-up, for choosing the appropriate personalized therapy and for adequate genetic counselling, carrier screening and pre-natal diagnosis.

In common disorders such as DM1 and FSHD, diagnosis is relatively straightforward, as clinical features guide the appropriate genetic tests to be performed. However, establishing the specific diagnosis in patients with other forms of muscular dystrophy remains difficult, despite the skill of the clinician and pathologist. This is attributable to the extremely wide, as well as overlapping, clinical and genetic heterogeneity among and between the different forms and sub-types.

Considering these difficulties and the knowledge that some forms are more prevalent than others in different populations, the general aim of this work was to establish the genetic profile and epidemiology of the main muscular dystrophies in Portugal. To that end, a large cohort of patients was studied, consisting of 615 unrelated probands/families with clinical presentation and/or histopathological findings consistent with D/BMD, LGMD or CMD. Referrals were from all over the Country, including the autonomous regions of Madeira and the Azores.

The approach was to carry out extensive molecular screening and obtain a detailed molecular characterization in as many patients as possible, so as to:

- determine the relative proportion of the different forms and sub-types, and their geographical distribution;
- identify “private” mutations in our population;
- identify *foci* of founder effects;
- establish new genotype-phenotype correlations, thereby widening the mutational and phenotypic spectra.

Given the large and ever-expanding number of candidate genes, molecular and clinical information was collated with the aim of establishing appropriate algorithms for future approaches to the diagnosis of muscular dystrophy, in our population.

It is envisaged that this work will constitute a valuable contribution in terms of proper patient care and well-informed reproductive choices in the respective families. However, considering further effective benefits for the patients themselves, and in view of the promising personalized therapies currently under on-going trials, the final aim of this work was to develop and implement a national patient registry, with trial-ready clinical, pathological and molecular data of registered patients.

3. RESULTS AND DISCUSSION

3.1 DYSTROPHINOPATHIES

3.1.1 Profile of *DMD* genetic variants in Portuguese patients

3.1.1.1 Study approach

Referrals for *DMD* testing were received from all over the Country, including the autonomous regions of Madeira and the Azores, usually referred by Paediatrics, Neuropaediatrics and Neurology clinics, and to a lesser extent by Genetic counselling and prenatal diagnostic clinics. The clinical and complementary examination data of the patients available from the start were thus highly variable, depending on the referral service, age and time interval of follow-up, family history, among others. A total of 503 probands were received, some of which for exclusion of dystrophinopathy. Although a negative test result could not categorically exclude this diagnosis, some of these cases were later found to be different forms of limb-girdle muscular dystrophy (Section 3.2).

For the overview of Portuguese D/BMD patients, the cohort included only those cases that fulfilled a combination of minimal diagnostic criteria such as male gender (if sporadic) or an X-linked pattern of inheritance, elevated CK levels, proximal muscle weakness at onset (especially of the lower limbs) and/or compatible muscle histology (dystrophic and abnormal immunohistochemical staining for dystrophin). One affected female showing complete absence of dystrophin (therefore “patient”, as opposed to manifesting carrier), and two obligate carrier female relatives of deceased patients (with well-founded suspicion of having a dystrophinopathy), were also included. In all, the cohort included index cases from 312 apparently unrelated families.

Due to the size and complexity of the *DMD* gene, as well as the diversity of mutation types, molecular analysis of the patients often required a combination of techniques to enable the detection of gross deletions and duplications as well as the more subtle variants such as point mutations, small insertions or deletions, del/ins variants and other sub-exonic rearrangements, as well as deep intronic lesions.

The study approach evolved over the years, following the trends of technical advances. Initially, only large deletions (involving one or more exons) could be detected, as this was carried out by Southern blotting and hybridization with a total of eight cDNA probes (required to cover the entire coding sequence). Multiplex PCR was introduced next, where two nineplex reactions for the most frequently deleted 18 exons (as observed by SB

analysis), enabled coverage of ~98% of all deletions known at the time [195, 196]. In fact, the conception of multiplex PCR was driven by the labour-intensive *DMD* testing – it was first designed, validated and used routinely in D/BMD diagnosis.

Both SB analysis and multiplex PCR could be adjusted to provide semi-quantitative assays for the detection of large duplications [197, 198]. These were substituted by more sensitive and less laborious techniques such as MAPH (multiplex amplifiable probe hybridization) [199] and MLPA (multiplex ligation-dependent probe amplification) [200], which incorporate both the principle of hybridization and of PCR. Again, the development of these techniques arose from the need to overcome the difficulties in *DMD* testing. Indeed, this was the first disorder for which a commercial MLPA kit was made available, and this technique is currently accepted as the gold standard for gross deletion and duplication screening, in the dystrophinopathy diagnostic setting.

The detection of the remaining smaller lesions that would account for a considerable number of patients could not circumvent *DMD* gene sequencing. The gene size, however, made this a challenging task, and so initially it was carried out mostly in a research context. As the detection of such variants began to gain clinical importance, the need was felt to include sequence analysis in the diagnostic setting.

Different working groups used a variety of preliminary screening strategies to avoid the laborious and costly sequencing of *DMD*. This included multiplexed single-strand conformational polymorphism (SSCP) analysis [201], denaturing high-performance liquid phase chromatography (dHPLC) [202], denaturing gradient gel electrophoresis (DGGE) [203, 204] and single-condition amplification/internal primer (SCAIP) sequencing [84, 205]. Given the technical experience and the platform available, multiplexed SSCP was initially chosen to pre-screen our patients. This was soon abandoned due to the exceptionally high polymorphic content of the gene, which yielded complex conformational profiles. Systematic sequencing of the 79 exons was then adopted, after being simplified by designing M13-tailed primers for the basic PCR and using an M13 universal primer for the asymmetric cycle sequencing reactions.

It also became evident that DNA-based strategies alone could not pick up all mutations, particularly deep intronic variants affecting pre-mRNA splicing [206-208], and so studies were complemented with cDNA profiling and sequence analysis of muscle transcripts.

The study of our patients accompanied these technical developments over time. With the sequential implementation of methods providing higher sensitivities (thus higher mutation detection rates), the earlier unconfirmed or poorly characterised cases were submitted to re-analysis, including mRNA studies when muscle specimens were available.

3.1.1.2 Results

General mutation profile

A dystrophinopathy was confirmed at the molecular level in a total of 308 probands, representing 91% (284/312) of the unrelated families (**Paper I**).

The distribution according to mutation type was in close agreement with that reported in the literature for most large cohorts [83, 84, 198], although slightly higher proportions of gross duplications and point mutations were found in our patients (Figure 3.1.1.1 A). This difference may reflect the fact that (i) in diagnostic laboratories, duplication and sequence analysis has not been obligatory in the routine workup, and (ii) re-analysis and extensive studies in our patients, including RNA-based methodologies, increased the detection rate of point mutations, especially sub-intronic lesions. In all, 175 different mutations were detected in our cohort, 39 of which had not been previously documented (**Paper I**).

Approximately 1/3 of the cases were sporadic, with no known family history of the disease. The *de novo* mutational events were ascertained via the mothers' non-carrier status in 82 cases (29%). The mutation profile of these neomutations followed the general cohort's distribution according to type (Figure 3.1.1.1 B). Given the low reproductive fitness, this substantiates the fact that *de novo* mutations are the prime factor maintaining the constant frequency of the disease in all populations. The slight discrepancy, with a relatively lower proportion of deletions among neomutations compared to the total cohort, may be explained by the characteristics that define the composition of the general patient sample: (i) deletions are comparatively more frequent in BMD than in DMD and (ii) usually only patients with BMD have offspring, thereby transmitting mutations derived from a single mutational event and maintaining the relative proportions (Figure 3.1.1.1 C) [83].

In a longitudinal descriptive study of a paediatric sub-population consisting of 97 patients, mostly from the north of Portugal and with follow-up in a multidisciplinary clinic of the *Hospital de Crianças Maria Pia*, BMD patients also presented a higher proportion of gross deletions, as compared to DMD patients [209]. Unlike the general cohort, this paediatric sample is unbiased for the offspring effect, although there is an underrepresentation of the late-onset spectrum of dystrophinopathies, including mild BMD-type cases.

All considered, the distribution among true sporadic cases, in which the neomutational occurrences have been demonstrated, are those that reliably reflect the proportion of *DMD* mutation types in the gene.

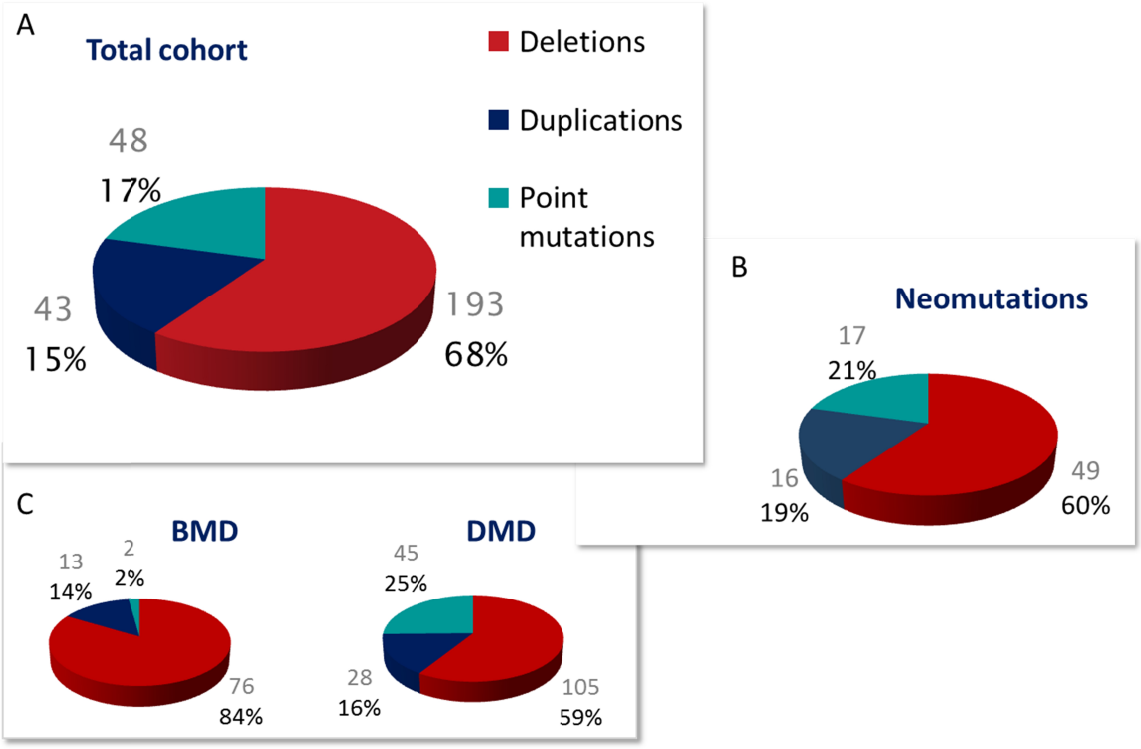

Figure 3.1.1.1 Distribution of independent *DMD* mutations according to type. A. Mutation type proportions in the cohort (284 unrelated patients). **B**. Mutation type proportions in cases resulting from *de novo* mutational events. **C**. Distribution of independent mutation types in patients with Duchenne *versus* Becker muscular dystrophy. This was ascertained in 269 unrelated patients, where phenotypes could be clearly distinguished, clinically and/or by dystrophin staining pattern in muscle biopsy. Deletions – whole-exon deletions; Duplications – whole-exon duplications; Point mutations – sub-exonic and sub-intronic lesions.

Gross deletions

Figure 3.1.1.2 shows the distribution of gross deletions (n=193) along the *DMD* gene, detected among the 284 unrelated families. The extensions of the deletions vary greatly, but there are two clear hotspots of exons involved; one near the 5' region of the gene, between exons 3 and 30, and a main hotspot near the centre of the gene, between exons 45 and 52. Exon 47 is the most commonly deleted exon, while the most recurrent deletion is that of exons 45-47. Mapping of the deletion extremities in this region shows breakpoint clustering between introns 44 and 55 (Figure 3.1.1.2 C). There is a clear preponderance of 5' breakpoints in intron 44; the 3' breakpoints are more evenly distributed in this central deletion hotspot, but the majority are found in intron 50.

In the 5' hotspot, deletion of exons 3-7 was the most recurrent, although only detected in three unrelated patients. These findings are in general agreement with the collective data obtained from the public locus-specific database for *DMD* (Leiden Mutation Database, http://www.dmd.nl; last accessed March 2015), currently with entries for >3000 unique variants recorded from world-wide sources. They agree to a slightly lesser extent with that

observed in other major cohorts, such as the French national registry (>2400 entries) or the joint data of the United Dystrophinopathy Project in the USA (>1000 records) [82-84]; here, for example, exon 45 is seen to be the most commonly deleted exon.

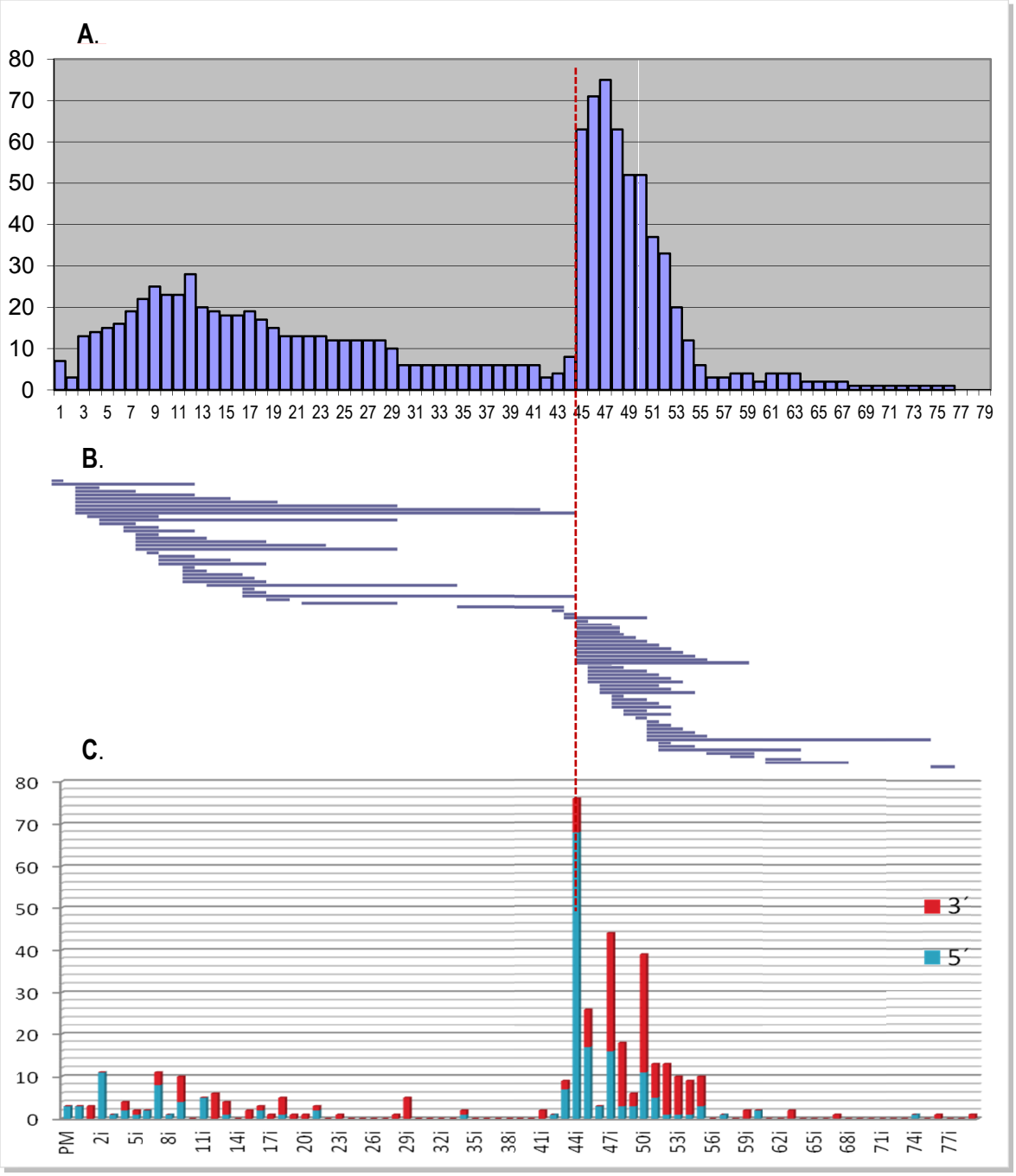

Figure 3.1.1.2 Profile of the 193 different gross deletions detected among the patient cohort. A. Frequency of exon involvement in deletions. **B**. Extension of individual gross deletions. Each different deletion is represented only once. **C**. Distribution of intronic breakpoints, indicating number of 5' (blue) and 3' (red) deletion endpoints found in each intron.

The two deletion hotspots coincide with the major meiotic recombination regions of the gene [210], and both processes have been attributed to intron composition. However, there appears to be no breakpoint clustering across most introns; in particular, in gross deletions involving intron 44, where the highest rate of 5' breakage is found, patients presenting the same whole-exon deletions are seen to differ at the intronic sequence level [211-216]. Several groups have searched the regions encompassing some deletion breakpoints in an attempt to elucidate the underlying mechanism(s). Although repetitive elements are a key feature of *DMD* introns, most studies show that these recombinogenic sequences are seldom associated with breakpoints of "simple" gross deletions, but rather with some of the more complex rearrangements within the gene [208, 212, 213, 215]. It therefore appears that homologous unequal recombination is unlikely to be a major mutation mechanism for these gross deletions. On the other hand, many of these studies highlight the frequent detection of 2-6bp micro-homologies at deletion breakpoint junctions [208, 216, 217], which are characteristic of the non-homologous end-joining (NHEJ) DNA repair mechanism, that promotes illegitimate recombination.

In this group of patients with deletions, there are two situations illustrative of genotyping pitfalls; one is the presence of two mutations running in the same family and the other is the co-occurrence of two *DMD* mutations in the same patient. These highlight the need for caution in diagnosis, as they have implications for family members in terms of carrier screening, genetic counselling and prenatal diagnosis (**Paper I**).

Gross Duplications

Gross duplications were detected in 43 (15%) unrelated families, which is a slightly higher proportion than has been documented for large cohorts (7-11%) [82-84]. As shown in Figure 3.1.1.3, the distribution of the involved duplicated exons along the *DMD* gene reveals a major duplication hotspot affecting exons 3 to 20. The concentration around this single hotspot region is also in agreement with documented data (http://www.dmd.nl), except that our results do not show a clear median around exon 20.

Contrary to what has been reported for most recurrent duplications to involve exons 3-7 and 5-7 (http://www.dmd.nl) or the single exon 2 [83, 84, 218, 219], each of these was detected only once in our cohort. Here, duplication of exon 44 was the most recurrent, albeit only in three unrelated families, and those involving exons 2-7, 3-19, 8-11, 61 and 65 were found in two unrelated families. As opposed to deletions, a higher number of duplications (21%) involved single exons, in line with previous findings [215, 220].

The majority of 5' duplication breakpoints were found in intron 2, but intron 7 showed a distinct cluster of both 5' and 3' breakpoints (Figure 3.1.1.3 C). If recombinogenic elements were to account for most gross rearrangements, then it would be expected that the profile of the distribution of exons involved in gross deletions should overlap with that of gross duplications. This is this only observed to a small extent in the 5' hotspot, supporting the hypotheses that various mechanisms are involved in these mutational events, with fundamental differences in the origin of deletions and duplications.

While there are several hypotheses on possible mechanisms involved in the generation of gross deletions, very few groups have looked at duplications. Nonetheless, sequence analysis around breakpoint junctions provides evidence for both homologous and non-homologous recombination. The former (involving mostly *Alu* repetitive elements) account for a minority of cases [221], whereas the latter occurs more frequently, either through unequal sister chromatid exchange [221] or through synthesis-dependent NHEJ [219].

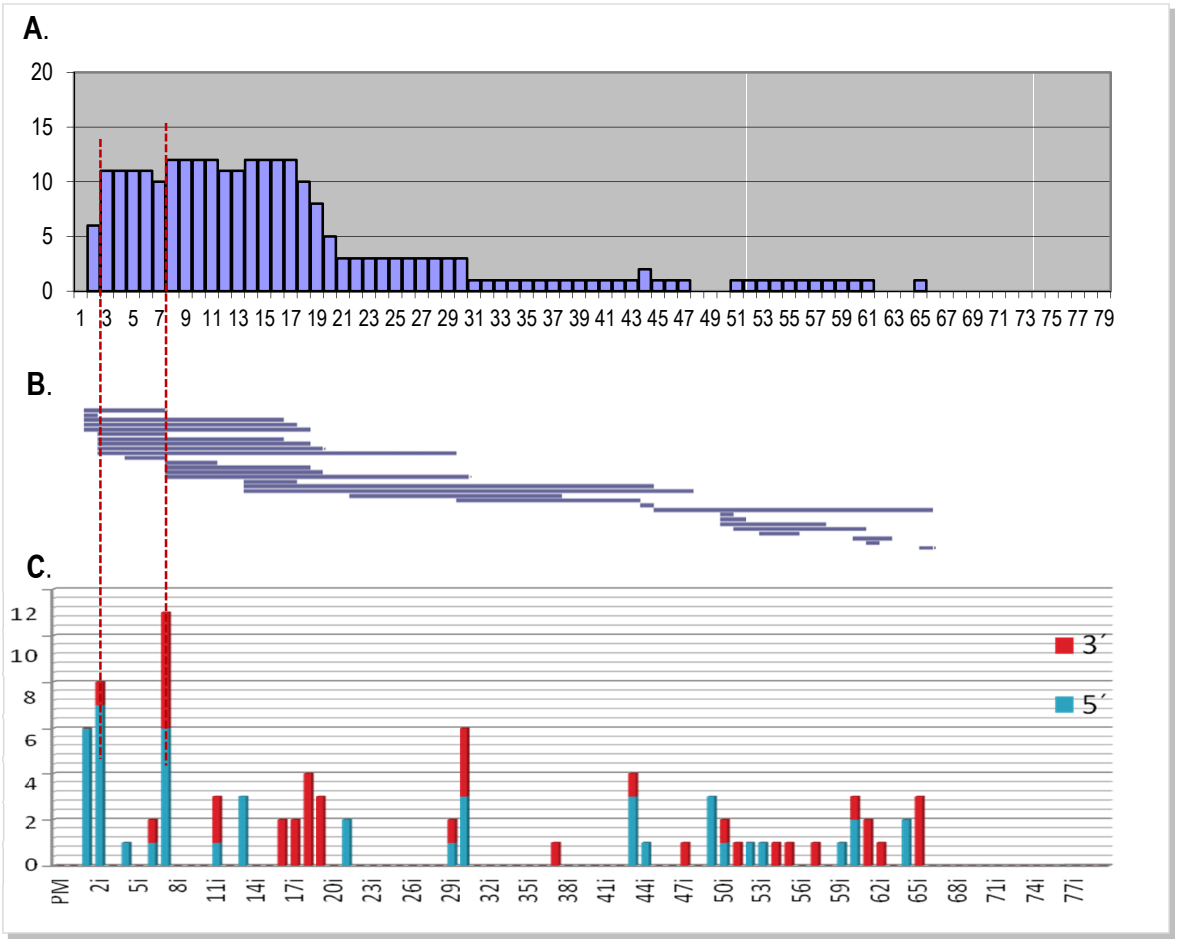

Figure 3.1.1.3 Profile of the 43 different gross duplications detected among the patient cohort. A. Frequency of exon involvement in duplications. **B**. Extension of individual gross duplications. Each different duplication is represented only once. **C**. Distribution of intronic breakpoints, indicating number of 5' (blue) and 3' (red) duplication endpoints found in each intron.

Point mutations

Forty-eight independent point mutations were detected in this cohort, with a distribution according to type as represented in Figure 3.1.1.4. Frameshifts and nonsense mutations were seen to be the most frequent types, similar to other large data collections [83, 84]. The numbers in these and other publications often consider all of the patients tested, whether related or not, whereas our results consider only unrelated cases, thereby reflecting a realistic profile of independent mutational events. None of our patients were found to carry missense mutations, which are also reported to be the least frequent, constituting 0-3% of all *DMD* mutations in large cohorts [83, 84, 222].

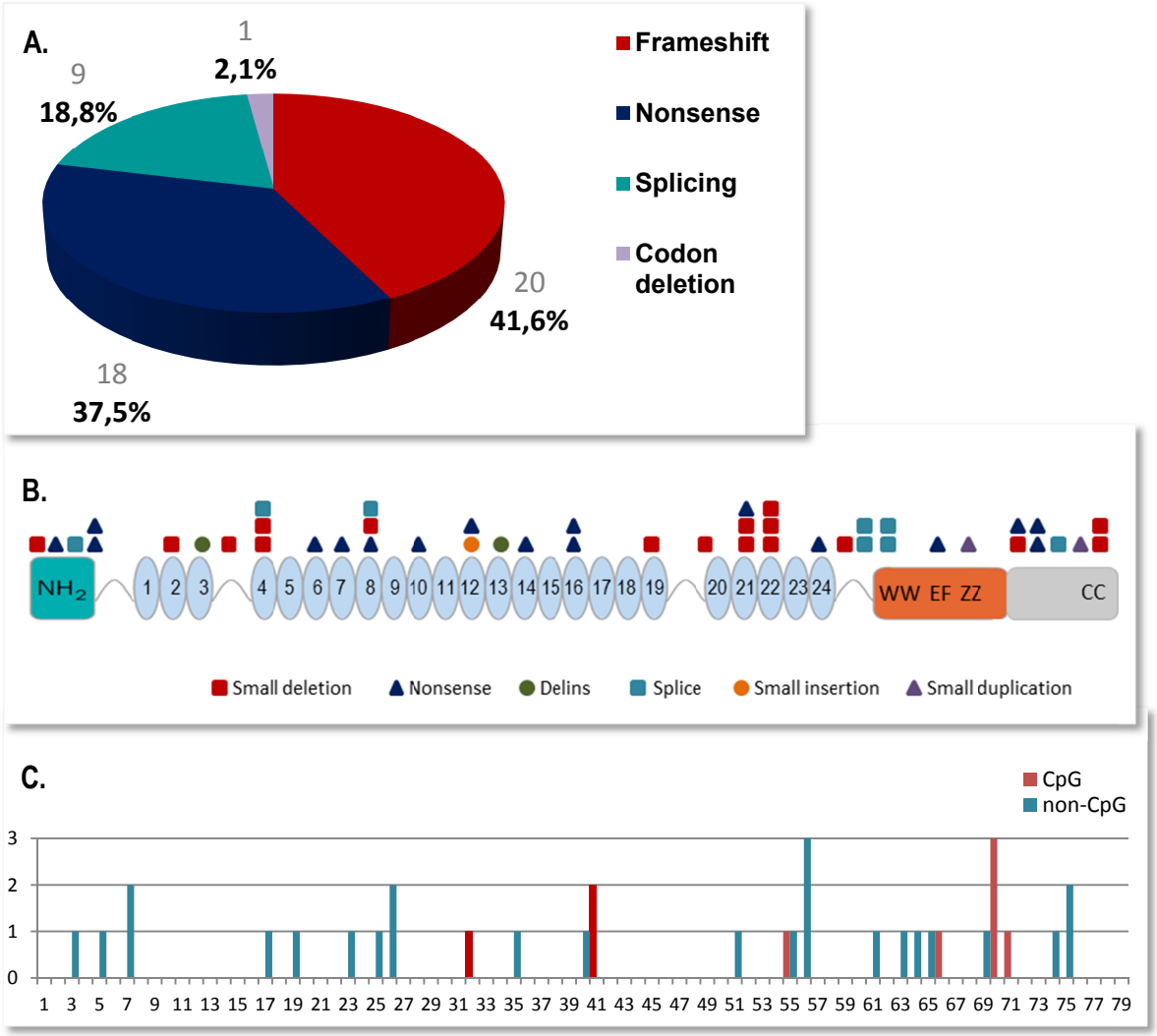

Figure 3.1.1.4 Distribution of the 48 different small lesions detected among the patient cohort. **A**. Distribution of small lesions according to type. (Frameshifts include all small insertions, duplications, deletions and delins leading to premature termination codons, but not those that arise due to splice site mutations). **B**. Distribution of small lesions along the *DMD* gene, represented on the protein. **C**. Distribution of single-base changes along the 79 exons of the *DMD* gene, distinguishing CpG from non-CpG mutation sites.

There are no distinct hotspots for small lesions along the gene, nor is significant clustering observed according to type. Only four recurrent mutations were found, in exons 18, 41, 56 and 70, which emphasizes the need to screen the whole gene sequence to identify these mutations.

Single-base changes, namely those giving rise to nonsense mutations and some of the splice site and frameshift mutations, were analysed for sequence context prevalence. CpG dinucleotides are known to be mutational hotspots due to frequent oxidative deamination of 5-methyl cytosine [223]. Contrary to what might have been expected, therefore, in our patients only 9 of the 34 single-base changes (26,5%) affected CpG dinucleotides. These were all transitions; a single G>A transition was a splice site mutation while the other 8 were C>T transitions, all of which converted an arginine codon into a stop codon (CGA>TGA). The latter accounted for half of all detected nonsense mutations (9/18), and were distributed among 5 of the known 28 CpG hypermutable targets for stop codon creation in the *DMD* gene [224].

The sequence context of the remaining changes was diverse, with no obvious consensus. Moreover, no nonsense mutations were found to derive from a C>T transition at a glutamine codon with conversion to an ochre chain termination codon (CAA>TAA) which, besides being the cause of muscular dystrophy in the common mouse model *mdx*, has also been reported several times in the literature, accounting for up to 24,5% of the nonsense mutations in dystrophinopathy patients [83, 225].

Among the cases with single-base changes leading to splicing mutations was a deep intronic C>A change, 9192bp downstream of exon 62. The substitution creates a splice sequence that outweighs the normal splice signal, thereby compromising correct pre-mRNA processing and leading to a pseudoexon inclusion (**Paper I**).

From previous reports of pseudoexon inclusions [83, 84, 206, 207], it appears that either DMD or BMD can result from out-of-frame pseudoexons, depending on the amount of residual normal transcript that is produced, ultimately depending on the relative strengths of the normal versus the newly generated splicing signals. Deep intronic lesions activating cryptic splice sites, or other complex rearrangements, may be more common than has been recognized. In the more recent data sets, they constitute about 0,5% of all mutations [83, 84], but this is clearly an underestimation because screening for these mutations currently relies heavily on RNA-based methods, requiring muscle specimens which are often not available for many patients.

Genotype-phenotype correlations

According to the reading frame rule [85], mutations that disrupt the open reading frame, leading to premature truncation, give rise to the severe DMD-type dystrophinopathy, whereas those that preserve the reading frame and enable read-through to the 3' end of the gene give rise to the milder BMD-type dystrophinopathy. In our cohort this held true for most cases, although analysis was limited by the heterogeneity and/or lack of phenotypic data available for all patients.

Among the nonsense mutations, 15/18 (83%) were found in patients with a DMD phenotype. This included a female who presented a severe course of the disease. A modification of the human androgen receptor assay (HUMARA) [226] was performed and a skewed X-inactivation pattern was observed, thereby providing evidence that her phenotype resulted from this heterozygous mutation (Figure 3.1.1.5).

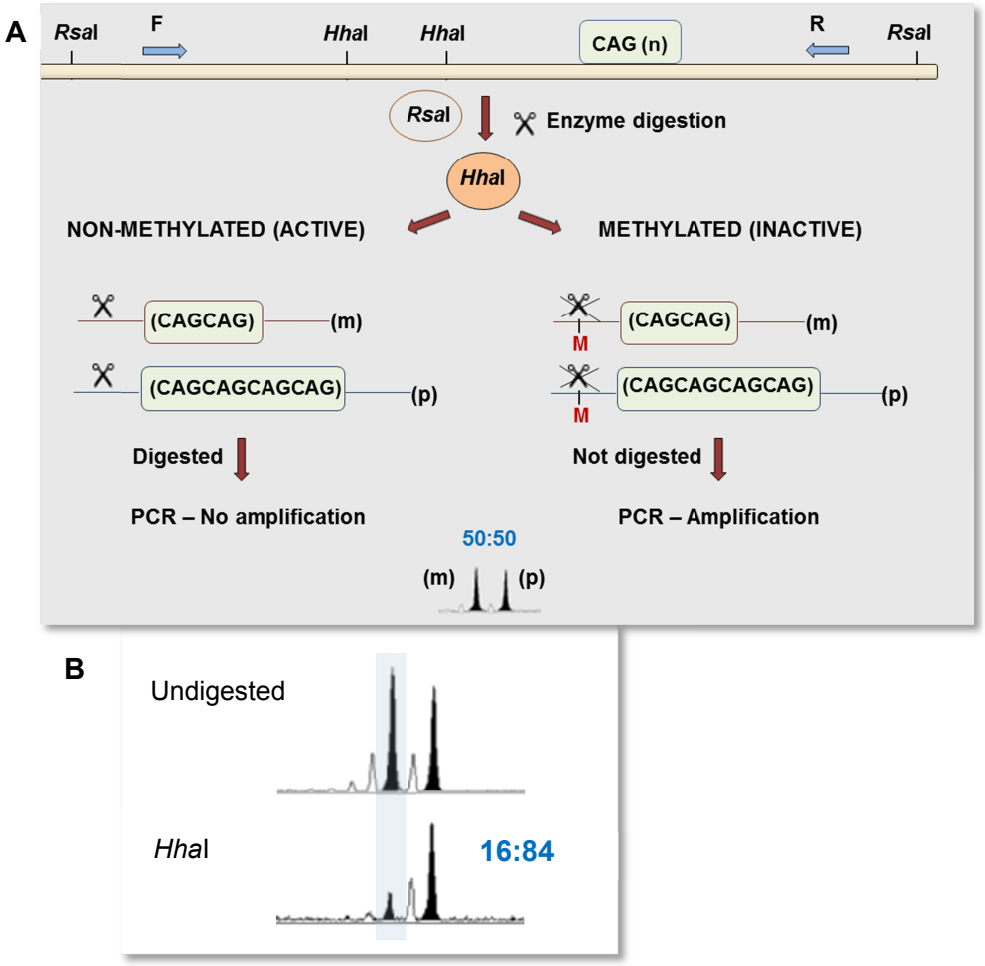

Figure 3.1.1.5 Analysis of the X-chromosome inactivation pattern. A. Description of the HUMARA assay used to detect skewing from the random X inactivation pattern. **B**. Profile observed in a female patient with a nonsense mutation and a DMD-like phenotype, showing almost complete skewing, with preferential inactivation of one of the X-chromosomes. *Rsa*I – restriction enzyme; *Hha*I – methylation-sensitive restriction enzyme; m – maternal; p – paternal.

Although theoretically nonsense mutations should give rise to normal amounts of truncated protein, very little or no protein is detected in the muscle, indicating that the transcripts might be degraded by nonsense-mediated mRNA decay or, as has been shown more recently, that the truncated proteins are unstable and/or non-functional due to the lack of the vital COOH domain [23, 74].

The milder BMD-type presentation in cases with nonsense mutations is usually explained by some degree of altered splicing whereby, either by resorting to cryptic splice sites or by means of whole-exon skipping, there is baseline production of in-frame mRNA that evades the premature truncation signal and maintains the COOH terminus intact. Many apparent exceptions to the reading frame rule can be explained by the activation of cryptic exonic or intronic splice sites, and the degree of severity often correlates directly with the amount of in-frame transcript that is produced. This was seen by cDNA studies in one of our BMD patients with a nonsense mutation (c.3281T>A), where two products were detected: one containing the predicted stop codon in exon 25 and a minor, shorter product corresponding to an in-frame transcript lacking exon 25 (**Paper I**).

In some mutations that coincide with splice sites, *mutation leakage* can lead to the production of variable amounts of normal transcript. This was observed in a patient with the splicing mutation c.3603+3A>T, predictably leading to a frame-shift with premature truncation. However, clinically he presented with strain-induced myalgia after the age of 3 and at 11 he showed signs of limb girdle weakness, consistent with a BMD-like presentation. Transcript analysis revealed the presence of a longer, abnormally spliced and out-of-frame transcript (with partial inclusion of intron 26) as well as residual amounts of normal full-length transcript (explaining the milder phenotype).

A further, although more particular, exception to the reading frame rule is exemplified by a BMD patient with an out-of-frame deletion involving exons 1 to 5 [predictably p.(0)]. Although this would be expected to result in severe disease, the patient maintained unassisted ambulation at age 23 and referred an affected cousin who had only become wheelchair bound in his fourth decade. No muscle specimen was available for cDNA studies; however, it was recently shown that alternative initiation of translation can take place at one of two AUG codons within exon 6 [87]. One may thus speculate that, having the muscle promoter and the exon 6 alternative initiation codons intact, normal amounts of dystrophin product is produced, albeit with compromised actin-binding activity [227, 228].

While the above examples of alternative splicing or translation initiation show phenotype amelioration compared to what is predicted, there are also cases of severe disease associated with mutations that are theoretically less detrimental. One such example in our

cohort is the in-frame codon deletion (c.10097_10099del) detected in a patient that was clinically classified as a severe/intermediate form. cDNA studies confirmed the removal of a single glycine residue at the beginning of the carboxy terminal domain (r.10097_10099, p.(Gly3366del)), with the maintenance of all known binding sites for interacting factors (see Figure 1.1.3.1). Although no specific function has been attributed to this region of the protein, it is one of the most highly conserved regions. A similar single codon deletion has been reported affecting the adjacent amino acid residue (p.Glu3367del), in a patient who also presents with DMD [229], corroborating the functional importance of this region. Secondary structure prediction places both residues at one extremity of an α-helix, so that even subtle deletions could cause helix displacement. In some other point mutations distributed along the gene, it has been shown that tertiary structure misfolding substantially lowers the functional capacity of the respective domains [74, 230-232].

The detection of whole-exon duplications is accepted as being sufficient to confirm the diagnosis dystrophinopathy. However, the gDNA techniques currently in practice provide information on copy number variation, but tell us nothing concerning the site or orientation of the duplicated gene segment. Without knowledge of its orientation, or whether it is present in tandem or interspersed, one cannot make assumptions regarding the reading frame. Although most turn out to be contiguous and direct, in some of these presumably “simple” duplications, studies at the mRNA level have revealed unexpected changes such as partial triplications, deletions/duplications and other complex rearrangements [216-220]. Additionally, some cases are confounded by exon skipping. It is therefore not surprising that whole-exon duplications have been reported to account for almost one third of the cases behaving as exceptions to the reading frame rule [233].

Examples from our cohort include the contrast of two patients with extensive in-frame duplications, one presenting a DMD phenotype (dup 3-29), with severe disability by the age of 8, and the other, with a larger duplication (dup 14-47), presenting as BMD with loss of ambulation only in the fourth decade. Another case concerns a two-exon duplication (dup 50-51), predictably in-frame yet associated with a severe clinical course and no dystrophin detected in the muscle biopsy. This duplication has been described previously in a patient who also presents a DMD phenotype [234].

No muscle specimens were available from our patients with whole-exon duplications, to elucidate the site, orientation or true nature of these rearrangements.

3.1.2 National Patient Registry

Due to the nature of rare diseases, research into the development of adequate therapies has been slow and discouraging. The intrinsic drawback is that these patients are dispersed and often incompletely characterized, making it difficult to find enough candidates that might be eligible to participate in clinical trials for testing potential therapies.

The creation of national and international registries for patients with orphan diseases has therefore been a major encouragement for industry to invest in the development of disease-specific therapeutic strategies that target specific genetic defects. To this end, the network of excellence designated TREAT-NMD (Translational Research in Europe – Assessment and Treatment of Neuromuscular Diseases), initiated a global patient registry; initially for DMD and later extended to other neuromuscular disorders. This global database collects encrypted (anonymised) information from the various national registries that have adopted the same data set of mandatory items, transversal for all registered patients. Besides contributing towards decision-making in industry, the trial-ready data facilitate patient recruitment by identifying all candidates for each specific mutation-centred therapeutic trial.

The genetic and clinical data collected in this work for a vast number of Portuguese dystrophinopathy patients from all over the Country, motivated the creation of the National D/BMD Patient Registry (**Paper I**). Besides constituting an important clinical and epidemiological research tool, it enables our patients to be recruited for on-going clinical trials, via the TREAT-NMD global database.

The National D/BMD Patient Registry is authorized by the *Commissão Nacional para a Proteção de Dados* and abides by the TREAT-NMD Registry Charter as well as national and European laws. It has been operational since 2012 and has catalysed the recent application for recognition of clinical trial sites in Portugal, also under the auspices of TREAT-NMD. The conception details and data profiles of the Portuguese National D/BMD Patient Registry have been documented as joint publications of the TREAT-NMD Steering Commission and registry curators [235, 236].

3.2 LIMB-GIRDLE MUSCULAR DYSTROPHIES

3.2.1 Profile of the LGMDs among Portuguese patients

3.2.1.1 Study approach

The LGMD profile in the Portuguese population was ascertained in patients referred from all over the Country over a period of 15 years. The cohort consisted of 256 index cases/families. Inclusion criteria were essentially based on compatible clinical presentation and/or muscle biopsy. Clinical phenotypes ranged from severe childhood-onset, adult-onset and distoproximal myopathy, to sub-clinical cases presenting only hyperCKemia. Muscle histology invariably revealed dystrophic features. Immunostaining, although performed in different hospital services and with different panels of antibodies, always included those directed against the three DYS domains and against the four SGs. In the more recent biopsies, staining against dysferlin, merosin (laminin-α2) and often also α-dystroglycan was included in the routine immunocytochemical analysis. None of the national neuropathology laboratories perform immunocytochemical analysis for emerin.

For most of the male patients seen to have reduced expression of the DGC proteins in muscle, screening for deletions and duplications in the *DMD* gene was first carried out, especially in young patients where clinical signs at onset are often indistinguishable between dystrophinopathy and many LGMD forms. The LGMD cohort included 28 such patients, found to be negative for *DMD* mutation screening.

Also included were 5 patients with an EDMD phenotype (in particular, presenting rigid spine and contractures), previously found to be negative for *EMD* mutation screening (XL-EDMD). These were primarily subjected to *LMNA* gene analysis, for confirmation/ exclusion of AD- or AR-EDMD.

Genetic testing for LGMD was performed for several genes using a combination of methodologies that included co-segregation analysis using linked polymorphic markers (Appendix II), gDNA and cDNA sequencing, MLPA screening and Western blot analysis. A total of 525 genetic tests were carried out for 13 different genes, namely: LGMD1A (*MYOT*, n=5), LGMD1B (*LMNA*, n=46), LGMD1C (*CAV3*, n=9), LGMD2A (*CAPN3*, n=87), LGMD2B (*DYSF*, n=105), LGMD2C (*SGCG*, n=94), LGMD2D (*SGCA*, n=46), LGMD2E (*SGCB*, n=39), LGMD2F (*SGCD*, n=18), LGMD2G (*TCAP*, n=6), LGMD2I (*FKRP*, n=55), LGMD2J (*TTN*, n=4) and LGMD2L (*ANO5*, n=11).

3.2.1.2 Results

Differential diagnosis was achieved in 195 unrelated families (76% positivity), where a total of 96 different mutations were identified, 44 of which had not been documented previously. The new variants were submitted to the respective locus-specific databases in the Leiden muscular dystrophy pages (www.dmd.nl/).

Figure 3.2.1.1 shows the distribution of the different subtypes among the cohort. The γ-sarcoglycanopathies were the most frequent subtype (~29%), clearly as a result of a founder effect with families of gypsy (Roma) ethnicity [237] and the presence of a frequent mutation thought to be of North African origin [238]. This was followed by the dysferlinopathies (~28%), with no particular hotspot along the 56 exons, except for two private mutations.

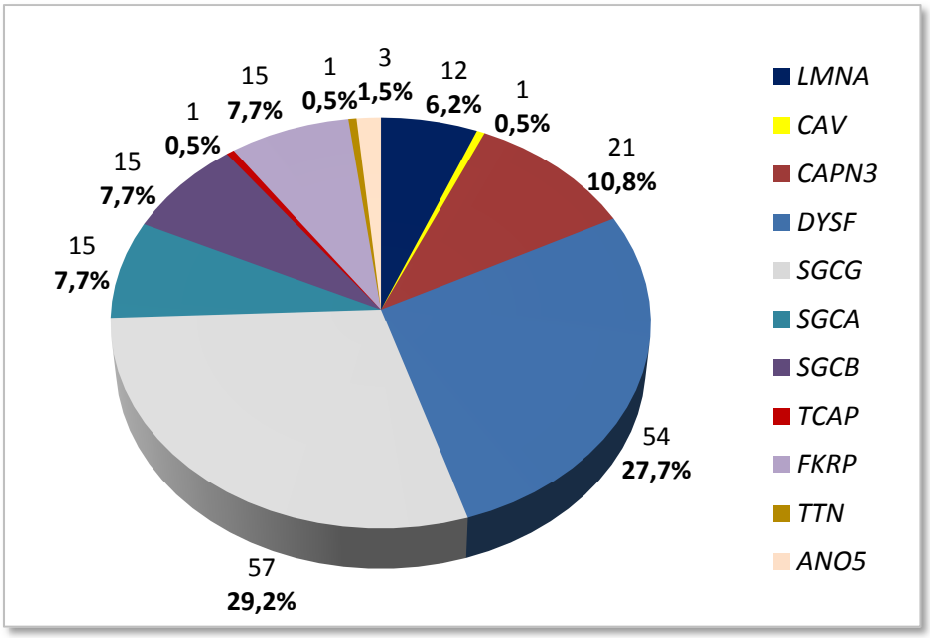

Figure 3.2.1.1 Distribution of subtypes among 195 unrelated Portuguese LGMD patients.

The dysferlinopathies were over twice as frequent as the calpainopathies, in contrast to what has been reported for most western countries. If one excludes possible founder effects of the common mutations detected in the *SGCG*, *DYSF* and *CAPN3* genes (Table 3.2.1.1), the dysferlinopathies still outweigh all other subtypes in terms of representation in the cohort (23 patients with other *DYSF* mutations versus 17 and 3 patients respectively with other *CAPN3* and *SGCG* mutations). Sample bias can be further ruled by the

observation of the same predominance of dysferlinopathies in a subset of 39 unrelated cases followed at the Coimbra University Hospital (**Paper II**).

Table 3.2.1.1 List of common mutations in the LGMD cohort.

LGMD Subtype / gene	Mutation		Nº of patients* / proportion in the subtype
	Genomic	Protein (predicted)	
LGMD2A / *CAPN3*	c.2306G>A	p.Arg769Gln	4 / 19%
LGMD2B / *DYSF*	**c.1180_1180+7del**	p.Glu353_Leu429del	7 / 13%
	c.5492G>A	p.Gly178Valfs*17	9 / 17%
	c.5509G>A	p.Asp1837Asn	9 / 17%
	c.5979dupA	p.Glu1994Argfs*3	7 / 13%
LGMD2C / *SGCG*	c.525delT	p.Phe175Leufs*20	20 / 35%
	c.848G>A	p.Cys283Tyr	34 / 60%
LGMD2D / *SGCA*	c.229C>T	p.Arg77Cys	10 / 67%
LGMD2E / *SGCB*	c.299T>A	p.Met100Lys	10 / 67%
LGMD2I / *FKRP*	c.826C>A	p.Leu276Ile	11 / 73%
		Total	**121 / 62%**

* Homozygous or heterozygous for the mutation. Mutations in bold are new with founder effects.

Table 3.2.1.1 lists the eleven most frequent mutations in the cohort, among seven genes, which together account for 62% of all molecularly characterized LGMD cases.

Two mutations alone were present in 95% of the patients with γ-sarcoglycanopathies. The variant c.848G>A was confined to families of gypsy ethnicity. This is a known private mutation among the gypsy Roma [237], linked to the microsatellite marker D13S232. Our first 15 positive families were screened for this polymorphism, and the disease was seen to always co-segregate with allele 5 (CA_{13}), supporting the common origin and founder effect among our patients. No other mutation or LGMD subtype was detected in patients of this ethnicity, who all presented a severe, DMD-like phenotype.

Similarly, the variant c.525delT (initially denoted Δ521-T) is known to be in linkage disequilibrium with allele 4 (CA_{17}) of D13S232 in North African patients, where it was originally described [239]. In our cohort, all patients carrying this mutation were Caucasians. Moreover, it was seen to co-segregate with alleles 3 (CA_{18}), 4 (CA_{17}), and 5 (CA_{13}), implying an association also with a central European origin. In this case, where 50% of the patients (10/20) were homozygous and the other 50% were compound heterozygotes for c.525delT, phenotypes were highly variable in terms of onset and progression. Moreover, homozygosity for c.525delT was found in patients with either infantile or adolescent onsets, and with equally variable clinical courses.

In the dysferlinopathies, four mutations were seen to be relatively frequent. Among these, c.1180_1180+7del (p.Glu353_Leu429del) and c.5492G>A (p.Gly178Valfs*17) were novel and with a founder effect. The former was shown to have a common origin with a patient diagnosed in Uruguay, and a migratory link was discovered (**Paper III**).

Studies on the latter variant revealed it to be a private mutation in our population. Moreover, new alternatively spliced dysferlin transcripts were identified, one of which (the ∆49/∆50 isoform) was found to have higher expression levels in peripheral blood leukocytes than in muscle, both patient and in control specimens (**Paper IV**). According to the working reference at the time, this variant was published as c.5429G>A.

Of note also is the exceptionally high frequency of the *FKRP* mutation c.826C>A (p.Leu276Ile), present in 11/15 LGMD2I patients. This follows the trend of other European populations, where it is known to be a relatively common mutation. In Denmark [240] and in the North American S-leut Hutterites [241] it accounts for all patients with LGMD2I.

Among the 12 unrelated cases of laminopathy (*LMNA* gene) were 4 of the 5 index cases that had previously been screened for XL-EDMD; these were characterized as AD-EDMD (n=1) and AR-EDMD (n=3), the latter all representing novel mutations. All 4 presented a severe phenotype, with onset ranging between 1 and 5 years of age.

One of the LGMD1B cases, with the novel missense mutation c.80C>T, pertained to a family with 12 affected members. The laminopathies may be associated with potentially fatal cardiac arrhythmias, and 8 family members with suggestive clinical phenotype of LGMD1B had suffered sudden death between the second and third decade of life. However, a second cardiac disorder was seen to co-segregate in this family, namely Brugada Syndrome (a channelopathy), resulting from a mutation in *SCN5A* [242].

IHC results from muscle biopsies enabled direct orientation of the molecular studies in 33% of suspected SG referrals and 62% of suspected DYSF referrals. Irregular staining for the SGs is known to often represent a secondary deficiency to underlying abnormalities in other structural components of the sarcolemma or cytoskeleton, and vice-versa; in particular, the dystrophinopathies often show deficiencies of the various SGs.

In the case of DYSF, complete absence in muscle IHC analysis invariably results from mutations in the *DYSF* gene, whereas reduced or irregular staining may be attributable to a primary defect in *DYSF, CAPN3* or *CAV*3 (implicated in DYSF trafficking) [243].

IHC analysis for calpainopathy has low sensitivity and specificity. Moreover, there are no frequent mutations or hotspots in this relatively large and highly polymorphic gene. Among the patients screened for *CAPN3* mutations, three presented only one mutated allele,

which was insufficient to provide a differential diagnosis. These restraints triggered the implementation of Western blot analysis in an effort to identify CAPN3 deficiencies, in terms of abundance and of autolytic function. Figure 3.2.1.2 exemplifies the semi-quantitative (A) and qualitative (B) studies, which also enabled the clarification of the three patients with a single mutated allele; two of these cases presented loss of CAPN3 autolytic function, thereby providing evidence of calpainopathy and justifying further molecular studies, whereas in the third case the diagnosis of calpainopathy was excluded.

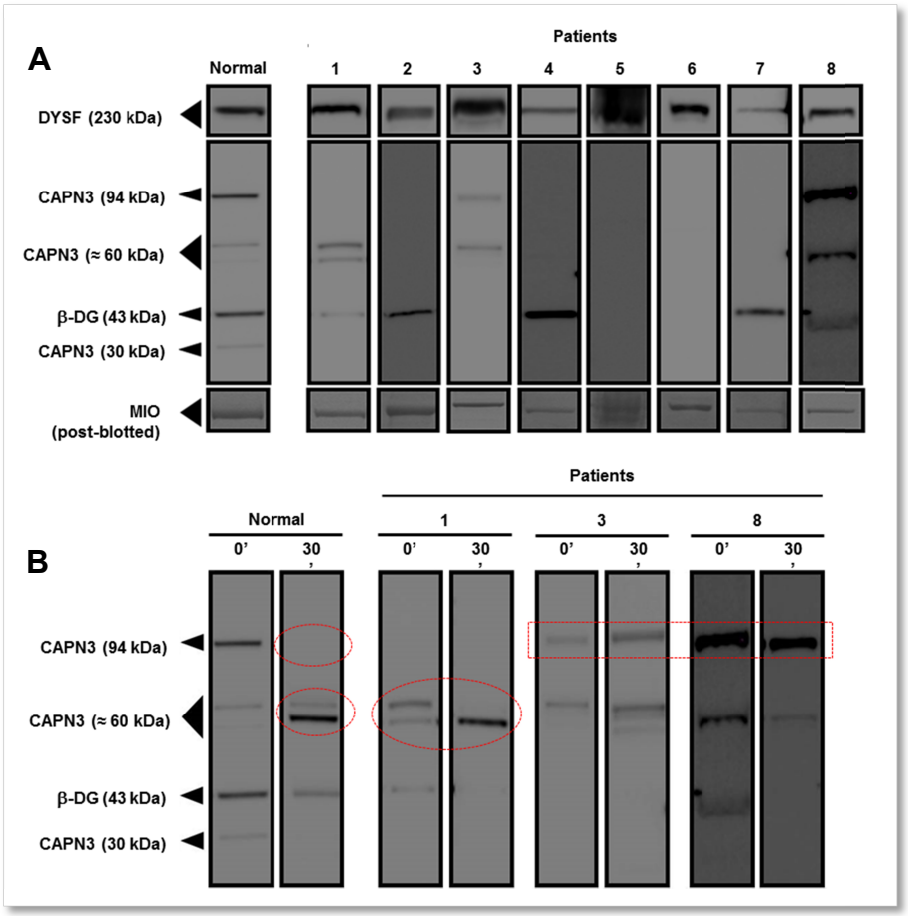

Figure 3.2.1.2 Study of calpain-3 abundance and autolytic activity by immunoblotting.
A. Semi-quantitative analysis of CAPN3 showing normal protein abundance in patients 1, 3 and 8 but no detectable protein in patients 2, 4, 6 and 7 (poor specimen quality for patient 5). **B**. Autolytic activity in the 3 patients with normal CAPN3 abundance. Patient 1 shows normal degradation (94 kDa to ~60 kDa products), whereas patients 3 and 8 lack autolytic activity.
DYSF - dysferlin; CAPN3 – calpain-3; β-DG - beta-dystroglycan; MIO - myosin.

In this cohort, the clinically suspected diagnosis was not confirmed in 61 cases (24%). Some of these were later characterized at the molecular level and shown to be several forms of Congenital Muscular Dystrophy (Section 3.3).

3.3 CONGENITAL MUSCULAR DYSTROPHIES

3.3.1 Profile of the CMDs among Portuguese patients

3.3.1.1 Study approach

The patient cohort consisted of 90 unrelated patients referred from all over the Country over the past 15 years. Inclusion criteria were (i) clinical signs such as congenital hypotonia, developmental delay and early onset progressive muscle weakness, (ii) the presence of severe dystrophic features on muscle biopsy, together with IHC results showing no involvement of products known to be deficient in certain LGMDs (specifically DYS, the SGs and DYSF) but often with abnormal staining for laminin-α2 and/or α-DG, and/or (iii) structural anomalies on brain MRI. None of the national neuropathology laboratories perform IHC analysis for collagen VI.

Among this group were 15 patients that had been screened for genes implicated in some LGMD subtypes, with phenotypes that could possibly represent milder forms of CMD. In particular, many of these undiagnosed cases presented clinical features such as spine rigidity and/or contractures, and marked dystrophic features in the muscle biopsies.

The molecular studies involved a combination of methodologies that included gDNA and cDNA sequencing, MLPA screening, long-range PCR, Southern-blot analysis and Western blot analysis. Overall, 188 genetic tests were carried out for 9 different genes, namely: MDC1A (*LAMA2*, n=65), UCMD1 (*COL6A1*, n=10), MDDGA1 (*POMT1*, n=22), MDDGA2 (*POMT2*, n=22), MDDGA3 (*POMGNT1*, n=22), MDDGA4 (*FKTN*, n=5), MDDGA5 (*FKRP*, n=12), RSMD1 (*SEPN1*, n=15) and L-CMD (*LMNA*, n=15).

The molecular diagnostic approach in general began with gDNA sequencing. In larger and/or more complex genes such as *LAMA2*, *COL6A1* and *SEPN1*, mutational hotspots were screened prior to sequencing of the entire gene. Complementary techniques were applied as deemed necessary for molecular elucidation, depending on sequencing outcomes. The choice of candidate gene(s) in the study of each patient was determined firstly by IHC results in muscle, whenever these were available and informative for laminin-α2 and/or α-DG staining, and secondly by specific clinical signs such as eye and/or brain involvement.

3.3.1.2 Results

Differential diagnosis was achieved in 67 unrelated families (~74,5% positivity). A total of 35 novel variants were identified: 28 in *LAMA2*, 1 in *FKRP*, 2 in *POMGNT1*, 2 in *COL6A1* and 2 in *FKTN*. These new variants were submitted to the existing LSBDs in the Leiden muscular dystrophy pages (www.dmd.nl/). For several genes without existing international databases, these were created and are currently still curated by us (Section 3.3.2).

Figure 3.3.1.1 shows the distribution of the different subtypes among the cohort. MDC1A was by far the most frequent form, accounting for ~78% of the molecularly characterized CMD patients. This was followed by the collagen VI-deficient UCMD and the lamin A/C-related CMD (L-CMD), while the least frequent were found to be the individual secondary dystroglycanopathies.

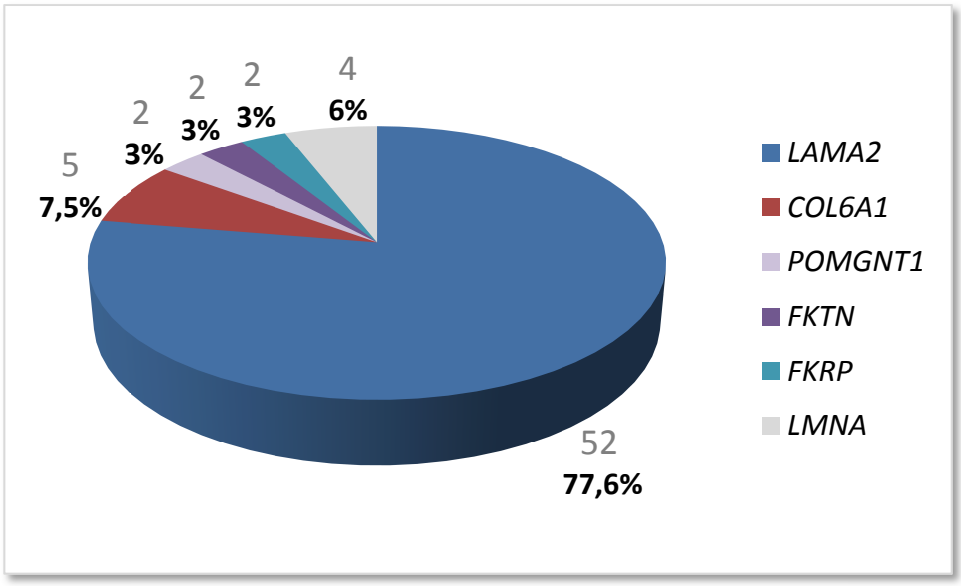

Figure 3.3.1.1 Distribution of subtypes among 67 unrelated Portuguese CMD patients.

MDC1A (also referred to as laminin-α2 deficiency or merosin-deficient CMD) is known to be one of the most common forms of CMD in Europeans, with reports of this comprising between 20 and 40% of all molecularly characterized CMD cases [108, 131, 132]. The exceptionally high prevalence of MDC1A among our patients may be attributable to a higher efficiency in *LAMA2* mutation screening; for example, our patients were screened for gross rearrangements, which is an approach that is not usually carried out in other

studies. Indeed, the first fully characterized gross rearrangement reported in the *LAMA2* gene was a multiexonic deletion detected in one of our patients (**Paper V**).

This out-of-frame deletion involving exon 56 (c.7750-1713_7899-2154del) was found to be one of the most frequent pathogenic variants amongst our patients, accounting for 23% (12/52) of the cases. This work demonstrated the clinical relevance of screening for gross rearrangements in the *LAMA2* gene, indirectly leading to the development of the respective MLPA commercial kit (P391-A1 and P392-A1 from MRC-Holland).

Since then, the first pathogenic large duplication and a further three large deletions were identified in our patient cohort (**Paper VI**).

The high number of differentially diagnosed merosinopathies can also be explained by the fact that inclusion criteria contemplated patients with partial laminin-α2 deficiency in muscle biopsies and/or abnormal brain MRI, regardless of whether or not other characteristic clinical signs were evident. These included patients presenting with later-onset and slower disease progression, including acquisition of independent ambulation. Two such patients with only subtle changes for laminin-α2 in IHC studies, but with alterations seen on brain imaging, were characterized at the molecular level with *LAMA2* mutations. These presented a particularly atypical combination of clinical signs; the first case was striking for the predominant CNS involvement (cognitive impairment and refractory epilepsy) but with normal muscular strength, while the second presented as a LGMD phenotype, with muscle weakness, rigid spine and an unusually severe cardiac involvement. The description of these atypical cases widened the spectrum of the *LAMA2*-related phenotypes (**Paper VII**).

Among the other CMD subtypes, of note is the detection of 2 cases resulting from mutations in the *FKRP* gene, commonly associated with the later onset LGMD phenotypes. At the time, very few of these cases with congenital presentation (MDDGA5 subtype) had been reported, and this is still considered a rare differential diagnosis. Both infants presented with muscle weakness, and one developed rigid spine and contractures. In both cases the muscle biopsies showed fibre size variation, necrosis and fibrosis, whereas brain MRIs were normal.

Another unexpected differential diagnosis in the cohort involved the two cases with Fukuyama CMD (FCMD). One of the two novel variants detected represents the first multiexonic duplication ever reported in the *FKTN* gene (**Paper VIII**).

3.3.2 Locus-specific databases

In chronological terms, the molecular study of the CMDs was first carried out on the genes known to be involved in the more severe forms, since a group of patients presented a full combination of inclusion criteria, with typical clinical signs such as ocular and cerebral involvement and suggestive muscle histology with reduced staining for α-DG. Mutation screening was thus initially carried out in three genes most commonly implicated at the time, namely *FKTN*, *POMT1* and *POMGNT1* [244].

The numerous variants detected, both apparently polymorphic and putatively pathogenic, made mutation screening a laborious task since no internationally recognized databases were available for mutations in these genes. To facilitate molecular diagnosis, and as advocated by the Human Variome Project Consortium [245, 246], we generated publicly available LOVD-powered Locus Specific Databases for these three genes, using the LOVD software [247] available through the Leiden Muscular Dystrophy pages (http://www.dmd.nl). The work involved collecting and reviewing all known DNA variants for these three genes from sources such as HGMD, OMIM and all scientific literature [244] (example in Appendix III).

Consequently, we were invited and appointed as curators of these three LSDBs, as well as of the LSDBs for *POMT2* and *FKRP* – the two other genes known at the time to be involved in secondary dystroglycanopathies. These are all recognised by the HGVS (Human Genome Variation Society) as the official international LSDBs for the respective genes and are accessible via http://www.dmd.nl/ at the following pages:

http://www.dmd.nl/nmdb/home.php?select_db=POMGNT1

http://www.dmd.nl/nmdb/home.php?select_db=POMT1

http://www.dmd.nl/nmdb/home.php?select_db=FKTN

http://www.dmd.nl/nmdb/home.php?select_db=POMT2

http://www.dmd.nl/nmdb/home.php?select_db=FKRP.

A subsequent collaboration with the Department of Electronics, Telecommunications and Informatics (DETI) of the University of Aveiro, resulted in the development of a user-friendly bioinformatics tool to annotate, analyze and compare sequence and protein data of human genes [248]. “Variobox” enables agile navigation and crossing of data retrieved from several sources, such as Protein Data Bank, UniProt, RefSeq and LRG (Locus Reference Genomic) databases, as well as from WAVe – a tool also developed by the DETI group, that centralizes and standardizes the data of all available LSDBs [249].

4. PUBLICATIONS

PAPER I

New variants, challenges and pitfalls in DMD genotyping; implications in diagnosis, prognosis and therapy.

Journal of Human Genetics (2014) 59, 454–464

www.nature.com/jhg

ORIGINAL ARTICLE

New variants, challenges and pitfalls in *DMD* genotyping: implications in diagnosis, prognosis and therapy

Rosário Santos[1,2,9], Ana Gonçalves[1,9], Jorge Oliveira[1], Emília Vieira[1], José Pedro Vieira[3], Teresinha Evangelista[4], Teresa Moreno[5], Manuela Santos[6], Isabel Fineza[7] and Elsa Bronze-da-Rocha[2,8]

Molecular characterization of patients with Duchenne or Becker muscular dystrophies is essential for establishing a differential diagnosis, allowing appropriate clinical follow-up, patient management and genetic counseling. In light of the recent mutation-based therapeutic approaches, *DMD* gene analysis has gained further relevance. Owing to the size and complexity of the *DMD* gene and the diversity of mutation types, molecular analysis is not always a straightforward task requiring the combination of several methodologies. Our national genetic diagnostic service genetically characterized 308 dystrophinopathy patients (284 unrelated families), leading to the identification of 175 distinct mutations, including 39 unpublished variants. These studies revealed several potential diagnostic pitfalls (because of technical limitations or related with *DMD*'s genetic heterogeneity) that may be overlooked even considering the international disease-specific diagnostic guidelines. Comprehensive analysis involved expression studies at the mRNA level, the identification of splicing changes and ultimately providing evidence for apparent exceptions to the reading-frame rule. Besides increasing the mutation detection rate, this detailed molecular characterization is indispensable for the identification of suitable candidates for the new mutation-centered therapies. As patient registries are internationally recognized as essential for clinical trial recruitment, this led us to develop the Portuguese Duchenne and Becker Muscular Dystrophy registry in collaboration with the Translational Research in Europe—Assessment and Treatment of Neuromuscular Diseases network.
Journal of Human Genetics (2014) **59,** 454–464; doi:10.1038/jhg.2014.54; published online 10 July 2014

INTRODUCTION

Duchenne and Becker Muscular Dystrophy (DMD and BMD, or D/BMD), collectively known as dystrophinopathies, are the most common neuromuscular disorders in childhood. The newborn male incidence of DMD ranges between 1 in 3500 and 6000,[1] whereas that of BMD is 1 in 12 000 and 30 000 male births.[1,2] DMD (MIM#310200) is characterized by progressive muscle wasting with onset at the age of 3–5 years, and patients being wheelchair bound at the age of 10–12 years, with death in their twenties often as a result of respiratory or cardiac insufficiency. Mental retardation is observed in approximately one-third of DMD patients.[3,4] BMD (MIM#300376) is a milder allelic form with a later onset and slower progression, with variable degrees of disease severity ranging from a more severe DMD-like presentation to an almost asymptomatic status maintained until the fifth or sixth decade of life.

Both DMD and BMD are caused by mutations in the *DMD* gene (MIM*300377), one of the largest known human genes, spanning about 2.4 Mb of genomic DNA.[5] The gene encodes several transcripts, the most relevant in the muscular disease being a 14-kb muscle-specific isoform. The protein product of the *DMD* gene—dystrophin—is part of the large dystrophin-associated glycoprotein complex. This structure is necessary for maintaining the link between the cytoskeleton and the extracellular matrix, playing an essential role in preserving the integrity of the muscle cell membrane.[6–8] The lack of dystrophin leads to sarcolemmal fragility, triggering a cascade of events that eventually result in muscle cell death.

The large size of *DMD* contributes toward a high mutation rate and it has been estimated that ~25–33% of all cases result from *de novo* mutational events.[9,10] The most common mutations in the *DMD* gene are the deletion or duplication of one or more exons, accounting all

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together for about two-thirds of D/BMD patients. These types of mutations can occur almost anywhere along the gene; however, most are clustered in two hotspot regions: a 'minor hotspot' spanning exons 2–20 and a 'major hotspot' spanning exons 45–53. The standard diagnostic approach, using techniques such as Multiplex Ligation-Probe Amplification (MLPA), Southern Blot and multiplex PCR, easily detects these mutations. The remaining 30% of pathogenic changes are essentially sub-exonic or sub-intronic, and include nonsense mutations, splice mutations, frameshifts (small deletions or insertions), mid-intronic variants that activate cryptic splice sites and, to a lesser extent, missense mutations. These are usually detected by *DMD* gene sequencing performed at the genomic and/or complementary DNA (cDNA) level.

DMD phenotype is usually caused by mutations that disrupt the reading frame leading to the complete loss of dystrophin expression, whereas BMD patients are generally associated with in-frame mutations leading to the production of abnormal yet semi-functional protein (with intact N and C termini).[11] This genotype–phenotype correlation is known as the 'reading-frame rule' and has been demonstrated in about 91% of cases.[12] However, several exceptions to this rule have been reported.[12,13] These exceptions are more commonly found in BMD patients and their frequency depends on the type of genetic defect involved: 15% of cases with *DMD* deletions[14] and approximately 30–34% of cases with duplications.[14,15]

Comprehensive genetic analysis and detailed molecular characterization of individual case reports and more extensive patient cohorts are essential not only for academic purposes, but also for the generation of useful information for disease prognosis and even for the development of new therapeutic approaches.

Several therapeutic strategies are currently being developed for DMD.[16] These include approaches not directly depending on *DMD* genotype of the patient, such as (i) myostatin blocking to increase fiber size and bulk, (ii) utrophin upregulation, to compensate the dystrophin deficiency and ameliorate symptoms, and (iii) gene therapy ('viral' and 'non-viral'), with the aim of introducing a non-native functional copy of the *DMD* gene. Other strategies intend to restore the reading frame or correct the native dystrophin; these include (i) stop codon read-through drugs, such as gentamycin and ataluren (PTC124), (ii) exon skipping using antisense oligonucleotides and (iii) *DMD* gene modification with meganucleases or zinc finger nucleases. Some of these experimental treatments currently under clinical trials are considered personalized approaches in the sense that their application and effectiveness will depend on the *DMD* gene mutation and its effect at the mRNA/protein level. Patient selection and recruitment for these clinical trials are facilitated by the development of detailed disease registries. To that end, the network of excellence TREAT-NMD (Translational Research in Europe—Assessment and Treatment of Neuromuscular Diseases) has been promoting the creation of national registries in several European countries and worldwide.[17] These national registries feed into a single global database managed by the network. For this integration, information regarding the *DMD* genotype profile of patients is required, as well as a clinical data set of items harmonized across all national registries.[18]

In this study, we present the profile of 39 unpublished *DMD* mutations detected in Portuguese patients in the course of our national diagnostic service. We exemplify the importance of detailed molecular characterization with special focus on the pitfalls and challenges regarding the molecular diagnosis of the dystrophinopathies.

MATERIALS AND METHODS

Patients

In the course of our genetic diagnostic service, provided on a national basis since 1989, 503 index cases/families were referred to our laboratory for *DMD* analysis. The present study describes a cohort of 312 unrelated cases (Supplementary Data I), with inclusion criteria based on compatible muscle biopsy, family history and/or clinical signs. It comprised 139 cases, including a female patient with irregular immunostaining for the three dystrophin domains (N-terminus, rod and C-terminus). The remaining 173 cases were selected on the basis of a highly suggestive clinical presentation and/or evidence of X-linked inheritance. These included two female relatives of deceased patients, who presented the at-risk haplotype.

The genetic studies had informed consent from the patients or their legal tutors, and laboratory registries were authorized by the National Committee for Data Protection.

Genomic DNA (gDNA) analysis

gDNA was extracted from peripheral blood by the salting-out method.[19]

The first studies relied on linkage analysis with polymorphic markers and Southern blotting and hybridization with cDNA probes.[20] After 1991, this was preceded by deletion screening using two multiplex PCR reactions[21,22] for 18 *DMD* fragments (17 exons plus the muscle specific promoter Dp427m/exon1). As of 2005, initial deletion/duplication screening has been carried out by multiplex PCR and MLPA analysis, the latter substituting Southern blotting. The two multiplexed PCR mixes were adjusted to include a further two exons (namely 21 and 27). PCR products were labeled with FAM or NED fluorochromes and resolved on an ABI 3130xl capillary sequencer (Applied Biosystems, Foster City, CA, USA) using Genescan 500 size standards (Applied Biosystems), and data analyzed with GeneMapper v4.0 (Applied Biosystems). MLPA analysis was carried out using P034 and P035 kits (MRC Holland, Amsterdam, the Netherlands), according to the manufacturer's recommendations. MLPA was done using 150 ng of gDNA obtained from peripheral blood, according to the manufacturer's instructions. PCR products were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The resulting fragments were analyzed by using GeneMarker v.1.5 software (Softgenetics, State College, PA, USA).

Detection of variants other than gross deletions or duplications, determination of breakpoint junctions and detailed characterization of mutations were carried out by partial or whole *DMD* gene sequencing—the latter introduced as a service in 2007. The 79 exonic sequences and flanking intronic borders were amplified by PCR using M13-tailed primers. Amplicons were purified with ExoSAP-IT® (USB, Cleveland, OH, USA) and sequenced using M13 universal primers and BigDye™ Terminator Cycle Sequencing Kit V1.1 (Applied Biosystems). Products were resolved on an ABI 3130xl Genetic Analyzer and mutation analysis was aided by Seqscape V2.5 (Applied Biosystems).

cDNA analysis

Expression studies at the mRNA level were required for the delineation of breakpoint junctions, the identification of splicing changes or the detection of alterations caused by deep intronic mutations. Total RNA was isolated from cryopreserved muscle specimens using the PerfectPure RNA Fibrous Tissue Kit (5 Prime, Hamburg, Germany). cDNA was obtained using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems). Specific primers were used to amplify either the mutated transcript region or the complete *DMD* cDNA sequence, using the bio-X-Act Long Range DNA polymerase kit (Bioline, Taunton, MA, USA). Resulting amplicons were sized on agarose gels and/or sequenced as described above.

Bioinformatics

For mutation nomenclature, the cDNA reference sequence NM_004006.2 was used, corresponding to *DMD* transcript Dp427m that encodes the main dystrophin protein found in skeletal muscle. Sequence variants were described following the recommendations of the Human Genome Variation Society.[23] Variant description and impact on the reading-frame was confirmed with Mutalyzer.[24] In our mutation validation strategy, we crosschecked each variant

with the Human Genome Variation Society-listed Locus-Specific Database for *DMD*, available in the Leiden Muscular Dystrophy pages. This Locus-Specific Database is currently subdivided into two databases, one for whole exon changes[25] (http://www.lovd.nl/DMD_d) and the other for smaller mutations[12] (http://www.lovd.nl/DMD). For new point mutations, the Human Splicing Analyser software (http://www.umd.be/HSF/) was used for *in silico* evaluation of their possible effect on splicing.

Patient registry

The Portuguese patient registry was implemented using the Leiden Open Variation Database (LOVD) software version 2.0.[26] LOVD was installed in a dedicated server, accessible only to the curators. Besides default LOVD columns, a set of custom database fields was used (listed in Supplementary data II), which included those defined by TREAT-NMD as mandatory or highly encouraged items in the network's global DMD registry.

In the organization model (Supplementary Data III), three clinical coordinators from geographically dispersed major hospitals (north, center and south of the Country, to facilitate patient access) are assigned to the collection of personal, clinical and pathology data as well as to the promotion of the patients' regular clinical (re)evaluation. Registry inclusion is completely voluntary and requires specific informed consent for registration purposes. All the information, namely, data sent by the clinician, signed consent forms and the genetic data obtained in the laboratory, is gathered by the registry curator and introduced in the D/BMD database after validation.

RESULTS

Mutation profile

In our cohort of 312 unrelated cases selected on the basis of inclusion criteria for *DMD* analysis, an underlying mutation was identified in 284 (91%) cases. Among the remaining 28 cases, where no *DMD* mutation was detected, 19 had revealed dystrophin deficiency in the muscle biopsy (Supplementary Data I). In two of these, with irregular immunostaining for dystrophin, muscle specimens were available and were used for cDNA analysis, but still no alteration was identified.

The mutation distribution by type was found to be 67.9% ($n=193$) deletions, 15.2% ($n=43$) duplications and 16.9% ($n=48$) sub-exonic and sub-intronic ('point') mutations.

Overall, 175 different mutations were characterized, 39 of which were undocumented variants (Table 1) detected in patients and in a female obligate carrier with deceased DMD family members. The majority ($n=24$) of these new variants are point mutations that create premature termination codons. Among these are 13 small deletions, 5 mutations affecting splicing (4 of these are depicted in detail in Figure 1), 3 nonsense mutations, 2 deletion/insertions and 1 insertion. The remaining new variants correspond to gross deletions ($n=8$, where 4 are predictably in-frame) and duplications ($n=7$, where 2 are predictably in-frame). All 39 newly described variants were submitted to the *DMD* Locus-Specific Database available in the Leiden Muscular Dystrophy pages, as encouraged by the Human Variome Project.[27]

gDNA sequencing of the entire *DMD* gene was carried out in 50 unrelated individuals (including one female patient and two carriers). Among these, muscle specimens were available for cDNA studies in 17 cases where further characterization was considered necessary. These included 12 cases with new variants and 2 with known mutations but where their effect at the mRNA level had not been described.

Overall, complete or targeted gDNA and cDNA sequencing enabled the detailed characterization of 44 cases with point mutations, mainly of the splicing, nonsense and frameshift types (small deletions or duplications). Four examples taken from the newly detected variants (Table 1) are shown in Figure 1. In patient 36, the c.9564-1G>A mutation abolishes the 3′ splice site of intron 65 causing skipping of exon 66 (Figure 1a). A similar mutation in patient 34 (c.9287-1G>A) located in intron 63 originates a partial exonic deletion at the mRNA level (Figure 1b). Although nonsense mutations are usually only studied at the gDNA level, they too may have additional and unsuspected effects on splicing. This is exemplified with patient 18 (Figure 1c), where, besides the predicted transcript with a stop codon (r.3281u>a), there is also residual production of a second transcript with an in-frame deletion that results from skipping of exon 25. This finding is in agreement with the apparently intermediate phenotype observed in the patient, who has shown a slowly progressive clinical course. The fourth example shows how full cDNA sequencing enabled the identification of the deep intronic mutation c.9224+9192C>A in patient 33 (Figure 1d). This single-nucleotide substitution promotes the inclusion of a small portion of intron 62 at the mRNA level, predictably originating a truncated polypeptide (p.His3076Leufs*37).

In four cases, targeted gDNA sequencing enabled the identification of the underlying cause of changes initially detected by MLPA ($n=3$) and multiplex PCR ($n=1$, patient 12 in Table 1 and Figure 2).

Genotyping pitfalls

The molecular diagnosis of our patients revealed potential genotyping pitfalls owing to either technical limitations and/or genetic heterogeneity. With MLPA, false negatives may result from partial exonic deletions that are not coincident with the ligation site of the respective exonic probes, as exemplified in Figure 2. In this case, both Southern blotting followed by hybridization with a cDNA probe (Figure 2a) and multiplex PCR (Figure 2b) suggested a deletion of exon 17 in patient 12. In contrast, MLPA results were not compatible with a deletion (Figure 2c). This apparent discrepancy was sorted out by genomic sequencing, where a 228-bp deletion that includes intron 16 and exon 17 was detected (Figure 2d). As the acceptor splice site was included in the deleted region, the mutation was further characterized at the mRNA level. Results demonstrated that normal splicing is compromised and that a cryptic acceptor splice site located upstream in intron 16 is alternatively used (Figure 2e). The resulting polypeptide would be truncated, if produced.

False-positive results with MLPA mainly arise from inadequate probe hybridization because of the presence of sequence variants (polymorphisms or point mutations) in targeted regions. Two representative cases are depicted in Figure 3. In the first, MLPA analysis suggested a deletion of exon 51 in patient 27. However, gDNA sequencing revealed a single base pair deletion (c.7425delC) in the ligation site for the respective MLPA probe (Figure 3a). The second case (not listed in Table 1) is one where, rather than absence, a signal reduction was observed with the MLPA probe for exon 70. Upon sequencing, this was explained by the presence of a nonsense mutation (Figure 3b), responsible for the observed sub-optimal ligation of the probe.

The high mutation rate of the *DMD* locus can also induce errors in genotyping. One such situation that may be overlooked is the presence of two distinct mutations running in the same family where, as exemplified in Figure 4a, the pedigree reflects the apparently normal X-linked transmission of a single mutation. In this family, referral was triggered by the request for prenatal diagnosis in a woman with family history of DMD, whose affected brother and maternal uncle were already deceased. A deletion encompassing exons 17 and 18 was identified in the male fetus (IV-1 in Figure 4a), but the pregnancy was carried to term as the same deletion (Figure 4b) was also detected in the fetus' 11-year-old maternal cousin (patient 13 in

Table 1 Novel *DMD* mutations found in Portuguese dystrophinopathies patients

Patient Id./ Gender	Exon	DNA change	RNA change	Protein	Origin/ inherit.	Disease	Onset	Phenotype at onset	CK levels (IU/l)	WCB(years)	MR	CI	IHC
Large deletions/duplications													
1-M	1_12i	c.(?_-244)_1482+?del	r.(0?)	p.0?	Germline/ familial	D/ BMD?	6 years	Motor development delay; positive Gowers' sign; calf hypertrophy; reduced reflexes in LL	14 640	—	N	Y	—
3-M	6i_9i	c.531-?_960+?del	r.(531_960del)	p.(fs*)	Unknown/ sporadic	IMD	10 years	Difficulty to walk; motor development delay	7545	12	Y	N	↓ DYS1,2 Normal DYS3
5-M	7i_30i	c.650-?_4233+?dup	r.(650_4233dup)	p.(fs*)	Unknown/ sporadic	DMD	4 years	—	23 000	—	—	N	Ø DYS1,2,3
6-M	11i_18i	c.1332-?_2292+?del	r.(1332_2292del)	p.(fs*)	Germline/ familial	DMD	17 months	Walking delay	21 000	10	Y	Y	Ø DYS1,2,3
7-M	11i_18i	c.1332-?_2292+?dup	r.(1332_2292dup)	p.(fs*)	Unknown/ sporadic	D/ BMD?	2 years	calf pseudohypertrophy; frequent falls; motor development delay	17 611	N (6)	N	—	Np
9-M	13i_34i	c.1603-?_4845+?del	r.1603_4845del	p.Val535_Lys1615del	Germline/ sporadic	BMD	25 years	Asymptomatic (elevated CKs)	↑	—	—	—	↓ DYS1 Normal DYS2,3
10-M	13i_47i	c.1603-?_6912+?dup	r.(1603_6912dup)	p.(dup)	Germline/ familial	BMD	PN	Calf hypertrophy	13 270	N (18)	N	N	Np
13-M[a]	16i_18i	c.1993-?_2292+?del	r.(1993_2292del)	p.(del)	Germline/ familial	BMD	—	—	↑	—	—	—	Np
16-M	21i_37i	c.2804-?_5325+?dup	r.(2804_5325dup)	p.(fs*)	Unknown/ sporadic	DMD	20 months	Positive Gowers' sign; Calf hypertrophy	30 000	?	?	N	Np
17-M	22i_55i	c.2950-?_8217+?del	r.(2950_8217del)	p.(del)	Germline/ sporadic	D/ BMD?	-	Muscle weakness and cramps	28 000	4	N	N	Np
21-M	29i_43i	c.4072-?_6290+?dup	r.(4072_6290dup)	p.(fs*)	Unknown/ sporadic	D/ BMD?	8 years	—	↑	—	—	—	Ø DYS1,2,3
25-M	49i_57i	c.7201-?_8547+?dup	r.(7201_8547dup)	p.(del)	Unknown/ sporadic	BMD	5 years	Difficulty to walk and climb stairs; proximal tetraparesis	10 000	N (21)	N	N	Normal DYS1,2,3
26-M	50i_74i	c.7310-?_10553+?del	r.(7310_10553del)	p.(fs*)	Germline/ familial	DMD	6 years	Global development delay; frequent falls	5320	9	Y	Y	Ø DYS1,2 ↓ DYS3
28-M[a]	51i_78i	c.7543-?_11046+?del	r.(7543_11046del)	p.(del)	Germline/ familial	D/ BMD?	3 years	Global development delay	9572	N (8)	Y	N	Ø DYS1 ↓DYS2,3
35-M	64i_65i	c.9362-?_9563+?dup	r.(9362_9563dup)	p.(fs*)	Germline/ familial	DMD	1 year	Hypotonia	10 919	N (13)	Y	N	Np
Point mutations													
2-M	3	c.183del	r.(?)	p.(Lys61Asnfs*14)	Unknown/ sporadic	DMD	4 years	Difficulty to walk	8900/ 26 000	12	N	N	Ø DYS1,2,3; ↓SGs
4-M	7	c.564C>A	r.(?)	p.(Cys188*)	Unknown/ sporadic	DMD	6 years	—	↑	—	—	—	Ø DYS1,2,3

Table 1 (Continued)

Patient Id./ Gender	*Exon*	*DNA change*	*RNA change*	*Protein*	*Origin/ inherit.*	*Disease*	*Onset*	*Phenotype at onset*	*CK levels (IU/l)*	*WCB(years)*	*MR*	*CI*	*IHC*
8-M	13	c.1529_1530del	r.(?)	p.(Leu510Hisfs*8)	*De novo/ sporadic*	DMD	2 years	Motor development delay	—	9	—	Y	Ø DYS1,2,3
11-M	16	c.1899_1901delinsCTTCTTTCAACACTGG	r.(?)	p.(Lys634Phefs*17)	Germline/ sporadic	DMD	4 years	Difficulty to climb stairs	12 200	10	N	Y	Ø DYS1,2,3
12-M	16i_17	c.1993-220_2000del	r.1993_2000del1993-251_1993-221ins	p.Ile665Phefs*20	Germline/ familial	DMD	3 years	Progressive proximal weakness; motor development delay	7970	9	Y	Y	Ø DYS1,2 ↓ DYS3
14-M	17	c.2111del	r.(?)	p.(Pro704Hisfs*25)	*De novo/ sporadic*	DMD	3 years	Difficulty to walk	6203	10	N	N	Ø DYS1,2,3
15-M	19	c.2380+1G>A	r.2293_2380del	p.Ala765Argfs*15	*De novo/ sporadic*	DMD	—	—	—	—	—	—	—
18-M	25	c.3281T>A	r.[3281u>a; 3277_3432del]	p.[Leu1094*; Leu1093_Gln1144del]	Germline/ sporadic	D/ BMD?	6 years	Positive Gowers' sign; Calf hypertrophy	20 000	N (10)	N	N	Np
19-M	26	c.3460G>T	r.(?)	p.(Gly1154*)	*De novo/ sporadic*	DMD	—	—	—	—	—	—	Ø DYS1,2,3
20-M	26	c.3550_3551del	r.(?)	p.(Asp1184Phefs*2)	Unknown/ sporadic	DMD	3 years	Positive Gowers' sign; Calf hypertrophy	11 282	N (10)	N	N	Np
22-F	35	c.4976insA	r.(?)	p.(Asn1659Lysfs*27)	Familial	Carrier	—	—	—	—	—	—	Np
23-M	38	c.5328_5332delinsTCCTTTGAAGGC	r.(?)	p.(Ser1777Profs*13)	Germline/ familial	D/ BMD?	—	—	—	—	—	—	—
24-M	49	c.7179_7180del	r.7179_7180del	p.Lys2393Asnfs*16	Germline/ sporadic	DMD	1 year	T21; motor development delay	7281	Never walked	Y	N	Ø DYS1,2,3
27-M	51	c.7425delC	r.(?)	p.(Phe2475Leufs*19)	Unknown/ sporadic	DMD	2 years	—	10 000	8	—	Y	Ø DYS1,2,3
29-M	55	c.8098_8099del	r.(?)	p.(Lys2700Valfs*9)	Germline/ familial	D/ BMD?	4 years	—	1860	11	—	—	Ø DYS1,2,3
30-M	55	c.8178del	r.(?)	p.(Asp2727Thr*5)	Unknown/ sporadic	D/ BMD?	8 years	—	—	—	—	—	—
31-M	56	c.8233del	r.8233del	p.Ile2745Leufs*19	Germline/ familial	DMD	6 years	Calf hypertrophy; global development delay	—	—	Y	Y	Ø DYS1,2,3
32-M	61	c.9131_9147del	r.0?	p.Asp3044Alafs*48)	Germline/ sporadic	DMD	7 years	—	6542	9	Y	Y	Ø DYS1,2,3
33-M	62i	c.9224+9192C>A	r.9224_9225ins9224+9144_9224+9189	p.His3076Leufs*37	Unknown/ sporadic	IMD	9 years	Myopathic syndrome; motor development delay; pseudohypertrophy	12 000/ 2690	13	N	Y	Ø DYS1,3 ↓DYS2
34-M	63	c.9287-1G>A	r.9287_9305del	p.Ala3096Aspfs*9	Unknown/ sporadic	DMD	2 years	Motor development delay; frequent falls	7630	—	Y	Y	Ø DYS1,2,3
36-M	65	c.9564-1G>A	r.9564_9649del	p.Gly3189Profs*13	Unknown/ sporadic	DMD	3 years	—	—	—	Y	—	↓ DYS1,2,3

Table 1 (Continued)

Patient Id./ Gender	Exon	DNA change	RNA change	Protein	Origin/ inherit.	Disease	Onset	Phenotype at onset	CK levels (IU/l)	WCB(years)	MR	CI	IHC
37-M	69	c.10086 + 2dup	r.[9975_10086del; 9808_10086del; 9975_10262del]	p.[Tyr3326Leufs*13; Ala3270_Pro3362del; Tyr3326_Ala3421del]	Unknown/ sporadic	DMD	2 years	Difficulty to stand up	9437	8	Y	N	Ø DYS1,2,3
38-M	75	c.10599del	r.10599del	p.His3533Glnfs*13	Germline/ sporadic	DMD	1 year	Difficulty to walk	28 336	N (7)	Y	N	Ø DYS1,2,3
39-M	75	c.10656del	r.10656del	p.Arg3554Glyfs*40	Germline/ sporadic	DMD	5 years	Myalgia in LL	8974	9	Y	Y	↓ DYS1 Ø DYS2,3 ↓SGs

Abbreviations: BMD, Becker Muscular Dystrophy; Carrier, asymptomatic carrier; CI, cardiac involvement; CK, creatine kinase; DMD, Duchenne Muscular Dystrophy; D/BMD?, undetermined (Becker or Duchenne); F, female; IHC, immunohistochemistry; Id, identification; IMD, intermediate muscular dystrophy; Inherit., inheritance; LL, lower limbs; M, male; MR, mental retardation; N, no; Np, not performed; PN, prenatal diagnosis; T21, trisomy 21; WCB, wheelchair bound age; Y, yes; Ø, absent; ↓, reduced; ↑, elevated[a] Family relatives.

Table 1, IV-2 in Figure 4a), who was clinically asymptomatic except for elevated creatine kinase levels. This newly documented mutation is consistent with the BMD phenotype as it is not predicted to cause a frameshift, according to the 'reading-frame rule'. More recently, an affected male first-cousin (patient 28 in Table 1, IV-3 in Figure 4a) presenting clinical features of DMD was referred for molecular studies. At 7 years of age, he presented physical and cognitive developmental delay, limb and facial muscle weakness and difficulty to stand-up. However, in this patient a distinct (and also novel) gross *DMD* deletion was detected, encompassing exons 52–78. This in-frame deletion extends into the cysteine-rich and also carboxy-terminal portion of the protein, which is essential for proper dystrophin function. The loss of these functional domains might explain the DMD phenotype. In view of these findings, all family members available for study were genetically reanalyzed. Interestingly, the mothers of all three patients (sisters III-4, -6 and -8) were found to be carriers of both mutations, one on each of the parental X chromosomes (Figures 4a and c). In light of these new genetic findings, the clinical reevaluation of these females was encouraged; however, there has been no further follow-up. Figure 5 shows a co-occurrence of two *DMD* mutations in the same patient (not listed in Table 1). This is an example of a patient with a severe DMD phenotype, presenting with delayed developmental and intellectual milestones, becoming wheelchair-bound by the age of 7 years. On referral for molecular diagnosis, two distinct changes were detected by MLPA: a gross deletion involving exons 46–50 and deletion of exon 79 (Figure 5a). Genomic sequencing of exon 79 revealed a deletion of 13 base pairs in the 3′-untranslated region (c.*23_*35del, Figure 5b). This mutation is located within the genomic sequence recognized by the MLPA probe, thereby explaining the absence of amplification of exon 79. Transcript analysis in the muscle specimen confirmed the presence of both alterations, designated as r.[6615_7309del; *23_*35del]. Although the former is a well-documented and recurrent out-of-frame mutation that also compromises expression of the Dp140 isoform, the *23_*35del mutation has been reported only twice in patients with a DMD-type phenotype.[28,29] This change lies in a highly conserved part of the 3′-untranslated region of *DMD* and predictably disrupts the reading frame of the Dp71b isoform (NM_004016.2), thought to have an important role during human neural development.[30] Moreover, as Dp71b and Dp140 are expressed in the CNS, the loss of both isoforms may have a cumulative effect on brain development,[30,31] thereby explaining the patient's mental impairment.

Patient registry

The detailed clinical and molecular data collected and described in this work for a significant number of dystrophinopathy families and the new emergent mutation-based therapies, motivated the authors to develop a national registry for patients affected with D/BMD, in collaboration with the TREAT-NMD network. The registry database is based on the LOVD system and is located in the 'Centro de Genética Médica Dr. Jacinto Magalhães', presently the genetics service of a public central hospital. The Portuguese registry follows the TREAT-NMD charter for patient database/registry, abiding by National and European legislation concerning data protection. Before implementation, this project was submitted for approval by the Institutional Ethics Committee and was granted authorization by the National Committee for Data Protection. The registry was launched in 2012 and since then the clinical coordinators have been actively gathering data from D/BMD patients. Until now (April 2014), only a small fraction of patients ($n = 18$) have been included in the database.[18]

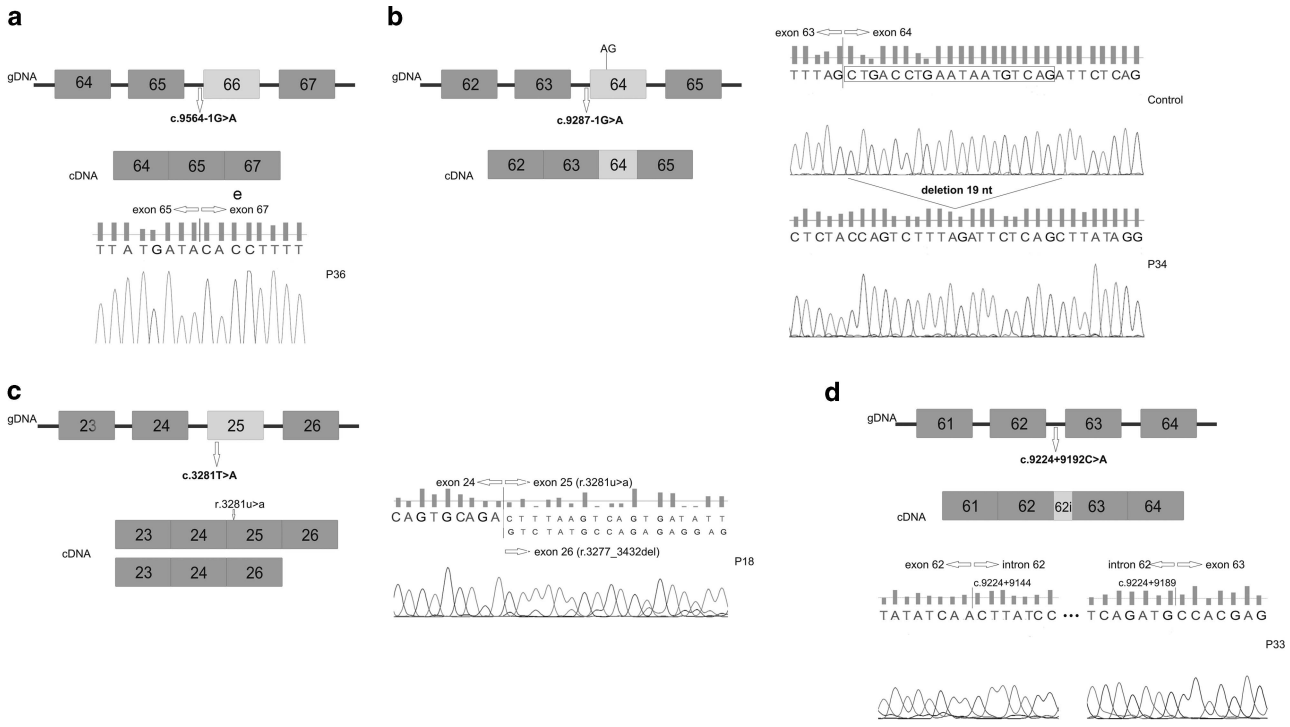

Figure 1 Importance of mRNA studies in *DMD* gene analysis. (**a**) In patient 36 (P36), a G to A substitution in the intronic 3′ splice site (intron 65) originated the skipping of exon 66. (**b**) In patient 34 (P34), a similar G to A substitution located in intron 63 originated a partial exon deletion because of the use of a cryptic exonic splice site. (**c**) Patient 18 (P18) presents a novel nonsense mutation in exon 25. Besides the transcript with the stop codon, a second transcript with an in-frame deletion (exon 25 skipping) is also produced. (**d**) Full complementary DNA sequencing showed a deep intronic substitution in patient 33 (P33). This change generates a stronger splice site score that completely overweighs that of the native splice site sequence, leading to the inclusion of a portion of intron 62 (46nt) at the mRNA level. gDNA, genomic DNA. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Regarding the registry's data content: (i) the average patients' age is 11.8 years; (ii) all patients, except one, have truncating mutations in the *DMD* gene; (iii) 11 patients still maintain independent ambulation; (iii) on average the beginning of wheelchair use is 10 years of age; and (iv) 4 patients require noninvasive artificial ventilation.

DISCUSSION

In the cohort totaling 312 unrelated cases that met the inclusion criteria for suspected dystrophinopathy, 284 were confirmed and characterized at the molecular level, corresponding to a mutation detection rate of 91%. Among the 28 cases with no *DMD* mutation detected, 19 had a muscle biopsy, which was compatible with D/BMD. Some of these are still likely to be dystrophinopathies because the molecular techniques used are not exhaustive for mutation detection. However, abnormal immunostaining for dystrophin may represent a secondary deficiency, particularly in disorders with overlapping clinical signs, as is often the case in certain sarcoglycanopathies.[32] Indeed, out of all 503 index cases referred for *DMD* testing since 1989, 22 cases were subsequently diagnosed with sarcoglycanopathy, and a further 10 were diagnosed with other forms of muscular dystrophy or myopathy.

DMD mutation proportions by type were found to be in agreement with that reported in the literature for large cohorts, with gross deletions in approximately 70% of the cases and with gross duplications and point mutations being equally accountable for the remainder.

A total of 175 different mutations were identified in our cohort, 39 (22%) of which had not been described previously. In this subset, the apparent discrepancy in mutation type proportions, with our higher frequency of new point mutations as compared with new large deletions and duplications, reflects a past sub-representation of point mutations in databases and mutation reports in the literature.

gDNA sequence-based interpretation alone may fail to predict the correct mutation outcome, even with the aid of bioinformatics algorithms. Some of the new variants in the present report are used to illustrate the importance of mRNA studies in helping to elucidate the mutation effect on RNA processing, to delineate breakpoint junctions and often to provide explanations for apparent exceptions to the reading-frame rule. In addition, transcript analysis may enable the detection of deep intronic splicing mutations that otherwise remain undetected or with unknown pathogenicity.

In our patient cohort, several cases highlighted the importance of extensive molecular studies for accurate diagnosis and concomitant appropriate genetic counseling. These potential pitfalls are either consequent to technical limitations or derive from the genetic heterogeneity of the *DMD* locus.

It is recognized that limitations due to sensitivity and experimental design of the various diagnostic techniques may lead to false-negative or false-positive results. As shown in the examples provided, these and other technical pitfalls may be avoided by combining different methodologies with complementary specificities. Indeed, our standard operating procedures in internal quality control for disease-specific molecular diagnoses include, whenever possible, a second alternative and complementary technique.

Genetic heterogeneity constitutes a second factor that can lead to erroneous diagnostic conclusions. Current international guidelines for D/BMD propose that carrier screening and prenatal testing should be orientated toward (thus limited to) the mutation previously

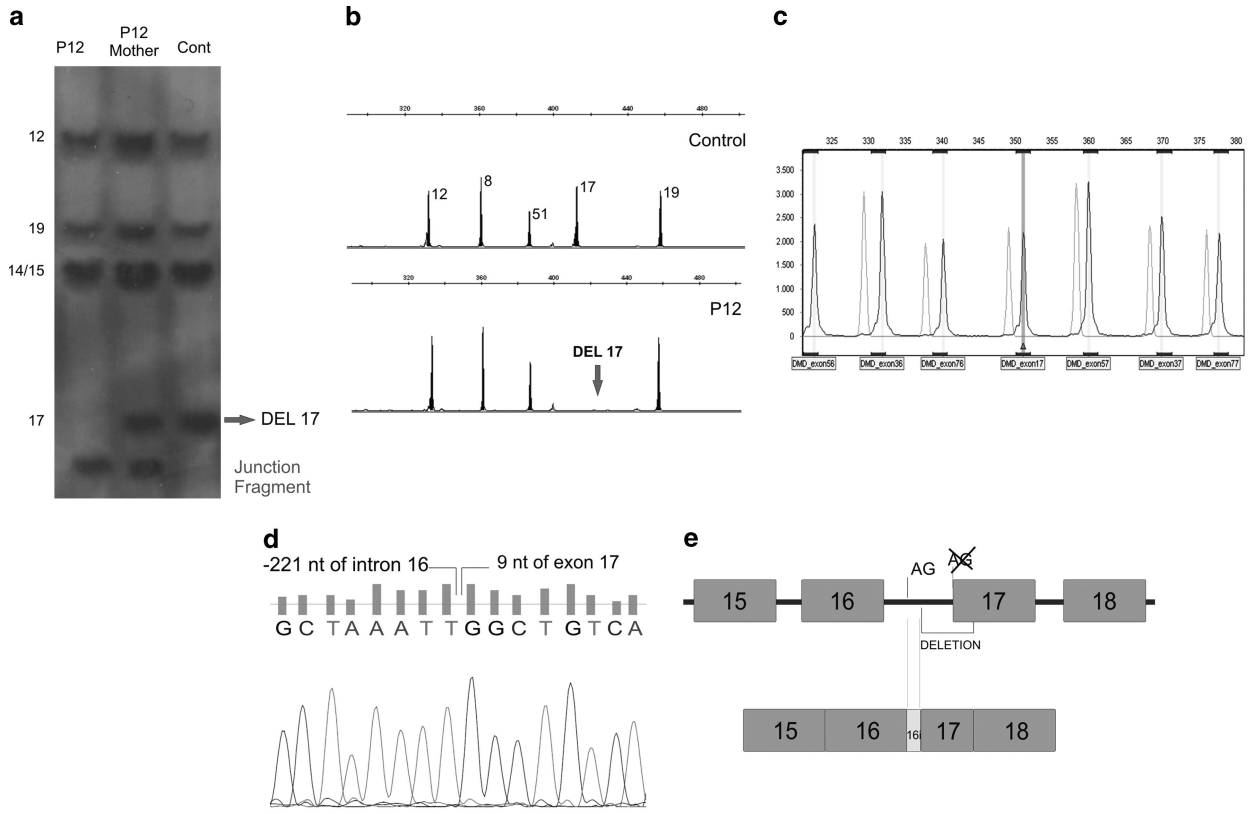

Figure 2 *DMD* deletions undetected by Multiplex Ligation-Probe Amplification (MLPA). (**a**) Southern blotting and hybridization using *Hin*dIII-digested genomic DNA (gDNA) and complementary DNA (cDNA) probe 30-2[22] showing a deletion of exon 17 and the presence of a junction fragment (patient 12 (P12) in Table 1). This fragment also elucidated the carrier status of the patient's mother (P12 mother). (**b**) No amplification was obtained for the fragment corresponding to exon 17 using the multiplex PCR technique. (**c**) MLPA analysis revealed the presence of exon 17 (probe P035). (**d**) Targeted gDNA sequencing revealed a deletion encompassing the last 220 nucleotides of intron 16 and the first 8 nucleotides of exon 17. (**e**) Schematic representation of resulting transcript. cDNA analysis confirmed that this mutation compromises the splicing process of the intron 16/exon17 boundary: the deletion encompasses the 3′consensus region (including the native AG splice site and the branch point), such that the pre-mRNA processing resorts to a cryptic acceptor splice site located upstream in intron 16. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

identified in the index case.[33] However, as shown here, the presence of the same molecular defect in all clinically affected family members should not be presumed categorically, even if the pedigree appears to follow a typical X-linked pattern of inheritance. Clinical diagnosis should be considered definitive only after the molecular confirmation using strategies with a complementary and/or wider scope of mutation detection, and all possible carriers should be tested in cascade.

The co-occurrence of two *DMD* mutations in the same patient is also exemplified. Here, the patient's mother was found to carry only one of the mutations, namely c.*23_*35del, but not the gross deletion of exons 46–50, thereby posing a dilemma for counseling and prenatal diagnosis. Further studies in other family members may help elucidate the degree of pathogenicity contributed by each variant, especially the 3′-untranslated region point mutation. Nonetheless, if only the common exon 46–50 deletion had been screened in the mother, her carrier status would have been incorrectly ascertained.

The detection of such cases is more problematic, as in practice it implies performing extensive screening of the *DMD* gene in each patient. To avoid this pitfall, physicians who request *DMD* screening, and genetic counselors in particular, should be aware of the specificity of the tests performed in each case and of their respective limitations.

Besides enabling a definitive diagnosis, obtaining a detailed genotype is particularly relevant for both candidate selection and treatment outcome evaluation in light of the emergent mutation-directed therapies for DMD, which are currently under clinical trials. In 2007, TREAT-NMD initiated a global patient registry for DMD.[17] This international database network cooperation functions with a twofold purpose—the patient profiles facilitate industry decision-making and simultaneously the trial-ready data contribute to the effectiveness of patient recruitment. This global database collects from national registries, thus each participating country adopted the same data set of mandatory items that are transversal in patient studies. Follow-up and clinical progression are updated regularly, ideally at least once a year.

Three basic models have been used for the deployment of DMD registries: (i) a patient self-report system (DuchenneConnect Registry Report;[34] United Dystrophinopathy Project[35]), where the patient or legal tutor replies to a specific disease questionnaire; (ii) a clinical reporting system (UMD-DMD database;[10] Czech DMD/BMD registry[36]), insuring that the data are collected and validated by physicians and molecular geneticists, or (iii) a mixed model that combines both strategies (New Zealand Duchenne Muscular Dystrophy Registry;[37] Japanese Registry of Muscular Dystrophy—Remudy[38]). The Portuguese DMD registry is currently using the clinical reporting model, which at the time was considered the most adequate, after discussions with professional groups and with the neuromuscular patients' association. However, the small number of

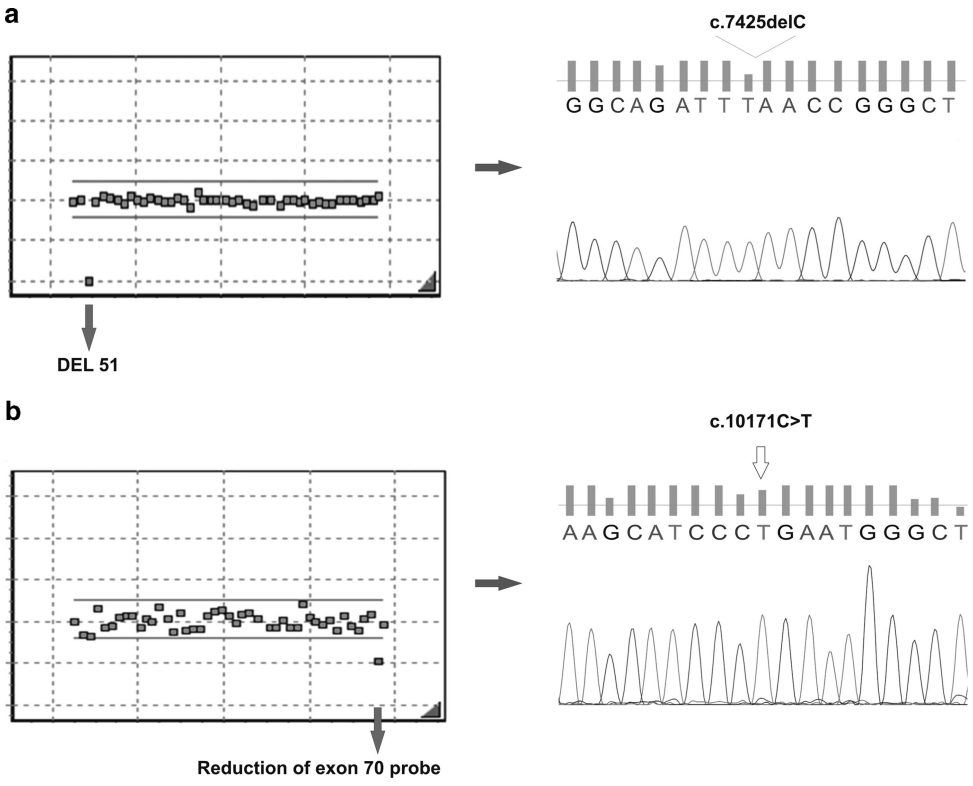

Figure 3 Point mutations initially identified by Multiplex Ligation-Probe Amplification (MLPA) as single exonic deletions. (**a**) MLPA analysis revealed a deletion of exon 51 in patient 27 (P27; Table 1). Direct sequencing of exon 51 showed a frameshift mutation (p.Phe2475Leufs*19) in the ligation site for the respective MLPA probe. (**b**) In another patient (not listed in Table 1), an apparent reduction of amplification with the exon 70 probe was observed. When sequenced, a previously reported nonsense mutation (p.Arg3391*) was detected in this exon, coincident with the probe's ligation site. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

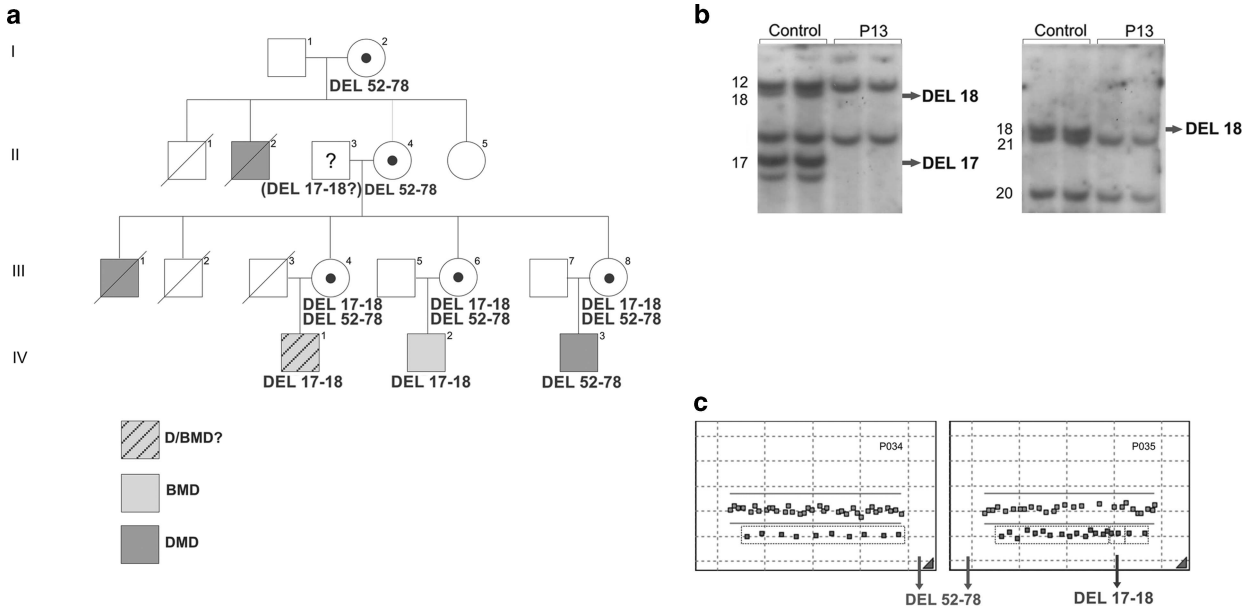

Figure 4 Two independent *DMD* mutations identified in one family. (**a**) Pedigree showing an X-linked transmission but also the presence of two different mutations associated with different phenotypes. (**b**) Southern blotting and hybridization using the complementary DNA probe 30-2 (genomic DNA digested with *Bgl*II and *Hin*dIII), revealing a deletion of exons 17 and 18 in individual IV-2 (patient 13 (P13)). (**c**) Multiplex Ligation-Probe Amplification (MLPA) analysis of a female carrier (III-4) showing the presence of both deletions (compound heterozygosity). BMD, Becker Muscular Dystrophy; DMD, Duchenne Muscular Dystrophy. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

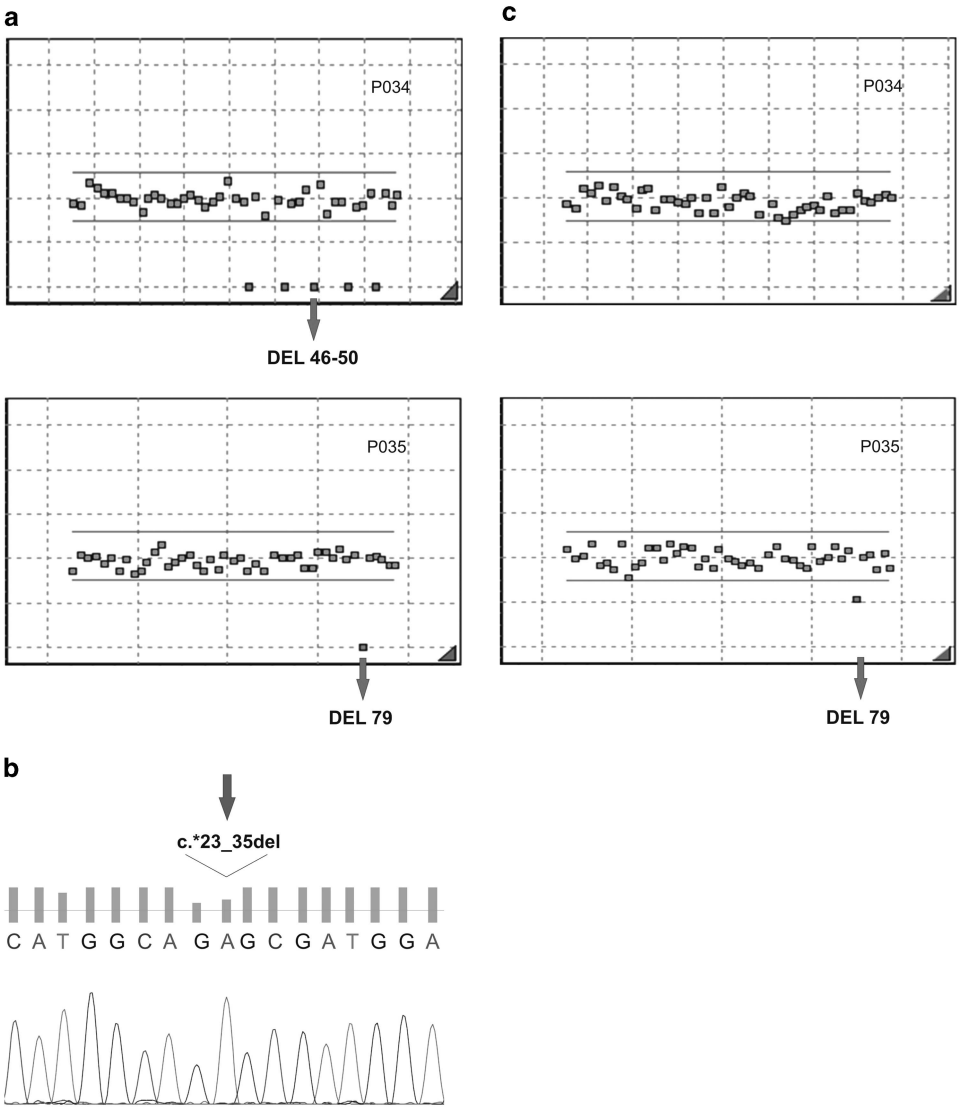

Figure 5 Two distinct *DMD* mutations identified in the same patient. (**a**) Multiplex Ligation-Probe Amplification (MLPA) analysis shows deletions of exons 46–50 and of exon 79. (**b**) Genomic DNA sequencing of exon 79 revealed a deletion of 13 nucleotides in a conserved part of the 3′-untranslated region (UTR), presumed to alter an alternative open reading frame that normally gives rise to transcripts lacking exon 78.[29,39] (**c**) Patient's mother presenting only the small 3′UTR deletion. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

patients registered thus far warrants reevaluation of this model, in an effort to facilitate and ensure a more widespread access of patients to the registry. One measure that is underway is the reference of the registry in all mutation-positive molecular reports. The implementation of clinical trial-sites for DMD in Portugal may also motivate patient registration. Some patients have been recruited on an individual basis, with the help of their clinicians, to ongoing clinical trials in other European countries. However, this strategy does not ensure equitable patient access to the trials. Considering the overall numbers for D/BMD patients in Portugal reported in this work, which were gathered through the genetic studies performed over the last two and a half decades, we believe it is justified to have an operational DMD registry in the country, as well as one or more clinical trial sites for this disease. Finally, the registry data itself can act as an important clinical and epidemiological research tool, to follow the natural history of the disease and to estimate the prevalence of D/BMD in our country.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)

PAPER II

Autosomal recessive limb-girdle muscular dystrophies diagnosed at Coimbra University Hospital.

Autosomal recessive limb-girdle muscular dystrophies diagnosed at Coimbra University Hospital

Distrofias Musculares das Cinturas autossómicas recessivas diagnosticadas nos Hospitais da Universidade de Coimbra

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Introduction

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of muscle diseases. Autosomal dominant (LGMD1) and recessive (LGMD2) forms are recognized, each one with several subtypes. In Portugal there are no studies reporting the relative distribution of the different subtypes of LGMD2.

Objective

To determine the subtypes of LGMD2 diagnosed and their relative distribution at the Neurology Department of the Coimbra University Hospital.

Material and Methods

The medical files of the patients with a diagnosis of LGMD2 were analysed and individual clinical, laboratory, pathologic and molecular data were recorded. The time frame of analysis was from 2000 to 2010.

Results

Forty-two patients from thirty-nine unrelated families were identified with a LGMD2 diagnosis. There were twenty-three female and nineteen male patients. Parental consanguinity was reported in eighteen patients (fifteen families). Their actual mean age is 44.6 years and the mean age of first symptoms was 23.2 years. The mean time from first symptoms to genetic diagnosis was 16.2 years. Twenty patients are wheelchair bound and seventeen can't raise the arms above the shoulder level. Three patients presented symptomatic dilated cardiomyopathy and twelve patients a restrictive respiratory syndrome, which was severe in five. The mean CK value was elevated in all LGMD2 subtypes. Immunohistochemistry suggested the specific diagnosis in twenty patients (LGMD2B: 11; LGMD2C-F: 9). Molecular studies performed in forty-one patients revealed 27 homozygous mutations, 11 compound heterozygous mutations and 3 heterozygous mutations. The LGMD2 subtypes diagnosed and the number of patients of each subtype was: LGMD2A: 5, LGMD2B: 16, LGMD2C-F: 9 (one patient without molecular study), LGMD2G: 1, LGMD2I: 7, LGMD2J: 1, LGMD2L: 3.

Conclusion

This retrospective analysis shows that most of the autosomal recessive LGMDs subtypes are represented in Portugal, being the LGMD2B subtype the most frequent. Rarer subtypes, like LGMD2G and J, were also found rare.

Key-words: Limb-girdle muscular dystrophies in Portugal; LGMD; autosomal recessive LGMD

Running title: AR LGMD at Coimbra University Hospital

Introdução

As Distrofias Musculares das Cinturas (DMC) constituem um grupo heterogéneo de doenças musculares. Existem as formas autossómicas dominantes (DMC1) e recessivas (DMC2), cada uma com vários subtipos. Em Portugal não há informação científica sobre a distribuição relativa dos diferentes subtipos de DMC2.

Objectivos

Avaliar os subtipos de DMC2 diagnosticados no Serviço de Neurologia dos Hospitais de Universidade de Coimbra e a sua distribuição relativa.

Material e Métodos

Análise dos processos clínicos dos doentes com o diagnóstico de DMC2 no período compreendido entre 2000 e 2010 e registo dos dados clínicos, laboratoriais, patológicos e moleculares individuais.

Resultados

Foram diagnosticados 42 doentes com DMC2, pertencendo a 39 famílias distintas e 23 eram do sexo feminino e 19 do sexo masculino. Consanguinidade parental foi identificada em 18 doentes, correspondendo a 15 famílias. A idade média actual é de 44.6 anos e a idade média dos primeiros sintomas de 23.2 anos. O tempo médio decorrido entre os primeiros sintomas e o diagnóstico molecular foi de 16.2 anos. Vinte doentes tinham perdido a marcha e dezassete não conseguiam elevar os braços acima do nível dos ombros. Três doentes apresentavam cardiomiopatia dilatada sintomática e sindroma respiratório restritivo foi diagnosticado em doze doentes, que era grave em cinco. O valor médio de CK estava elevado em todos os subtipos de DMC2. O estudo imunohistoquímico foi sugestivo do diagnóstico específico em vinte doentes (LGMD2B: 11; LGMD2C-F: 9) e o estudo molecular realizado em 41 doentes revelou mutações homozigóticas em 27 doentes, mutações em heterozigotia composta em 11 doentes e mutações em heterozigotia simples em 3 doentes. Os subtipos de DMC2 diagnosticados e o número de doentes por cada subtipo foram: DMC2A: 5, DMC2B: 16, DMC2C-F: 9, DMC2G: 1, DMC2I: 7, DMC2J: 1, DMC2L: 3.

Conclusão

Esta análise retrospectiva revela que a maioria dos diferentes subtipos de DMC2 estão presentes em Portugal, e o subtipo DMC2B foi o mais frequentemente diagnosticado. Os subtipos mais raros, como DMC2G e J, também foram diagnosticados, sendo o seu número muito reduzido.

Palavras-chave: Distrofias musculares das cinturas em Portugal; DMC; DMC autosómica recessiva

Título de cabeçalho: DMC AR nos Hospitais da Universidade de Coimbra

Introduction

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of inherited muscle disorders. Classified in two forms according to the mode of heredity[1], currently they comprise seven autosomal-dominant (LGMD1A to G subtypes) and fourteen autosomal-dominant subtypes (LGMD2A to N subtypes) and seventeen of them have their protein products identified. The LGMD1 is relatively rare and represents probably less than 10% of all LGMD cases.

LGMDs are characterized by muscle weakness and wasting of the scapular and pelvic girdle muscles with preservation of the facial muscles and high CK values. It is recognized extreme variability of the age of onset, the degree of muscle weakness, the pattern of muscular involvement and the level of disability and co-morbidities, like cardiac and respiratory involvement[2].

LGMDs have been the subject of numerous reviews and individual reports. Variable relative distribution of the different subtypes of the LGMD2 form have been presented, probably reflecting different ethnic backgrounds and geographic origins[3,4]. Some studies have found that the subtypes LGMD2A[5], 2C-F[6] and 2I[7] were the most common in each of the different countries where the studies were conducted, with small differences among them.

In Portugal, individual clinical cases[8,9] and small clinical series[10,11] about the individual LGMD2 subtypes have been published, but the relative distribution of the different subtypes of the LGMD2 has not been studied yet.

The Outpatient Neuromuscular Clinic of the Coimbra University Hospital is the reference center for the study of adult neuromuscular diseases in the central region of Portugal, a geographical area with a population of about 1.5 million people.

Here we describe the clinical, laboratory, pathologic and molecular data of a group of patients with LGMD2.

Material and Methods

Patient Population

The medical files of the Outpatient Neuromuscular Clinic of the Coimbra University Hospital were scrutinized to look for patients with a LGMD2 diagnosis. The time frame of the analysis was from 2000 to 2010. To be included in the study they had to have definite weakness on clinical examination primarily of the shoulder-girdle and pelvic muscles, a muscular biopsy showing dystrophic (or myopathic) features and a molecular study confirming the clinical and/or pathologic diagnosis of LGMD2. Patients with a diagnosis of dystrophinopathy or other neuromuscular disease confirmed by immunohistochemical or molecular genetic studies (FSHD, myotonic disorders, inflammatory muscle diseases and mitochondrial myopathy and glycogen or lipid storage myopathies) were excluded, as well as patients with weakness and wasting in the scapular and pelvic girdle muscles in which the etiologic investigation had been inconclusive. The LGMD2 subtypes are individually designated by the accepted nomenclature (1). LGMD2A indicates calpainopathy (*CAP-3* gene), 2B dysferlinopathy (*Dysf* gene), 2G telethoninopathy (*TCAP* gene), 2I α-dystroglycanopathy (*FKRP* gene), 2J titinopathy (*TTN* gene) and 2L anoctaminopathy (*ANO5* gene). In the text, the sarcoglycanopathies comprising the subtypes LGMD2C (γ-*sarcoglycan* gene), D (α-*sarcoglycan* gene), E (β-*sarcoglycan* gene) and F (δ-*sarcoglycan* gene) are collectively referred LGMD2C-F subtype and only when necessary each one is referred individually. The LGMD2 subtypes H, M and N are not mentioned in the text because they were not diagnosed in the time frame of analysis. Two brothers of patients with a LGMD2 diagnosis (LGMD2B and LGMD2A), without muscle biopsy but with a positive molecular study, were included in the analysis. Seven other patients were included, four with initial distal weakness later proven to be caused by pathogenic mutations in one of the LGMD2 genes and the other three patients, one of of gypsy descend with a suspected LGMD2C by immunohistochemistry (did not perform the appropriate molecular study) and the other two patients of the LGMD2C-E subtype confirmed by molecular studies requested by our centre, had muscle biopsy performed elsewhere and only the final impression of the pathological data was available.

Fifty-eight patients with limb-girdle muscular dystrophy diagnosis were identified. Sixteen had no definite diagnosis (unclassified LGMD). Forty-two patients with a definite LGMD2 diagnosis were included in the study, corresponding to 39 families.

Clinical Evaluation

The following data was recorded from the medical file of each patient: **1- historical features:** present age, gender, birthplace, race, ethnicity, consanguinity, family history of neurological disorders, age of first symptoms, initial site of first symptoms (lower limbs - proximal or distal, upper limbs - proximal or distal), age of muscular biopsy, age of molecular genetics diagnosis, time of muscular biopsy and molecular genetic study from the age of first symptoms, concurrent medical diseases and current medication; **2- physical evaluation:** height, weight, vital signs, records of involvement of other organ systems, muscular hypertrophy; **3- functional tests:** walking (independent, with support, impossible), arising from a chair (independent, with

hand support, impossible), raising arms (normal, shoulder level, below shoulder level, impossible), cardiovascular and respiratory functions (electrocardiogram, echocardiogram and functional respiratory evaluation). The highest serum creatine kinase value present on the medical file of each patient was recorded.

Muscle Biopsy Evaluation

The reports of the muscle biopsies were evaluated by one of us (L.N.) who recorded and graded the most prominent features: variability of fibre diameter (normal; increased, if superior to 3%), internal nuclei (absent; increased), necrotic fibres (absent; present: rare or frequent), basophilic fibres (absent; present), predominance of fibre type, connective tissue (normal; increased: focal or generalized), fat infiltration (absent; present: focal or generalized) inflammatory infiltrates (absent; present - localization), vascular abnormalities and the presence of special histopathologic features (lobulated fibres, rimmed vacuoles).

The muscle biopsies were routinely processed accordingly to procedures already described (10), namely: the biopsy fragments were frozen in isopentane chilled in liquid nitrogen and kept at a -70ºC. The transverse and longitudinal cryostat sections were cut 8µ thick, and stained by histochemical (H/E, PAS, Red-oil and Trichrome Gomori) and histoenzimatic routine methods (NADH-TR, SDH, ATPase pH4.35 and pH9.4) and cut 4µ thick for immunohistochemistry study (IHC) with antibodies against dystrophin (*dys* 1, *dys* 2, *dys* 3), α, β, δ and γ sarcoglycans, dysferlin, merosin, α-dystroglican and emerin (all from Novocastra). The intensity of staining with each antibody was graded from zero (absent) to 3+ (normal expression). Control human skeletal muscle was included with patient material on each glass slide immunostained in the study. At the time of the muscle biopsy some antibodies were not commercially available and if clinically indicated these were later studied in the biopsy fragments kept frozen at -70ºC in the laboratory. This happened in four muscle biopsies from patients with the LGMD2B subtype, which were evaluated with antibodies anti-dysferlin after the molecular diagnosis and all the muscle biopsies from patients with the LGMD2I subtype were evaluated with antibodies anti α-dystroglican after the molecular diagnosis.

A total of 38 reports of muscle biopsies were available and evaluated (two patients clinically affected, one brother of a LGMD2A female patient and a sister of a LGMD2B female patient did not perform muscle biopsy). Two patients had a muscle biopsy performed elsewhere and only the final impression with the results of the IHC (two LGMD2C-F patients) was available.

Molecular Studies

The molecular genetics studies were requested directed by the clinical examination and/or the results of muscle biopsy protein findings and were performed at the Molecular Genetics Unit of the Institute of Jacinto Magalhães, Porto.

1. gDNA analysis: Genomic DNA was extracted from peripheral blood by the salting-out method (12). Normal or M13-tailed primers used to amplify all the coding exons and directly flanking intronic sequences, were designed with aid of Primer Express (Applied Biosystems, Foster City, CA). Amplicons were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced with the respective normal or M13 universal primers, using the Big-DyeTM Terminator Cycle Sequencing Kit V1.1. The products were resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis was aided by SeqScape V2.5 software (Applied Biosystems, Foster City, CA) and Alamut V2.1 (Interactive Biosoftware, Rouen).
2. Transcript analysis: Total RNA was extracted from peripheral blood and/or muscle biopsies of patients and controls using TRIzol isolation reagent (Invitrogen, CA), and reverse transcribed using either Superscript One-Step RT-PCR with Platinum Taq (Invitrogen, CA) or the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City CA). Primers were designed according to case-specific interrogations. Amplicons resolved on 1% w/v agarose gels were eluted and sequenced in both directions, as described above.
3. Mutation characterization: Sequence variants in each gene were described according to the mutation nomenclature recommendations of the Human Gene Variation Society (HGVS) (13), using cDNA reference sequences filed under the following respective accession numbers: CAPN3 – NM_000070.2+BX499738.1; DYSF – M_001130978.1; MYOT - NM_006790.2; TCAP – NM_003673.3; ANO5 – NM_213599.2; FKRP – NM_024301.2+AC008622.6; SGCA – NM_000023.1 +L34355.1; SGCB – NM_000232.3+CN483961.1; SGCG – NM_000231.2; SGCD – NM_000337.4; FCMD - NM_139309.2; POMT1 - NM_007171.2; POMGNT1 - NM_017739.2.

Undocumented variants, for which pathogenicity could not be ascertained by transcript analysis or co-segregation studies, were evaluated by performing a population screen (100-150 anonymised control samples) using single-strand conformation analysis, high resolution melting-curve analysis or direct sequencing.

Results

A- Clinical Evaluation

1- Historical features (Table 1)

A total of 42 patients, representing 39 unrelated families were enrolled in the study. There were 23 (54.7%) female patients and 19 (45.2%) male patients, all of Caucasian race, with a Portuguese female of gypsy descent. Parental consanguinity was identified in 18 patients [(32.7%), (LGMD2A: 2, LGMD2B: 8, LGMD2C-E: 2, LGMD2I: 5, LGMD2J: 1)] corresponding to fifteen families. Family history for similar neuromuscular disorder was positive in four LGMD2 subtypes: two affected brothers in each of the subtypes LGMD2A, LGMD2B and LGMD2L, and two patients in the LGMD2I subtype were second-degree cousins.

The mean age of enrolment was 44.6 years and the mean age of first symptoms was 23.2 years. The mean age of first symptoms was below the age of twenty in 24 patients (57.1%), with 9 patients showing the first symptoms in the first decade, with 6 of these belonging to the LGMD2C-F subtype. Only five patients had their first symptoms at or after the age of 50. The mean age of first symptoms was lowest in the LGMD2C-F and higher in the LGMD2L subtypes. The mean age of molecular diagnosis was 38.5 years, with the LGMD2C-F and LGMD2L subtypes presenting the lowest and highest mean age, respectively, and reflecting the difference of onset of the disease of each subtype. The mean time from first symptoms to muscle biopsy and molecular diagnosis was 13 years and 16.2 years, respectively. Only one patient with the LGMD2G and another one with the LGMD2J subtypes were found, which are very rare outside the countries where they were first described (14,15) and the age of and the time to molecular diagnosis of these two subtypes were 50/30 years and 24/2 years, respectively. All patients, except two without weakness, reported the initial site of first motor symptoms in the legs, 36 proximally and 4 distally.

2- Physical Evaluation (Table 1)

No medical condition was identified, like endocrine or metabolic diseases that could explain or be related to the weakness. Fourteen patients presented calf hypertrophy (33.3%), distributed by the subtypes LGMD2A, LGMD2C-F (one of these patients also had macroglossia), LGMD2D, LGMD2I and LGMD2G.

3- Functional Tests (Table 2)

Twenty-one patients (50%) were able to walk without support, one with support and twenty were confined to a wheelchair. According to the LGMD2 subtypes, the LGMD2C-F subtype presented the highest percentage of patients wheelchair bound (77.7%), but the subtypes 2A, 2B and 2I (13 in a total of 28 patients) also presented a significant percentage of wheelchair bound patients (46.4%). The patients of the LGMD2G, J and L subtypes were able to walk without support. The ability to rise from a chair without hand support was preserved in 16 patients; three patients needed hand support and twenty-three patients were unable to rise from a chair. According to the different subtypes the LGMD2C-F (8 out of 9) and 2I (5 out of 7) subtypes were those with the highest percentage of patients with this motor disability.

The number of patients with ability of raising the arms above their head was 13, three patients from LGMD2L subtype and six from the LGMD2B subtype. Only one patient from each of the other subtypes was able to raise the arms above head (except the patient from LGMD2G subtype). Seventeen patients with the highest percentage of patients belonging to the LGMD2B and 2C-F subtypes were not able to raise the arms above head. Twelve patients (28.5%) were able to raise the arms to shoulder level.

From the historical data and the last three functional tests, it is clear the progressive nature of the disease, with progressive severity of the weakness with time in the lower limbs, and the upper limbs being involved later (or if present since the beginning of the disease not so severely affected as the lower limbs). The severity of weakness of the upper limbs and the resulted disability was less severe than that found in the lower limbs.

The patients from the LGMD2B, 2G, 2J and 2L subtypes had no signs or symptoms of cardiac or respiratory insufficiency, and this is in accordance with the experience of other centres. These medical complications were found in 12 patients from the LGMD2A, 2C-F and 2I subtypes. Combined symptomatic dilated cardiomyopathy and severe restrictive respiratory syndrome (needing intermittent ventilator support) was present in one patient of the LGMD2D subtype and in two patients of the LGMD2I subtype. Five patients of the LGMD2C-F subtype had a severe restrictive respiratory syndrome (LGMD2C: 2, 2D: 2, 2E: 1) with one patient of the LGMD2D subtype needing intermittent ventilator support. Four other patients (LGMD2A: 2 and LGMD2I: 2) had a restrictive respiratory syndrome of variable severity, with only one patient from the LGMD2A subtype needing intermittent ventilator support.

The individual CK values were elevated in 40 patients (it was normal in two patients, one from the LGMD2B subtype and the other from LGMD2C subtype) and the mean CK value was elevated (2866 UI/L). The highest individual CK value was recorded in a patient of the LGMD2B subtype (25 648 UI/L) and the highest mean value was also recorded in

Table 1. Patient population and clinical evaluation (historical features and physical evaluation)

	Sex F/M	Mean Age (years)			Mean age of molecular diagnosis (years)	Time to (years)		Calf hipertrophy
		Actual	1st symptoms	1st symptoms by decade		Biopsy	Molecular diagnosis	
All patients (n = 42)	23/19	44.6 ± 15.1 (21 - 75)	23.2 ± 16.4 (4 - 69)	1st - 9 / 2nd - 15 / 3rd - 10 / 4th - 1 / 5th - 2 / 6th – 3; 7th - 2	38.5 ± 15 (12 – 74) (n = 39)	13 ± 9.95 (2 – 32) (n = 40)	16.2 ± 11 (2 – 43) (n = 41)	14 (34.4%)
LGMD2A (n = 5)	3/2	44.2 ± 12.8 (31 - 62)	14.9 ± 14.9 (8 - 46)	1st - 1 / 2nd - 3 / 5th - 1	40.6 ± 14.2 (30 – 59)	17.7 ± 7.2 (11 – 24) (n = 4)	19.8 ± 11.5 (9 – 38)	1
LGMD2B (n = 16)	7/9	46.5 ± 17.2 (26 - 75)	28.3 ± 17.3 (15 - 69)	2nd - 8 / 3rd - 4 / 4th - 1 / 6th - 1 / 7th - 2	41.4 ± 19 (21 – 74)	8,6 ± 7.9 (3 - 32) (n = 15)	13.1 ± 11.6 (3 – 43)	
LGMD2C-F (n = 9)	7/2	35.5 ± 10 (21 - 49)	11.6 ± 8.15 (4 - 28)	1st - 6 / 2nd - 2 / 3rd – 1	29.5 ± 111.7 (12 - 46) (n = 8)	16.3 ± 9.2 (4 - 30)	18.5 ± 8.4 (5 – 29)	5 (one with macroglossia)
LGMD2I (n = 7)	5/2	43.4 ± 9.57 (34 - 61)	18.1 ± 10.4 (6 - 30)	1st - 2 / 2nd - 2 / 3rd - 3	37.5 ± 9.53 (29 - 56)	15.1 ± 10.7 (2 – 27)	18.8 ± 10.7 (4 - 30)	7
LGMD2G (n = 1)	1/0	50	20	3rd - 1	50	30	30	1
LGMD2J (n = 1)	0/1	25	22	3rd - 1	24	2	2	
LGMD2L (n = 3)	0/3	69 ± 5.6 (65 - 70)	50 ± 7 (45 – 55)	5th - 1 / 6th - 2	68 ± 3.6 (64 - 71)	13.3 ± 9.45 (6 - 24)	17 ± 8 (9 - 17)	

LGMD: Limb-girdle muscular dystrophy; n: number of patients; F: female: M: male; 1st: first; 2nd: second; 3rd: third; 4th: fourth; 5th: fifth; 6th: sixth; 7th: seventh; ±: standard deviation

Table 2. Clinical evaluation: functional tests

	Walking			Rising from a chair			Raising Arms			Cardiovascular Respiratory Systems		CK (UI/L)
	Independent	With support	Impossible	Without support	With support	Impossible	Above head	Shoulder level	Below shoulder level			
All patients (n = 42)	21 50%)	1 (2.3%)	20 (47.6%)	16 (38%)	3 (7.1%)	23 (54.7%)	13 (30.9%)	12 (28.5%)	17 (40.4%)	3 (7.1%)	9 (21.4%)	2866 ± 4869 (n = 38)
LGMD2A (n = 5)	2	- -	3	2	- -	3	1	3	1		BiPAP (n = 1) SRS (n = 1)	941 ± 570.5 (279 - 1859)
LGMD2B (n = 16)	9	1	6	9	1	6	6	3	7	- -	- -	4975 ± 6520 (37 - 25648)
LGMD2C-F (n = 9)	2	- -	7	1	- -	8	1	2	6	DCM + BiPAP (n = 1)	BiPAP (n = 1) SRS (n = 4)	1724 ± 2500 (265 - 7376)
LGMD2I (n = 7)	3	- -	4	2	- -	5	1	4	2	DCM + BiPAP (n = 2)	SRS (n = 2)	1383.5 ± 875.6 (500 - 3045)
LGMD2G (n = 1)	1	- -	- -	- -	- -	1	- -	- -	1	- -	- -	476
LGMD2J (n = 1)	1	- -	- -	1	- -	- -	1	- -	- -	- -	- -	639
LGMD2L (n = 3)	3	- -	- -	1	2		3	- -	- -	- -	- -	3793 ± 1081 (2696 - 4858)

LGMD: Limb-girdle muscular dystrophy; n= number of patients; DCM: dilated cardyomyopathy; SRS: severe respiratory syndrome; BiPAP: bilevel positive airway pressure; ±: standard deviation

the LGMD2B subtype (4975 UI/L) and the lowest mean CK value was recorded in the LGMD2A subtype (941 UI/L).

B- Histopathology and Immunophenotypes

Reports of thirty-eight muscle biopsies were analysed. The majority of the muscles biopsies were dystrophic. One of the four muscle biopsies from patients of the LGMD2A subtype was considered myopathic and the muscle biopsies from patients of the subtypes 2J and 2L were also myopathic, without increased connective tissue or fat infiltration.

Before the antibodies anti-dysferlin became available, muscle biopsy from one patient of the LGMD2B subtype showed histopathologic findings that were suggestive of Inclusion Body Myositis, another of metabolic disease and one was considered normal. The muscle biopsies from patients of the LGMD2I subtype had no distinctive morphologic features and all of them were dystrophic. The muscle biopsy from the patient with the LGMD2G subtype presented lobulated fibres and rimmed vacuoles together with a muscular dystrophic pattern[9]. Lobulated fibers were also present in muscle biopsy from a patient of the LGMD2A subtype. Endomysial focal inflammatory infiltrate was found in the muscle biopsies of the two brothers with the LGMD2L subtype. The muscle biopsies from patients of the LGMD2B subtype had several distinctive histopathologic features, including frequent necrotic fibres (n=8) with four of these presenting focal inflammatory infiltrates and rimmed vacuoles. The presence of these three special histopathology features in the same muscle specimen was identified in two muscle biopsies. Frequent basophilic fibres (n=6) were identified together with necrotic fibres in five cases, and three of these also had focal inflammatory infiltrates.

In LGMD2B immunohistochemistry with anti-dysferlin antibodies revealed irregular immunostaining in two cases and absence in the others. In the LGMD2C-F subtypes, an abnormal imumunostaining of the sarcoglycans was present in all of them. In two muscle biopsies, all the sarcoglycans were absent (LGMD2D and LGMD2E). One muscle biopsy showed the absence of γ-sarcoglycan accompanied the reduced immunostaining of the other three sarcoglycans (LGMD2C), and in the other six muscle biopsies only one sarcoglycan was absent, with normal immunostaining of the other three. The diagnosis of the LGMD2I subtype was done by molecular study in all patients. The antibodies against α-dystroglican only became available after the molecular diagnosis, and when applied to the muscle specimens, the results of IHC were in accordance with the molecular ones. In our centre antibodies against telethonin, titin and Anoctamin 5, as well as Western blot analysis, are not available yet.

The diagnosis of the individual LGMD2 subtypes was possible by immunohistochemistry in twenty patients (47.6%) (LGMD2B: 11, LGMD2C-E: 9). All the other LGMD2 subtypes were diagnosed and confirmed by molecular studies (except one patient of the LGMD2C-F subtype).

C- Genotypes (Table 3)

Forty-one patients had an informative genetic test [the gipsy descent patient did not perform molecular study (suspected LGMD2C by IHC)]. Twenty-seven patients had a homozygous mutation. Of these, seven were frameshift mutations (LGMD2B: 3, LGMD2C: 1; LGMD2J: 1; LGMD2L: 2), seventeen were missense (LGMD2B: 6, LGMD2C: 1, LGMD2D: 2, LGMD2E: 1, LGMD2I: 7), one was a nonsense mutation (LGMD2G), another one was an aberrant splicing (LGMD2L) and the last one a deletion (LGMD2B). Eleven patients had compound heterozigoty (LGMD2A: 3, LGMD2B: 5, LGMD2C: 1, LGMD2D: 2). Mutations in heterozigoty were found in the LGMD2A (n=2) and LGMD2B (n=1) subtypes, all of the missense type.

The mutations in the *CAP-3* gene were found in exons 1 (n=3), 5, 9, 11, 22, in the *DYSF* gene (n=21) in exons 53 (n=4), 12 (n=4), 49 (n=4), 48, 18, 39, 29, 52, 15, 37, 7 and 50. All the missence mutations in the FKRP gene were located in exon 4, with only one with a different exon location of the mutation. The mutations in the sarcoglycans genes (n=11) were found in exons 6 (LGMD2C: 2, LGMD2D: 1, LGMD2E: 1), 3 (LGMD2D: 4) and 7 (LGMD2C: 2, LGMD2D: 1). The different types of sarcoglycanopathies diagnosed were divided in LGMD2C (n=3), LGMD2D (n=4) and LGMD2E (n=1) subtypes. The mutations in the *TCAP* and *TTN* genes were located in the exons 2 and 363, respectively and in the ANO5 gene the mutations were located in exons 18 and 5 (n=2).

In the present study, LGMD2B was the most common subtype diagnosed (38%), followed by sarcoglycanopathies as a group (21.4%), LGMD2I (16.6%) and LGMD2A (11.9%). The LGMD2G and 2J are very rare autosomal recessive LGMDs and ANO5 gene mutations only recently were found in LGMD2 patients (16), so the number of patients with this subtype might be higher than what it was found.

Discussion

Since the first time a genetically confirmed LGMD2 was presented at the Portuguese Society of Neurology[16], the number of LGMD2 subtypes increased significantly in the subsequent ten years. The gene defects discovered affect various sites throughout the muscle fibre, including the nuclear envelope, sarcomere, sarcoplasm and sarcolemma. The frequent clinical phenotype overlap among the different LGMD2 subtypes makes it difficult to come up with an immediate and accurate diagnosis of a specific LGMD2. Immunohistochemistry and Western blot analysis first and molecular studies later are, in most cases, necessary for a definite diagnosis.

The present study does not intend to give an estimate of the prevalence of the different subtypes of LGMD2 in the central region of Portugal, but the relative distribution of the different LGMD2 subtypes diagnosed in our centre. Recently, other hospitals from the central area of Portugal began to carry out independent investigation and diagnosis of patients with LGMD. While some of these patients have

Table 3. Molecular data

	Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level
LGMD2A			**LGMD2B**			**LGMD2C-F**			**LGMD2I**		
1	exon 1 heterozygous c.295T>A	p.Trp99Arg	6	- Intron 26 + exon 49 c.[2801+1G>A; c.5509G>A] - exon 53 c.5999G>A	(p.?) + p.Asp1837Asn p.Arg2000Gln	1	not available		1	exon 4 homozygous c.826C>A	p.leu276Ile
2	exon 1 c.60delA exon 22 c.2306G>A	p.Pro62fsX35 p.Arg769Gln	7	exon 49 homozygous c.5509G>A	p.Asp1837Asn	2	γ sarcoglycan gene exon 6 homozygous c.525delT	p.Phe175fsX20	2	exon 4 homozygous c.826C>A	p.leu276Ile
3	exon 5 heterozygous c.637C>T	p.His213Tyr	8	- exon 29 c.3115C>T - exon 52 c.5813_5821dup CAGCCAAGA	p.Arg1039Trp p.Thr1938_1940 Lysdup	3	α sarcoglycan gene exon 3 homozygous c.229C>T	p.Arg77Cys	3	exon 4 homozygous c.826C>A	p.leu276Ile
4	exon 9 c.1116-1G>A exon 11 c.1468C>T	p.Trp373ThrfsX59 + p.Trp373_Trp398del p.Arg490Trp	9	exon 12 c.1180_1180+7del exon 15 c.1379_1381del	p.Glu353_Leu429del p.Arg460del	4	α sarcoglycan gene exon 3 c.229C>T exon 7 c.850C>T	p.Arg77Cys p.Arg284Cys	4	exon 4 homozygous c.826C>A	p.leu276Ile
5	exon 1 c.60delA exon 22 c.2306G>A	p.Pro62fsX35 p.Arg769Gln	10	exon 49 homozygous c.5509G>A	p.Asp1837Asn	5	α sarcoglycan gene exon 3 homozygous c.229C>T	p.Arg77Cys	5	exon 4 homozygous c.826C>A	p.leu276Ile
LGMD2B			11	exon 53 homozygous c.5979dupA	p.Glu1994ArgfsX3	6	β sarcoglycan gene exon 6 homozygous c.323T>G	p.Leu108Arg	6	exon 4 homozygous c.545A>G	p.Tyr182Cys
1	exon 53 homozygous c.5979dupA	p.Glu1994ArgfsX3	12	exon 12 homozygous c.1180_1180+7del	p.Glu353_Leu429del	7	α sarcoglycan gene - exon 3 c.229C>T - exon 6 c.739G>A	p.Arg77Cys p.Val247Met	7	exon 4 homozygous c.826C>A	p.leu276Ile
2	exon 12 c.1180_1180+7del exon 53 c.5979dupA	p.Glu353_Leu429del p.Glu1994ArgfsX3	13	exon 37 homozygous c.4003G>A	p.Glu1335Lys	8	γ sarcoglycan gene exon 7 c.629A>G	p.His210Arg	**LGMD2J**		
3	exon 48 homozygous c.5429G>A	p.Gly178ValfsX17	14	exon 12 homozygous c.1168G>A	p.Asp390Lys	9	-γ sarcoglycan gene - exon 6 c.525delT - exon 7 c.629A>G	p.Phe175LeufsX20 p.His210Arg	1	exon 363 homozygous c.100185delA	p.Lys33395AsnfsXp9
4	exon 18 c.1620delA exon 39 c.4200dupC	p.Glu541SerfsX86 p.Ile1401HisfsX8	15	exon 7 homozygous c.757C>T	p.Arg253Trp	**LGMD2G**			**LGMD2L**		
5	exon 49 homozygous c.5509G>A	p.Asp1837Asn	16	exon 50 heterozygous c.5626G>A	p.Asp1876Asn	1	exon 2 homozygous c.157C>T	p.Gln53X	1	exon 18 homozygous c.2012ª>G	P.Tyr671_Val667delinsPhe
									2	exon 5 homozygous c.1991dupA	p.Asn64lysfsX15
									3	exon 5 homozygous c.1991dupA	p.Asn64lysfsX15

been referred to our centre for a definite diagnosis and included in the present study, others are diagnosed locally and not referred to us, so the number, form and subtype of LGMD are not of our knowledge. LGMD2B subtype was the most common diagnosis (38%), followed by the sarcoglycanopathies as a group (21.4%), LGMD2I (16.6%) and LGMD2A (11.9%) subtypes. Different authors have reported data about the relative distribution of LGMD2 subtypes. In Brazil, sarcoglycanopathies were found to be the most common, followed by dysferlinopathies[18]. In Italy, several authors found the LGMD2A subtype as the most common one, followed by dysferlinopathies[5,19]. In the USA, dysferlinopathies represented the largest subtype, followed, with similar data, by LGMD2I and LGMD2C-F[20]. In Denmark, LGMD2I was the most common subtype, with LGMD2B representing only 2% of the population studied[7]. The relative distribution of the different subtypes in our study, with small differences, might be considered similar to the above cited data. The only exception that deserves some comment is the relatively low number of patients with LGMD2A. It is possible that some cases might have been missed when the clinical phenotype was not a typical one. The use of Western bolt analysis could have helped the diagnosis of LGMD2A, but it is not available in our centre. It is important to remember the experience

reported by other centres with this technique showing that reduced amounts of calpain-3, sometimes even its absence it is not followed by the identification of pathogenic mutations in the *CAPN3* gene[21]. Another possible explanation is that LGMD2A, like sarcoglycanopathies, is more common in the paediatric than in adult population[22], which forms the majority of the population included in our study. In two cases of LGMD2A and one of LGMD2B subtypes only one mutation was found. Regarding the first one, this finding is not rare and some authors suggest that calpainopathy could be transmitted in an autosomal dominant mode. For dysferlinopathy, other reports have been published in which only one mutation was found and several explanations, related to the methodology of the molecular study and the nature and location of the mutation, were proposed[23,24].

Phenotype-genotype correlations have been attempted for all the LGMD2 subtypes. No general rules can be applied or a direct consequence can be drawn from the type and location in the gene of the mutation and the severity of clinical phenotype. The direct consequence of the defective protein over the integrity of the sarcolemma, the destabilization of the dystrophin-glycoprotein complex and the efficacy of the repair mechanisms of the injured sarcolemma are probably more important in determining the severity and progression of the muscular dystrophy than the mutation itself.

The mean times to muscle biopsy and molecular diagnosis were significantly high. The first one might be explained by insidious nature of most LGMDs and consequent delay of the patient and family in searching for medical care. Sometimes the delay in referring patients to specialized medical centre is a consequence of the rarity of the disease and the difficulty of the general practitioner in diagnosing a muscle disorder. Usually the molecular diagnosis is made after performing the muscle biopsy, so it is reasonable the longer mean time to reach a molecular diagnosis. Another reason for the delay in molecular diagnosis is that in some LGMDs the molecular diagnosis was not available at the time of the clinical evaluation and muscle biopsy, as it happened in LGMD2I and 2L subtypes.

It is important to have a specific molecular diagnosis, since it gives us the opportunity to inform the patients about the probable natural history of the disease, the probability of other members of the family to be clinically affected and to be more active in preventing and treating medical complications which in some subtypes are relatively common. It is impossible to perform molecular investigations for all the genes responsible for the known LGMD2 so, probably, in some patients the appropriate test is not requested. Since 1995, when mutations in the proteolytic enzyme calpain-3 were identified in patients with an autosomal recessive LGMD[25] pathogenic mutations in other genes and in different chromosomes have been progressively found in patients with previously undiagnosed LGMD2. It is reasonable to think that new genes coding for proteins not yet known to be responsible for LGMD2 will be found in the future and so, it is expected that the number of patients and the subtypes of LGMD2 will be higher in the future.

The diagnostic process of LGMDs needs to encompass many variables (clinical, laboratory, pathologic, etc) which difficult the elaboration of a diagnostic algorithm useful in the clinical practice. Each patient should be evaluated individually and the result of each test should be critically considered in accordance to clinical signs and symptoms. ■

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PAPER III

Novel ancestral Dysferlin splicing mutation which migrated from the Iberian peninsula to South America.

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Novel ancestral *Dysferlin* splicing mutation which migrated from the Iberian peninsula to South America

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Abstract

Primary dysferlinopathies are a group of recessive heterogeneous muscular dystrophies. The most common clinical presentations are Miyoshi myopathy and LGMD2B. Additional presentations range from isolated hyperCKemia to severe functional disability. Symptomatology begins in the posterior muscle compartment of the calf and its clinical course progresses slowly in Miyoshi myopathy whereas LGMD2B involves predominantly the proximal muscles of the lower limbs. The age of onset ranges from 13 to 60 years in Caucasians. We present five patients that carry a novel mutation in the exon12/intron12 boundary: c.1180_1180 + 7delAGTGCGTG (r.1054_1284del). We provide evidence of a founder effect due to a common ancestral origin of this mutation, detected in heterozygosity in four patients and in homozygosity in one patient.

Keywords: Primary dysferlinopathies; Miyoshi myopathy; LGMD2B; HyperCKemia; Founder mutation

1. Introduction

Primary dysferlinopathies, a heterogeneous group of autosomal recessive muscular dystrophies, are caused by mutations in the 55-exon gene encoding the protein dysferlin (*DYSF,* 2p13, MIM#603009) [1,2]. Dysferlin is a member of a class of homologous proteins called "ferlins". It is a 230-kDa protein located at the sarcolemma, not associated with the dystrophin-glycoprotein complex [3,4]. It

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seems to play an essential role in membrane repair in skeletal muscle fibers [5,6].

Three main clinical entities have been described in different ethnic groups: Miyoshi myopathy (MM, MIM# 254130), limb girdle muscular dystrophy (LGMD2B, MIM#253601) and distal myopathy with anterior tibial onset (DMAT, MIM#606768) [7,8]. Additional presentations ranging from isolated hyperCKemia to severe functional disability have also been described [9].

The onset of MM and LGMD2B is generally in the teens or early adulthood. In MM, the muscles primarily affected are the soleus and the gastrocnemius muscles. Common early symptoms include inability to stand on tip-toe, hop on one leg, run and difficulty to climb stairs [7]. As the disease progresses the weakness involves the thigh and upper limb muscles [10]. In Caucasians, the age of onset of the disease ranges from 13 to 60 years [11]. At onset, LGMD2B involves predominantly the proximal muscles of the lower limbs. The distal muscles of both arms and legs are spared as the muscular dystrophy progresses [12]. DMAT is characterized by anterior tibial muscle weakness which rapidly progresses to the lower and upper proximal muscles [8].

In general, these dystrophies have very high creatine kinase (CK) levels and are slowly progressive [10]. On average, about 15 years after onset, the patients have to use a cane or crutches and they are wheelchair-bound approximately 22 years after disease onset [10,13]. There is also considerable phenotypic heterogeneity among the patients who have the same mutation in the *DYSF* gene, therefore MM or LGMD2B can be observed within the same family. Modifier genes can be held responsible for this [10,14,15].

Absence or reduced dysferlin in muscle, determined by immunohistochemistry or immunoblotting, as well as *DYSF* mutation detection, enables to confirm the diagnosis [10]. More than 400 different sequence variants have been reported in the *DYSF* gene (Leiden Muscular Dystrophy pages © Locus Specific Database; http://www.dmd.nl/DYSF/) revealing a high allelic heterogeneity. Occasionally, the presence of founder mutations has been suspected or described in distinct geographic regions or ethnic groups, such as Italy [15], Spain [16], Portugal [17], Libyan Jewish population in Israel [18] and in the Jews of the Caucasus [19].

We present the clinical and molecular characterization of a novel *DYSF* deletion affecting a splicing site, detected in five unrelated patients. The patients, one Uruguayan and four Portuguese, share the same haplotype background for this mutation, suggesting a common ancestry and a possible founder effect.

2. Patients and methods

2.1. Medical histories

The study includes five patients, one from Uruguay and four from Portugal, with genetically-proven dysferlinopathy on muscle biopsies and genetic analysis. Both studies were performed after written informed consent was provided. We ascertained that all the patients were seen prospectively. Muscle involvement was evaluated clinically using the Medical Research Council (MRC) grading scale. Since patient 1 is the first reported Uruguayan case diagnosed with a primary dysferlinopathy, a more detailed clinical description is provided.

2.2. Histological and ultrastructural studies

Skeletal muscle biopsies were obtained from the quadriceps and the deltoid muscles in patient 1. Cryostat sections (9 μm) of snap-frozen muscle tissue were stained for hematoxiline-eosin (HE), PAS, Gomori Trichrome, ATPase, NADH, and succinic dehydrogenase (SDH). Sections of muscle biopsies were immunolabeled with anti-desmin, anti-myotilin monoclonal (Dako, Glostrup, Denmark), and dysferlin monoclonal antibodies (Hamlet-1 and Hamlet-2 Novocastra, Newcastle upon Tyne, UK). For electromicroscopy muscle were fixed in 2.5% glutaraldehyde buffered with cacodylate, post fixed in osmium tetroxide and imbedded in Durcupan (Fluka/Sigma–Aldrich, St. Louis, MO). Semithin sections were stained with toluidine-fuchsin. Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate. Zeiss EM 10 electron microscope (Carl Zeiss, Overkochen, Germany) was used to examine the sections, collected on copper grids.

2.3. Western blot analysis

Multiplex Western blot analysis was performed on muscle as previously described [20]. The other proteins involved in DMD and LGMD such as dystrophin, sarcoglycans and Calpain-3 were tested in the same gel. The dysferlin monoclonal antibodies used (NCL-Hamlet) were from Novocastra, Newcastle upon Tyne, UK.

2.4. DYSF mutational analysis

2.4.1. gDNA studies

The coding regions and flanking intronic boundaries of the *DYSF* gene were subjected to mutation screening, either by DHPLC analysis and subsequent sequencing of abnormally eluted fragments, as previously described [21] (patient 1), or by direct sequencing using M13-tailed intronic primers (patients 2–5). Sequencing data analysis was assisted respectively by the Sequencher® software (Gene Codes Corporation, Ann. Arbor, MI, USA) and the SeqScape V2.5 software (Applied Biosystems, Foster City, CA), using the dysferlin cDNA reference sequence NM_003494.3.

2.4.2. cDNA studies

In order to study the effect of the c.1180_1180 + 7del mutation on *DYSF* splicing, cDNA studies were conducted by reverse transcription PCR (RT-PCR). Briefly, total RNA was extracted from a muscle biopsy from patient 3

and from a control individual using the Versagene RNA Purification kit (Gentra, Minneapolis, MN). RT-PCR was performed using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) and the following specific primers designed to amplify exons 11–18: c.11F: 5′ GCT ACC TGA AAA CAA GCC TTT G 3′ and c.18R: 5′ GGA CTG CCA TAG AGG TTG ATG T 3′. PCR products were resolved on 1% w/v agarose gels and sequenced in both directions.

2.5. Haplotyping

Haplotypes were constructed with the analysis of intragenic microsatellite markers Cy172-H32 and 104-sat, as well as the extragenic microsatellite markers D2S2113, D2S291, D2S2112 and D2S2111. Amplicons were sized following automated capillary electrophoresis using the program GeneMapper V4.0 (Applied Biosystems, Foster City, CA). Further information was obtained with the following intragenic single nucleotide polymorphisms (SNPs): c.1827T > C, c.2583T > A, c.4008C > A, c.4887–37C > T, c.5768–16T > C and c.5859A > C (primer sequences available upon request). Allele phases in the patients were established by analysing both parents. Haplotype frequencies were determined amongst the Portuguese population, using 20 anonymous, unrelated and non-consanguineous singleton families, selected at random, as described elsewhere [17]. These control samples revealed 77 independent haplotypes for the regions encompassing markers Cy172-H32 to D2S2112.

3. Results

3.1. Patients

Patient 1 is a 26-year-old engineer who is the second son of non-consanguineous parents. He had completely normal motor milestones and at the age of 12 years he engaged in competitive sports such as swimming, soccer, basketball and handball. At the age of 15 years, he noticed he no longer had the strength to run, jump, ride a bicycle or while playing soccer he could not tackle an opponent. These limitations progressed slowly. On the physical examination, two months after the onset, slightly decreased strength in the distal muscles of lower limbs was found. The serum enzyme levels were CK 38.700 IU/ml, creatine kinase MB 1580 IU/ml, 830 IU/ml, glutamyl oxaloacetic transaminase (GOT), glutamyl piruvic transaminase (GPT) 610 IU/ml, lactate dehydrogenase (LDH) 3230 IU/ml. The EMG revealed a myopathic pattern with reduced number of normal motor units in both tibialis anterior muscles, the left vastus lateralis and the left extensor digitorum brevis muscles. Low amplitude abnormal polyphasic motor units recruitment was recorded in all the studied muscle groups. Motor and sensory nerve conduction studies were normal in the lower limbs. As inflammatory myopathy was diagnosed in the first biopsy he was prescribed prednisone 40 mg/day for two months without clinical response.

One year after the onset, the patient stated he had fatigability of the proximal muscles of the upper limbs. The physical examination showed hyperlordosis, wasting and weakness of the glutei muscles. He was prescribed prednisone 60 mg and azathriopine 150 mg/day. He was then treated with Iv-IG. He was treated for six months but the treatment failed to improve his clinical condition. The

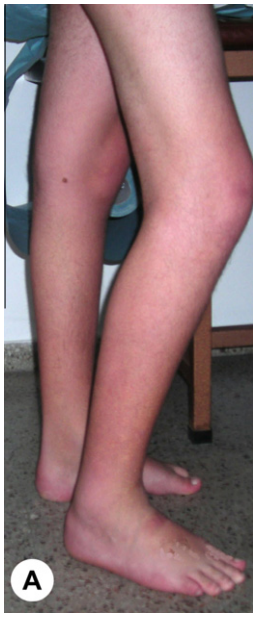

Fig. 1A. The knee joint is thrust backwards and locked in a hyperextended position in order to stabilize it.

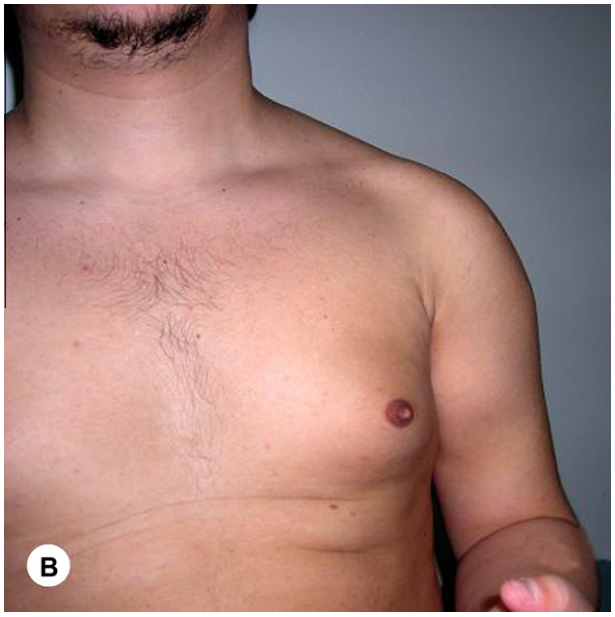

Fig. 1B. Bowl-shaped appearance of the left biceps brachia.

serum enzymes levels showed: CK 12577 IU/ml, CK-MB 560 IU/ml, GOT 207 IU/ml, GPT 240 IU/ml, LDH 1278 IU/ml, even though he had stopped practicing sports. His symptoms continued to evolve gradually over the following years.

The ultrasound imaging showed alterations in the left gastrocnemius, where the adipose tissue had substituted the muscle tissue.

The MRI of the lower limbs showed an increased signal change at the lower third of the internal left gastrocnemius of the left lower limb, comprising less than 30% of the volume of the muscle. There was an increased density at this level showing a replacement of muscle by connective and adipose tissues. Neurovascular structures and normal muscle were still present at the periphery.

On examination, at age 19, there was no muscle weakness of the face, nor of the upper limbs; the strength of these muscles (upper limbs) and both quadriceps muscles was normal, amyotrophy areas and bowl-shaped appearance stood out in the left quadriceps and both gastrocnemius muscles. He could not stand on tiptoe. There was no pain by palpation of the muscles. Deep tendon reflexes were normal except for knee reflexes which were difficult to obtain. Miyoshi myopathy was diagnosed on clinical grounds.

At 20 years, on examination the patient could not arise from squatting position. Both quadriceps muscles had bowl-shaped appearance and wasting. Muscle strength had decreased in the glutei and iliopsoas muscles. In the lower limbs the loss of strength was progressive, especially in the distal muscles, where paresis, paralysis and tightening of the heel cords were found. Severe paresis of the proximal muscle groups was also found. Steppage gait was observed.

The patient is currently overweight, walks with crutches and has abandoned the practice of swimming. Both knee joints are thrust backwards and locked in a hyper-extended position in an attempt to stabilize them (back-kneeing). Severe wasting of calf muscles is observed (Fig. 1A). He cannot sit down from the supine position without help.

On examination, facial and sternocleidomastoid muscles are normal. Trapezeii and serrate muscles (4/5), supraspinatus, infraspinatus, latissimus dorsi and pectoralis muscles (3/5), deltoid and triceps brachii muscles (5/5), supinator long muscles (4/5), radial muscles, lumbricals, dorsal interosseii muscles and finger flexor muscles (4/5). Bowl-shaped appearance was observed in both biceps brachia muscles (Fig. 1B). The upper limbs deep tendon reflexes are normal.

Major glutei muscles (1/5), psoas, sartorius, tensor fascia lata, quadriceps, semimembranosus and semitendinosus muscles (3/5). Peroneii, tibialis anterior and tibialis posterior muscles (2/5). Severe paresis of feet muscles: extensor muscles (1/5) and flexor muscles (2/5). Lower limbs deep tendon reflexes are absent. CK: 7700 IU/ml. ECG, echocardiogram and respiratory function have been normal during the ten-year follow-up. Clinical data, including that of patients 2–5, are summarized in Tables 1 and 2.

3.2. Skeletal muscle biopsies

3.2.1. Light microscopy

The first muscle biopsy of patient 1 showed with HE: increased fiber-size variability, numerous internalized nuclei, fiber splitting, necrotic and regenerative fibers. Lymphocytes and macrophages were observed surrounding and invading necrotic fibers. (Fig. 2A) NADH showed fiber-size variability and fiber splitting. No immunohistochemical techniques were available. The second biopsy showed a similar histopathological pattern. By immunohistochemistry the proteins of the dystrophin-glycoprotein complex and myofibrillar proteins were normal (not shown), whereas dysferlin was absent in most fibers (Fig. 2B).

3.2.2. Electron microscopy

Small membrane gaps were observed in necrotic fibers. The basal lamina had multilayered areas (Fig. 2C). A few papillary projections and aggregates of subsarcolemmal vesicles, some of them filled with electron dense material,

Table 1
Summary of the clinical data of the patients.

Patient	Current age (yr)	Sex	Clinical diagnosis	Age at onset (yr)	Physical development	Psychological development	Symptoms at onset	Clinical course
1	26	M	MM	15	Normal milestones	Normal (reached university level)	Decreased strength in LL: gastrocnemius, tibialis post., biceps femoralis	Faster than expected.Had to use crutches at 23.
2	61	M	LGMD	33	Normal milestones	Normal	Difficulty to walk; weakness in pelvic girdle muscles	Rapid (considered invalid to work at 42)
3	22	M	MM + LGMD	17	Normal milestones	Normal (high school student)	Frequent falls; difficulty to run and rise from a chair	Progressive
4	61	M	MM + LGMD	35	Normal milestones	Normal	Difficulty to walk	Slowly progressive
5	57	F	MM + LGMD	24	Normal milestones	Normal	Decreased strength and muscle pains in lower limbs	Rapidly progressive, loss of ambulation at 36

yr: years; M: male; MM: Miyoshi myopathy; LGMD: limb girdle muscular dystrophy

Table 2
Summary of the muscular features, biopsies of the patients and molecular diagnosis.

Patient	Muscle involvement	CK (U/l)	ECG	Muscle biopsy	Western blot	EMG	Mutational findings
1	Progressed to UL Asymmetric atrophy	38.730 (at onset)	N	1st biopsy (age 15) inflammatory myopathy. 2nd biopsy: (age20) dysferlin absent	Dysferlin absent	Myopathic pattern	Exon 12/ Intron 12:c.1180_1180 + 7delAGTGCGTG (r.1054_1284del; p.Glu353_Leu429del) heterozygous and Exon 50:c.5601C > A(p.Phe1867Leu) heterozygous
2	Progressed to UL	nd	N	1st biopsy (age 38): inflammatory infiltrates. 2nd biopsy (age 52):dystrophic; dysferlin absent	nd	Myopathic pattern	Exon 12/ Intron 12: c.1180_1180 + 7 delAGTGCGTG (r.1054_1284del; p.Glu353_Leu429del) heterozygous and Exon 24: c.2393T > C (p.Leu798Pro) heterozygous
3	Gastrocnemius Tibial anterior	14.282 (age 19)	N	Dystrophic; dysferlin absent.	nd	Myopathic pattern	Exon 12/ Intron 12: c.1180_1180 + 7 delAGTGCGTG (r.1054_1284del; p.Glu353_Leu429del) heterozygous and Exon 15: c.1379_1381del (p.Arg460del) heterozygous
4	Slowly progressed to upper limbs	1.860 (age 53)	N	Markedly dystrophic; dysferlin absent	nd	Myopathic pattern	Exon 12/ Intron 12: c.1180_1180 + 7 delAGTGCGTG (r.1054_1284del; p.Glu353_Leu429del) heterozygous and Exon 53:c.5979dupA (p.Glu1994ArgfsX3) heterozygous
5	Proximodistal; progressed to UL 5 years later; Neck flexion weakness	1.023 (age 41)	N	Dystrophic; dysferlin absent.	nd	Myopathic pattern	Exon 12/ Intron 12:c.1180_1180 + 7 delAGTGCGTG (r.1054_1284del; p.Glu353_Leu429del)homozygous

CK – creatine kinase; ECG – electrocardiography; EMG – electromyography; ; LL – lower limbs; UL – upper limbs; N – normal; nd – not determined.

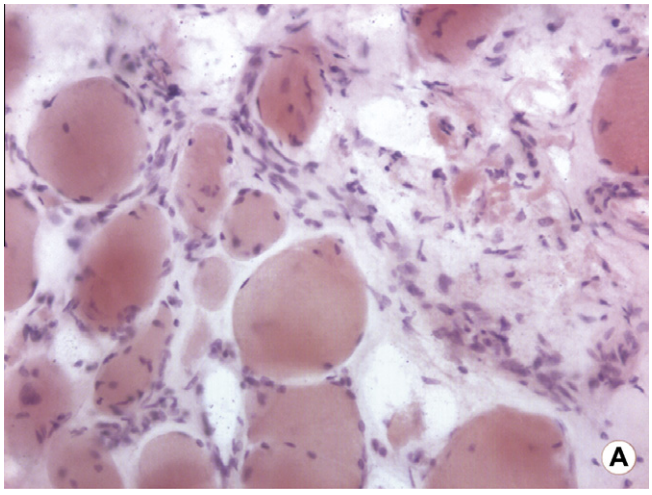

Fig. 2A. HE: Variation in fiber size (10–110 μm), internal nuclei, endomysial connective tissue with incipient fibrosis, mild lymphocyte infiltrate. (Magnification 40×).

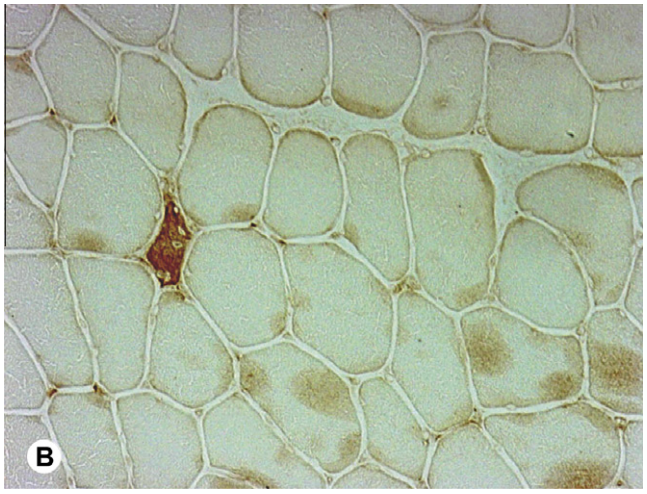

Fig. 2B. Absence of dysferlin in most of the fibers. Patchy sarcolemmal and diffuse cytoplasmatic dysferlin (Magnification 10×).

were also occasionally seen (Fig. 2C). There were also non-specific sarcoplasmic alterations such as focal disruption of myofilaments filled with mitochondria, streaming of *Z* line, large empty vacuoles and lipid droplets (Fig. 2D).

3.3. Western blot

The Western blot showed traces of dysferlin whereas dystrophin, sarcoglycans and calpain were normal (Fig. 3).

3.4. DYSF gene analysis

Causal mutations were detected in both alleles of all five patients (Table 2). Four patients were heterozygous and the fifth was homozygous for the mutation c.1180_1180 + 7delAGTGCGTG (Fig. 4A). This eight nucleotide deletion, located in the exon 12/intron 12 boundary, encompasses the donor splice site. The effect of this mutation was investigated by cDNA analysis of *DYSF* transcripts

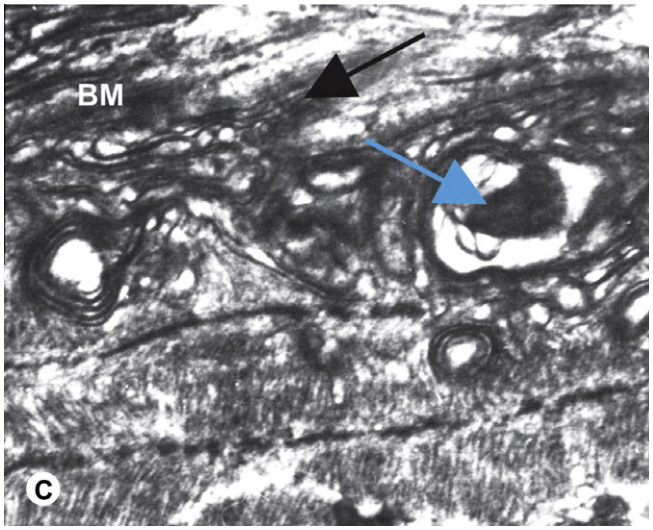

Fig. 2C. A multilayered basal membrane (BM). Myofibrillar disruption and degenerating myelin figures next to the sarcolemma (blue arrow). Papillary projections (black arrow) (Magnification 20000×).

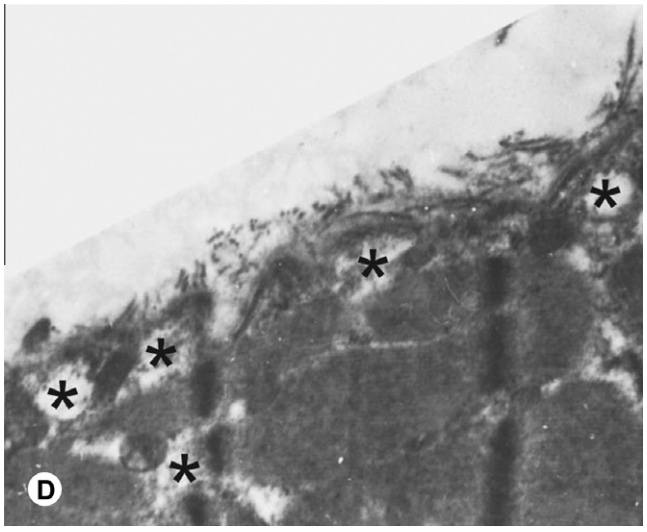

Fig. 2D. (*) Accumulation of vesicles in the subsarcolemmal region (Magnification 20000×).

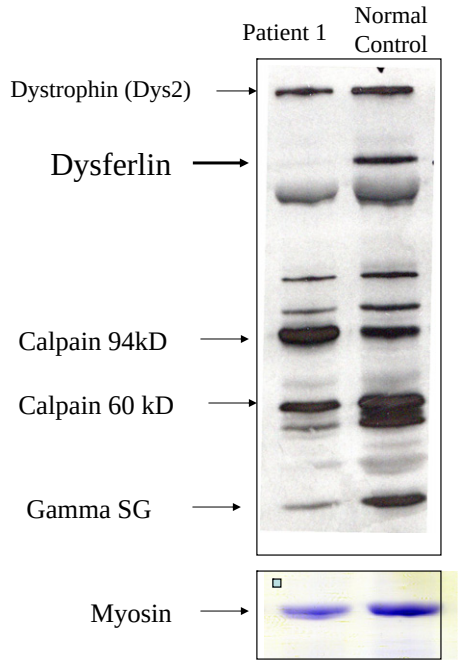

Fig. 3. Multiplex Western blot showing only traces of dysferlin in patient 1.

in patient 3 (Fig. 4C). Results showed that the mutation gives rise to a broader splicing defect than originally expected promoting the full skipping of exons 12 and 13 (r.1054_1284del) predictably translated into an in-frame deleted protein (p.Glu353_Leu429del). However, as all patients presented with severely reduced or absent dysferlin levels on Western-blot analyses, we conclude that the resulting in-frame deleted protein would be unstable and degraded.

Three previously unreported mutations were identified in the course of this joint study. The novel point mutation in exon 50 (c.5601C > A), was found in heterozygosity in patient 1. This is a single base pair transversion (C > A) predicted to cause a missense change affecting a highly conserved phenylalanine amino-acid residue (p.Phe1867Leu) (Fig. 4A) This change has not been previously described in the literature and databases and was not retrieved in a series of 100 control chromosomes. Bioinformatics analysis using SIFT (score 0.09) and Polyphen (score 2.403) also suggested that this missense mutation is pathogenic. Mutational analysis in the patient's asymptomatic parents showed that the mutation in exon 12/intron 12 has been inherited from his father, whereas the mutation located in exon 50 has been inherited from his mother. Moreover, only the mutation in exon 50 was detected in the patient's two siblings.

The missense mutation c.2393T > C (p.Leu798Pro), detected in patient 2, is located in exon 24 (Fig. 4B). It was considered pathogenic based on SIFT (score 0) and Polyphen (score 1.945) analysis, as well as the fact that it was not detected in 200 control chromosomes. The single codon deletion in exon 15 (c.1379_1381del), detected in patient 3, presumably deletes a single amino acid (p.Arg460del) (Fig. 4B). This arginine residue is located in one of the protein's C2 domains, and is conserved in all vertebrates except the dog. In patient 4, a known duplication was detected in exon 53 (c.5979dupA) presumed to cause a frameshift (p.Glu1994ArgfsX3) [22] (Fig. 4B).

3.5. Haplotype analysis

Haplotypes encompassing the *DYSF* locus, using markers spanning a region of approximately 2.8 Mb, are shown in Fig. 5. All patients were found to share a common haplotype background between markers Cy172-H32 and D2S211. This haplotype block had not been detected

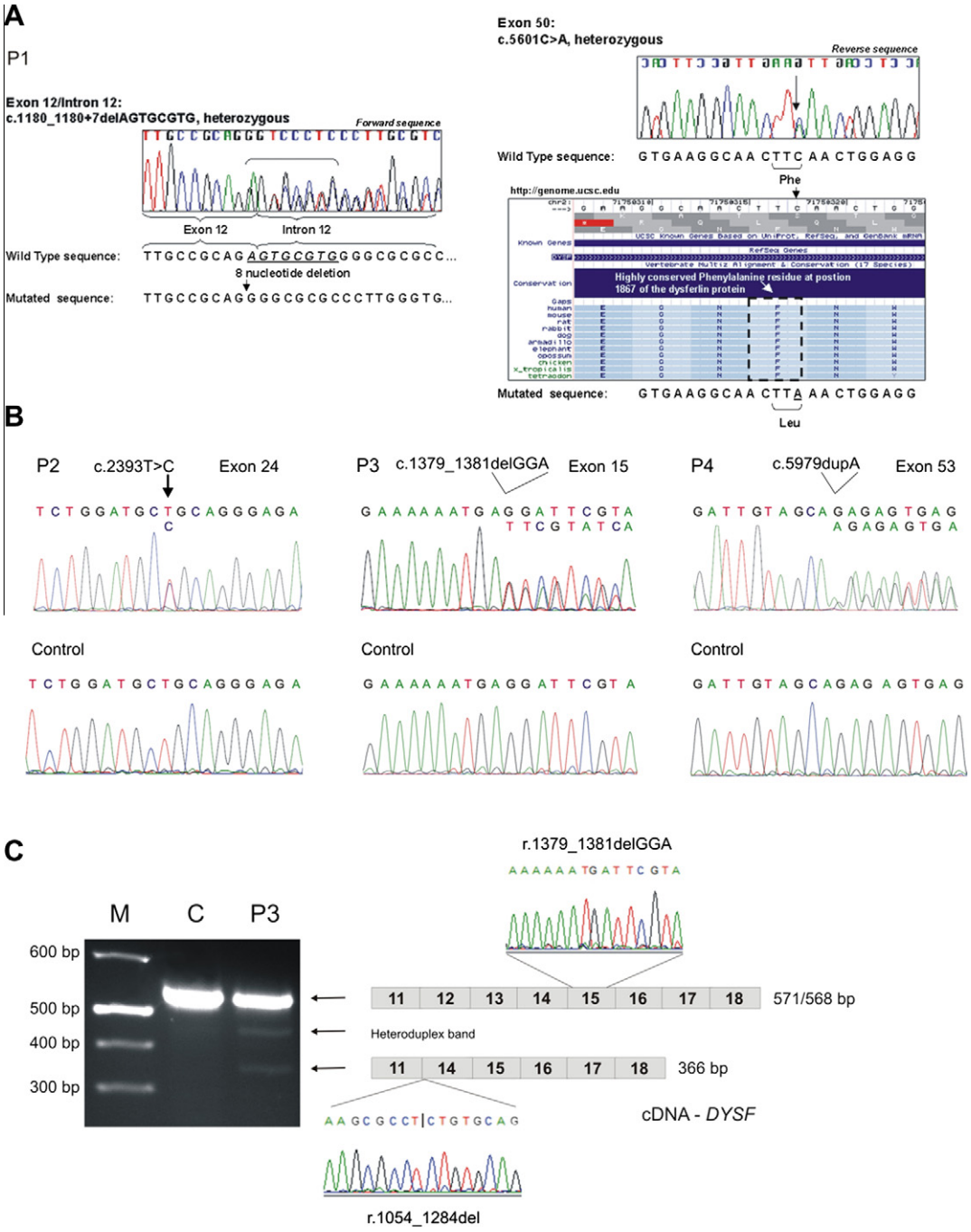

Fig. 4. Molecular *DYSF* analysis. (A) Compound heterozygous mutations c.[1180_1180 + 7del] + [5601C > A] identified in the Uruguayan patient (P1). (B) Other mutations found in compound heterozygosity in patients P2, P3 and P4. (C) Characterization of c.1180_1180 + 7del mutation at the mRNA level in Patient 3 (P3) revealing the presence of an aberrant *DYSF* transcript resulting from skipping of exons 12 and 13.

amongst the 77 most common independent haplotypes of the Portuguese population. Patient 5, who was homozygous for the c.1180_1180 + 7delAGTGCGTG mutation, was also homozygous for this haplotype. Patients 3 and 4 share a different allele for the telomeric flanking marker D2S2113. In all cases, the analyses of the parents confirmed that the haplotype shared by the patients segregated with the c.1180_1180 + 7delAGTGCGTG mutation (results available upon request).

4. Discussion

It is noteworthy that this novel mutation has not been detected in other populations; its apparently "private" nature is also consistent with a founder effect of a single mutational event. In fact, in the five patients, analyses with polymorphic markers revealed a haplotype block that was identical in state, in -the spanning of the *DYSF* gene, from markers Cy172-H32 to D2S2111 (Fig. 5). According

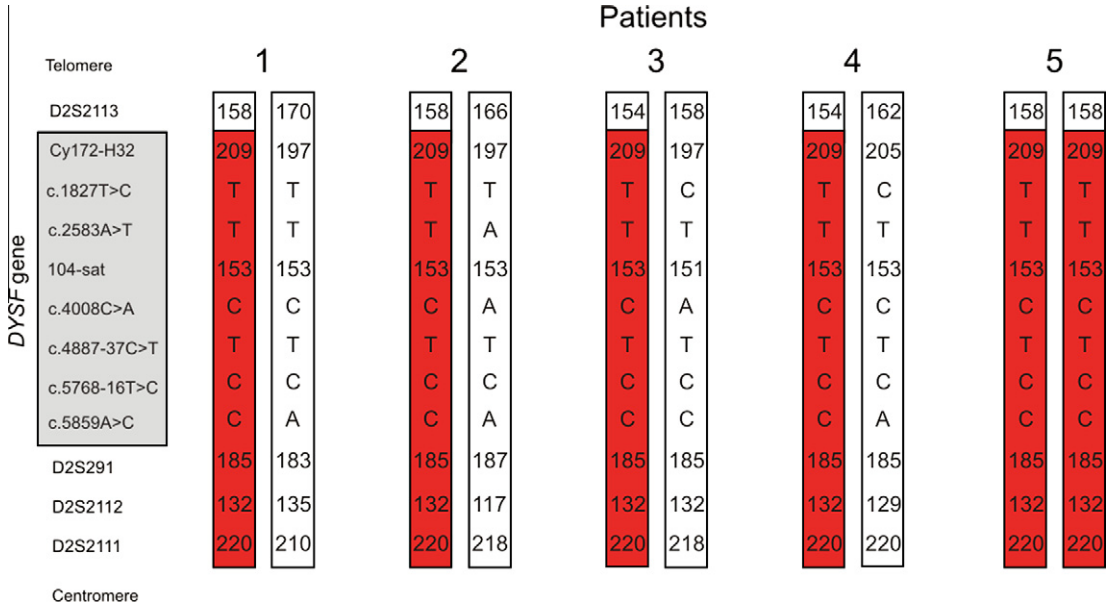

Fig. 5. Haplotypes of the patients with the founder effect.

to a previous survey [17], this particular haplotype is rare in the Portuguese population (<1.3%), thus it is highly unlikely that these mutated *DYSF* alleles represent recurrent events.

As mentioned previously, several possible founder effects in *DYSF* have been reported in the literature. Some of these have arisen within specific ethnic groups (native Canadian and Jews) [14,18,19] possibly due to endogamy. To our knowledge, only three mutations: c.2875C > T (p.Arg959Trp); c.5713C > T (p.Arg1905X); c.5429G > A (p.Gly1781ValfsX17) were experimentally demonstrated as being associated with a specific *DYSF* haplotype, and all of these were found in Mediterranean Latin populations [15–17].

In our study the c.1180_1180 + 7delAGTGCGTG mutation was identified in heterozygosity in four patients. Patient 5 is homozygous and there is no proven inbreeding in her family, so we must infer that there is homozygosity by descent.

As this mutation has been detected in 4 out of the 41 diagnosed Portuguese patients with dysferlinopathy so far, it can be postulated that this mutation is widespread in the Portuguese population because it has been found in around 10% of the patients with dysferlinopathies and it will probably also be found in patients from the former Portuguese colonies.

The fact that the same mutation in the *DYSF* gene exon12/intron12 boundary c.1180_1180 + 7delAGTG CGTG, has been found on the same haplotype background in five unrelated families, suggests that it derives from a common mutational event which is not very recent as it is observed by the recombination between the mutated gene and the telomeric flanking marker D2S2113 (Fig. 5).

We think that there must be more patients with primary dysferlinopathies that are misdiagnosed due to the fact that there is great clinical heterogeneity.

There is inter and intra-familial variability in the patients with dysferlinopathy, thus phenotypic heterogeneity can be found among the patients who have the same mutation in the *DYSF* gene [7–10]. In our patients we only observed either MM or LGMD2B.

The Uruguayan patient's paternal ancestors were from Galicia (in the northwest of Spain, and bordered by Portugal to the south), and from Brazil (a former Portuguese colony). As patient 1 and his father shared the same mutation in exon12/intron12 with the four Portuguese patients, it seemed highly probable that it derived from a common ancestor.

Patient 1 showed the salient clinical features of MM: age, muscle weakness and atrophy which involved the distal muscles of lower limbs and later on spread to the thigh and glutei muscles. In the lower limbs, the atrophy of the muscles is asymmetrical, more severe on the left limb as it was described by other authors [10]. In dysferlinopathies, CK value is very high in the active phase of the disorder and it decreases with greater disability as it has been reported previously [12]. At onset his serum CK was 38,730 UI/ml and currently its value is 7700 UI/ml. We can infer that the strikingly high CK value was probably because he had practised sports intensively since he was 12 years old.

It is frequent to diagnose inflammatory myopathy instead of dysferlinopathy [2,9,23]. The inflammatory histological pattern observed in the first biopsy led the pathologists to diagnose an inflammatory myopathy. Immunohistochemistry and immunoblotting were performed in the second biopsy and they showed the typical features of dysferlinopathy (Fig. 2A and 2B).

The ultrastructure showed areas of small subsarcolemmal microvesicles, multilayering of basal membrane as well as papillary projections (Fig. 2C and 2D) which are in agreement with previously reported data [24,25].

The MRI showed a distinct and consistent pattern of mild muscle involvement of the internal left gastrocnemius muscle which can be staged at level two of the scoring system used by Mercuri et al. [26].

The pharmacological treatment performed after the first biopsy had no results in patient 1, as it was previously observed [10,27,28].

Inflammation and severity of the disorder correlate with a more rapid progression [9,29]. The clinical course in patient 1 has been faster than expected as he had to use crutches eight years after onset. Patient 2 was disabled to work nine years after onset. Patients 3 and 4 present a mixed pattern (proximodistal). This was clear in patient 3, but in patient 4 it was uncertain at onset. Although patients 3 and 4 share the same ancestral mutation, the disorder had a protracted course, especially in patient 4. Patient 5 evolved more rapidly than the rest of the patients since she was wheelchair-bound 12 years after onset. The faster course of the disorder in this patient could partially be due to the fact she is homozygous for the common mutation, even though genetic variability is thought to be mostly due to genetic modifiers [10,14,30].

We think that there must be more patients with primary dysferlinopathies that are misdiagnosed due to the extensive clinical heterogeneity.

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PAPER IV

Private dysferlin exon-skipping mutation (c.5492G>A) with a founder effect reveals further alternative splicing involving exons 49-51.

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SHORT COMMUNICATION

Private dysferlin exon skipping mutation (c.5492G > A) with a founder effect reveals further alternative splicing involving exons 49–51

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The allelic muscle disorders known as limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy and distal anterior compartment myopathy result from defects in dysferlin—a sarcolemma-associated protein involved in membrane repair. Mutation screening in the dysferlin gene (*DYSF*) enabled the identification of seven Portuguese patients presenting the variant c.5492G > A, which was observed to promote skipping of exon 49 (p.Gly1802ValfsX17). Several residually expressed products of alternative splicing also involving exons 50 and 51 were detected in the leukocytes and muscle of both patients and normal controls. Quantitative transcript analysis confirmed these results and revealed that Δ49/Δ50 transcripts were predominant in blood. Although the patients were apparently unrelated, the c.5492G > A mutation was found in linkage disequilibrium with a particularly rare haplotype in the population, corroborating the hypothesis of a common origin. Despite the presence of the same mutation on the same haplotype background, onset of the disease was heterogeneous, with either proximal or distal muscle involvement.

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Keywords: alternative splicing; clinical heterogeneity; dysferlin; exon skipping; founder effect

INTRODUCTION

Dysferlin is involved in calcium-dependent vesicle trafficking and membrane fusion, playing an important role in muscle fibre repair.[1,2] Mutations in the dysferlin gene (*DYSF*, MIM*603009) lead to a clinically heterogeneous set of muscular dystrophies collectively known as dysferlinopathies. These may present as a predominantly proximal limb-girdle muscular dystrophy type 2B[1] or as predominantly distal forms, with early involvement either of the gastrocnemius, as in Miyoshi myopathy,[3] or of the anterior tibial muscles, as in distal anterior compartment myopathy.[4] Despite these distinctions, there appears to be no strict correlation between the type or location of the mutation and the clinical phenotype, as the same mutation has been described in both proximal and distal forms, even in an intrafamilial context.[5–8]

Over 400 variants have been reported to date, with pathogenic mutations widespread across this large gene. Moreover, an alternative promoter and alternatively spliced transcripts have recently been described,[9–11] which add complexity to mutation screening and interpretation.

Here we describe the characterization of the novel exon-skipping mutation c.5492G > A, with an apparent founder effect in our population, in which studies revealed further alternative splicing in this region of the gene, even in normal controls.

MATERIALS AND METHODS

Patients

The seven unrelated male patients had elevated serum creatine kinase levels and compatible clinical phenotype and/or muscle pathology (data summarized in Table 1). Informed consent was obtained for molecular studies in all the patients.

Mutation screening

In genomic DNA, all 56 exons and flanking intronic regions were cycle-sequenced using the BigDye Terminator Cycle Sequencing Kit V1.1 and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

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Table 1 Clinical–pathological data of the seven dysferlinopathy patients

Patient/ gender	*Onset/current age (years)*	*Presenting symptoms*	*Muscle involvement and progression*	*CK ($U\,l^{-1}$)*	*Muscle dysferlin*	*Cons.*	*Genotype*
I/M	18/34	Difficulty running and climbing stairs	Proximal tetraparesis	7960	Absent	N	c.[5492G>A]+ [5492G>A]
II/M	14/48	Unknown	Proximal muscular weakness in the lower limbs	8280	Absent	N	c.[5492G>A]+ [5492G>A]
III/M	12/23	Difficulty running	Proximal weakness and extreme pseudohypertrophy of the calves	14340	Reduced	U	c.[5492G>A]+ [5657delG]
IV/M	13/19	Fatigue; hyperCKemia; cardiac arrhythmia	Proximal muscular weakness in the lower limbs	5730	Absent	Y	c.[5492G>A]+ [5492G>A]
V/M	18/30	Difficulty walking on toes	Distal muscular weakness in the lower limbs	4950	ND	Y	c.[5492G>A]+ [5492G>A]
VI/M	18/25	Difficulty running and climbing stairs	Proximal weakness in the upper and lower limbs; generalized atrophy of the lower limbs	4470	Absent	Y	c.[5492G>A]+ [5492G>A]
VII/M	18/21	Muscle pain following exercise	Proximal muscular weakness in the lower limbs	8460	Absent	Y	c.[5492G>A]+ [5492G>A]

Abbreviations: CK, creatine kinase; Cons., consanguinity; M, male; N, no; ND, not determined; U, unknown; Y, yes.
Muscle biopsies were subjected to routine histochemical evaluation as well as immunohistochemical staining for dystrophin, merosin, dysferlin, and α-, β- and γ-sarcoglycans.

Analysis was aided by the SeqScape V2.5 software (Applied Biosystems) using the reference cDNA sequence filed under GenBank Accession Number NM_001130978.1.

Transcript analysis

Total RNA was extracted from peripheral blood and/or muscle biopsies of patients and controls using TRIzol isolation reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed using either Superscript One-Step RT-PCR with Platinum *Taq* (Invitrogen) or the High Capacity RNA-to-cDNA Kit (Applied Biosystems).

Gross changes around exon 49 were detected using the following primers designed in exons 47 and 53, respectively: c.(47)F-5′-CAGCAGCATAGAGTCAAG-3′ and c.(53)R-5′-CACCAGCCCTTCACTGTTTT-3′. Amplicons resolved on 1% w/v agarose gels were eluted and sequenced in both directions, as described above.

For quantitative transcript analysis, six TaqMan probes and primers were used: (i) human glyceraldehyde-3-phosphate dehydrogenase gene-specific set, as an endogenous control, (ii) a target region spanning the exon 48–49 junction (assay ID Hs01002534_m1; Applied Biosystems) and (iii) specifically designed probes and primers for alternatively spliced transcripts, namely for exon 48–50, 48–51, 49–51 and 48–52 junctions (Supplementary Table S1). Multiplex reactions were prepared in triplicate using TaqMan Gene Expression Master Mix and amplified on a 7500 Fast Real-Time PCR System (Applied Biosystems). Quantification was done by using the comparative ΔΔCt method.[12]

Haplotyping

Haplotypes were constructed using intragenic and flanking microsatellite markers (Cy172-H32 and 104-sat;[2,3] D2S2113, D2S2604, D2S291, D2S2112 and D2S2111) as well as intragenic single-nucleotide polymorphisms (c.1827T>C, c.2583T>A, c.3972C>T, c.4008C>A, c.4068C>T, c.4950-37C>T, c.5831-16T>C and c.5922A>C). Control samples consisted of 20 anonymized, unrelated and non-consanguineous singleton families from a randomized population sample. Only the parents were considered for ascertainment of haplotype frequencies (offspring were used to determine allele phase), providing a total of 80 control chromosomes for analysis. Data were analysed with Arlequin 3.01 software using the haplotype inference option and by the estimation of allele frequency at the loci.[13]

RESULTS AND DISCUSSION

Mutation analysis

Direct sequencing of *DYSF* led to the detection of the novel mutation c.5492G>A, which was found in homozygosity in all patients except in patient III. This variant, which was not detected in 240 control alleles (Supplementary Figure S1), could be either an amino acid substitution (p.Arg1831Lys) or a splicing mutation, as it coincided with the last nucleotide of exon 49. The possible effect was predicted with the aid of the Genscan program (http://genes.mit.edu/GENSCAN.html) and ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), and the scores of normal and mutated donor splicing sequences were further calculated according to Shapiro and Senapathy.[14] These algorithms favoured exon 49 excision due to the abolishment of a donor splice site and/or the disruption of an ESE element responsive to SRp40.

Transcript analysis in muscle tissue and/or peripheral blood revealed a smaller fragment, present in all seven patients, which was found to be missing in the entire sequence of exon 49 (Δ49). The aberrantly spliced transcript (r.5404_5492del) determines a shift in the reading frame with subsequent peptide truncation (p.Gly1802ValfsX17).

Alternative splicing of exons 49, 50 and 51

In addition to the predominant Δ49 transcript, weak smaller bands were detected in muscle and leukocyte mRNA samples, both in patients and in controls. These were found to correspond to alternatively spliced products involving also exons 50 and 51 (Figures 1a and b). Quantitative analysis confirmed the presence of these transcripts, although with different expression levels in blood and muscle (Figure 1c). Comparison of expression levels across groups and specimen types showed that the Δ49/Δ50 isoform was more frequent in blood, in patients and in controls, whereas Δ49/Δ50/Δ51, an in-frame isoform, was detected mostly in control muscle.

Indeed, exons 50 and 51 seem to be amenable to modulation as suggested by the low splice probability scores of the donor and acceptor sites (according to Shapiro and Senapathy[14]). Modulation-prone splicing was first described in this gene for an in-frame Δ17 isoform, which appears to be tissue- and differentiation-specific.[10] More recently, Pramono *et al.*[11] described transcript variants with further in-frame splicing combinations, including the newly identified exons 5a and 40a, generated under either of the two promoters. Despite the maintenance of the reading frame, all of these variants were far more abundant in blood, as was observed in our case.

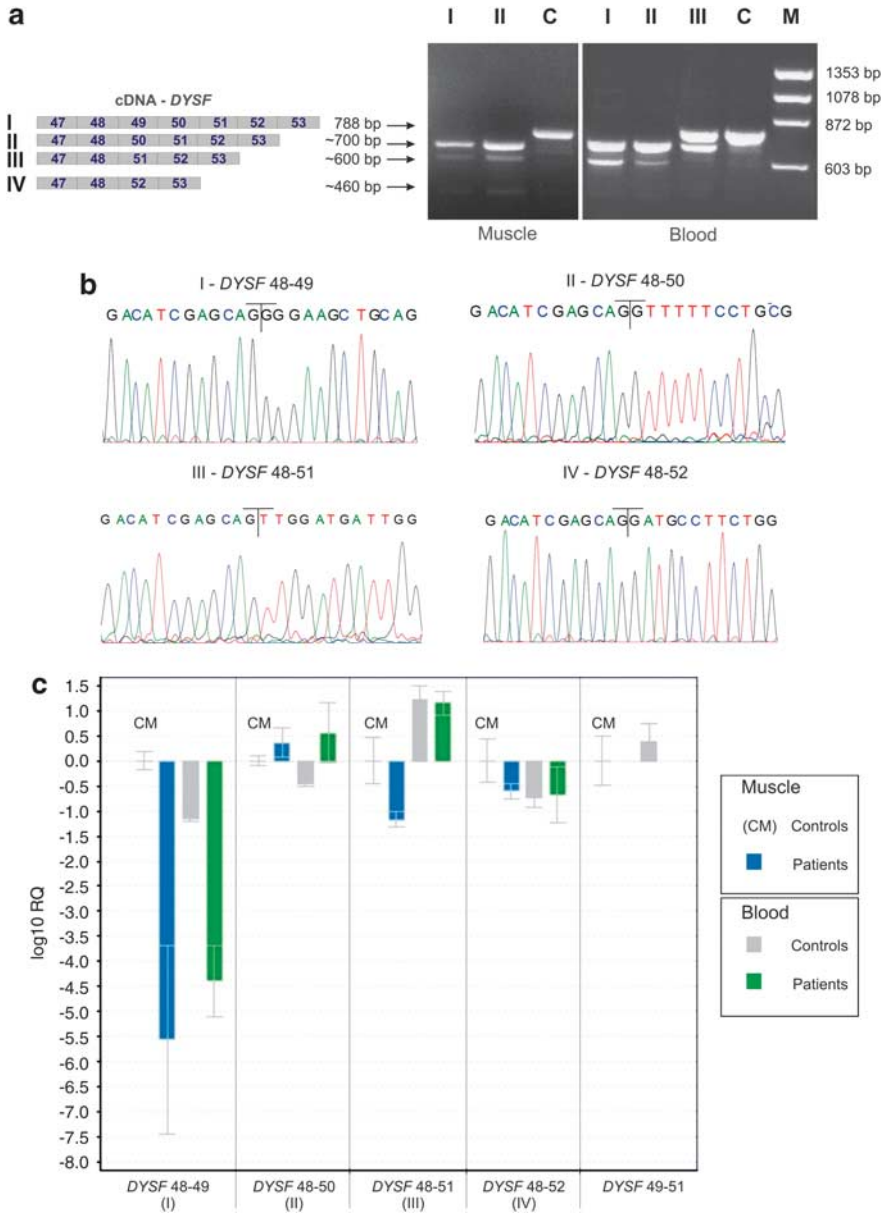

Figure 1 Dysferlin transcript analysis in the muscle and blood of patients and controls. (**a**) Conventional RT-PCR analysis of transcripts in patients (I, II and III) and a normal control (C). Grey bars represent the results observed by sequencing. (**b**) Electropherograms of junction sequences in the alternatively spliced RT-PCR products depicted in (**a**). (**c**) Quantitative real-time PCR using different Taqman probes designed to specifically detect junction fragments in normal (*DYSF* 48–49) and alternatively spliced transcripts (*DYSF* 48–50, 48–51, 48–52, 49–51). For Δ50 transcripts (*DYSF* 49–51), no signal was obtained in the patient samples. Quantification analysis was performed by the comparative ΔΔCt method using the muscle control samples (CM) as a calibrator.

Haplotype analysis

As all of the patients reside or have ancestry in a confined region in the northern interior part of Portugal, we tested the hypothesis of a founder effect for the c.5492G>A mutation. This mutation was found to be associated with the same *DYSF* gene haplotype in a stretch encompassing the gene and spanning approximately 1.4 cM (Figure 2). Sixty distinct haplotype blocks were distinguished in the 77 control chromosomes with informativity (Supplementary Table S2) and only one control chromosome shared the haplotype associated with the c.5492G>A mutation, thereby demonstrating its very low frequency (1.3%) in the population. Moreover, when analysis was extended to include the closest flanking extragenic markers D2S2604 and D2S291, none of the 77 control chromosomes presented the mutation-associated haplotype, thereby corroborating the hypothesis of a single mutational event leading to a founder effect. Previous reports on founder mutations in the *DYSF* gene include a nonsense mutation (p.R1905X) in the historically isolated Spanish town of Sueca and a missense mutation (p.R959W) in Italy.[8,15]

Concluding remarks

Although all our patients developed essentially a limb-girdle phenotype, some degree of heterogeneity was observed, especially at onset, with patient V initially presenting with distal muscle weakness in the lower limbs. Patient IV was being investigated by cardiology for

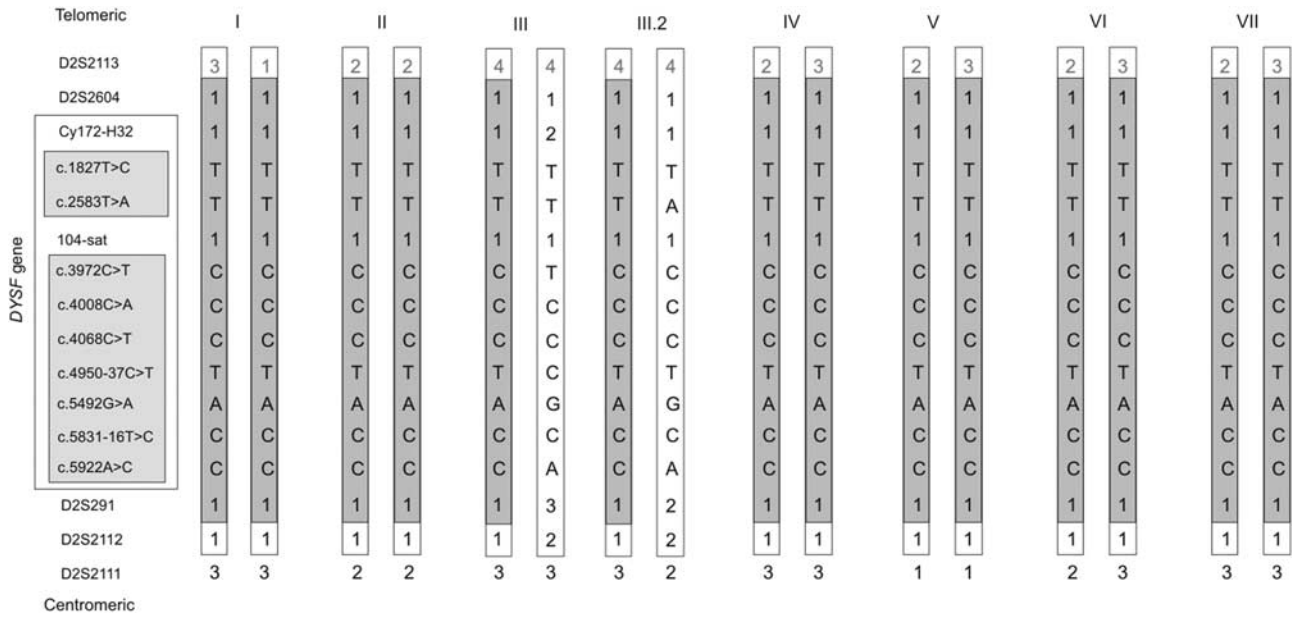

Figure 2 Haplotypes associated with the c.5492G>A mutation, detected in the studied patients. The mother of patient III is also shown (III-2). A full colour version of this figure is available at the *Journal of Human Genetics* journal online.

cardiac arrhythmia at the time of diagnosis, and only began to manifest proximal lower limb weakness at the age of 16.

Although splice mutation leakage and possibly alternative splicing may explain the varying degrees of severity, other modifying factors must necessarily determine the different manifesting patterns of the disease. Elucidation of the parallel and interacting mechanisms involved in membrane repair should provide insight into the clinical heterogeneity observed among dysferlinopathy patients with the same mutation, even on the same haplotype background, as reported here.

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PAPER V

LAMA2 gene analysis in a cohort of 26 congenital muscular dystrophy patients.

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Short Report

LAMA2 gene analysis in a cohort of 26 congenital muscular dystrophy patients

Oliveira J, Santos R, Soares-Silva I, Jorge P, Vieira E, Oliveira ME, Moreira A, Coelho T, Ferreira JC, Fonseca MJ, Barbosa C, Prats J, Aríztegui ML, Martins ML, Moreno T, Heinimann K, Barbot C, Pascual-Pascual SI, Cabral A, Fineza I, Santos M, Bronze-da-Rocha E. *LAMA2* gene analysis in a cohort of 26 congenital muscular dystrophy patients.
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Congenital muscular dystrophy type 1A (MDC1A) is caused by mutations in the *LAMA2* gene encoding laminin-α2. We describe the molecular study of 26 patients with clinical presentation, magnetic resonance imaging and/or laminin-α2 expression in muscle, compatible with MDC1A. The combination of full genomic sequencing and complementary DNA analysis led to the particularly high mutation detection rate of 96% (50/52 disease alleles). Besides 22 undocumented polymorphisms, 18 different mutations were identified in the course of this work, 14 of which were novel. In particular, we describe the first fully characterized gross deletion in the *LAMA2* gene, encompassing exon 56 (c.7750-1713_7899-2153del), detected in 31% of the patients. The only two missense mutations detected were found in heterozygosity with nonsense or truncating mutations in the two patients with the milder clinical presentation and a partial reduction in muscle laminin-α2. Our results corroborate the previous few genotype/phenotype correlations in MDC1A and illustrate the importance of screening for gross rearrangements in the *LAMA2* gene, which may be underestimated in the literature.

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Key words: congenital muscular dystrophy – gross deletion – *LAMA2* – laminin-α2 – MDC1A

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Congenital muscular dystrophy type 1A (MDC1A) is the most frequent form of congenital muscular dystrophy (CMD) in the European population, accounting for 30–50% of the cases (1–3). Major clinical symptoms include muscle weakness, raised serum creatine phosphokinase, no independent ambulation, cerebral white matter abnormalities and, in the most severe cases, respiratory insufficiency, which often leads to death in early childhood (4).

The *LAMA2* gene (MIM#156225), consisting of 65 exons and a 9.5-kb open reading frame, is implicated in MDC1A (5, 6). Two alternative transcripts have been described (accession numbers NM_000426 and NM_001079823); both isoforms are functional and encode the α2 chain of laminin-211 (α2-β1-γ1), laminin-221 (α2-β2-γ1) and laminin-213 (α2-β1-γ3) [nomenclature reviewed in (7)]. Laminin-211 trimer is predominantly expressed in skeletal muscle, cerebral white matter and Schwann cells and is a major component of the basal membrane (8). It binds to α-dystroglycan and the integrin-α7-β1 complex, establishing a connection between the cytoskeleton and the extracellular matrix [reviewed in (9)].

MDC1A patients usually have complete absence of laminin-α2 staining in muscle biopsies associated with the presence of truncating mutations. Patients presenting partial deficiency of laminin-α2 may also have mutations in *LAMA2*, or this deficit may be secondary to mutations in other genes acting in the α-dystroglycan glycosylation pathway [reviewed in (10)].

To date, 94 distinct mutations have been reported in the *LAMA2* locus-specific database (LSDB) (http://www.lovd.nl/LAMA2), the majority of which are small out-of-frame deletions (31.9%) and nonsense mutations (29.8%). Others include splice mutations (16.0%), missense substitutions (14.9%) and small duplications (7.4%). There is no noticeable mutational hotspot.

Since the identification of the *LAMA2* gene, a few reports have described a significant number of patients with mutations (11–14). However, the mutation detection rates were lower than could perhaps be expected considering the clinical presentation and/or total absence of laminin-α2 in muscle, used as selection criteria. This has been attributed to factors such as technical difficulties inherent to mutation screening in large genes, the inclusion of patients with other clinically indistinguishable forms of CMD, or the existence of heterozygous deletions/duplications that are not detected by standard polymerase chain reaction (PCR)-based screening methods. This study describes the molecular characterization of a group of 26 MDC1A patients. A total of 18 distinct *LAMA2* mutations were detected corresponding to 96% of the disease alleles. In particular, a single new large exonic deletion, which accounted for 10 disease alleles, is described in detail.

Materials and methods

Patients

The study included a total of 26 CMD cases from Portugal (patients 1–11, 13–17, and 22–26), Spain (patients 12 and 18–20) and Switzerland (patient 21) who met defined clinical criteria for MDC1A, compatible magnetic resonance imaging (MRI) anomalies and/or revealed changes in muscle laminin-α2 immunostaining (Table 1). The parents were screened, whenever possible, to confirm mutation allelism or homozygosity. None of the families were consanguineous. Informed consent was obtained for the molecular studies.

Table 1. Clinical–pathological data of the congenital muscular dystrophy patients

Patient	Sex	Age	Age of onset	Clinical presentation	Highest CPK (U/l)	Pattern/ progression of weakness	Best motor achievement	Contractures	Central nervous system involvement/ seizures	Magnetic resonance imaging	Laminin-α2 in muscle[a]
1	M	19 years	At birth	Hypotonia and feeding problems	1410	Severe generalized weakness and scoliosis	No motor milestones achieved	All joints	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
2	M	7 years	At birth	Hypotonia and poor spontaneous movements	2697	Generalized weakness with proximal predominance	Assisted trunk control	Knees and hips and limited ankle movements	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
3	F	7 years[b]	4 months	Hypotonia and areflexia	3265	Proximal weakness	Sat unsupported	No	No cognitive delay and no seizures	White matter changes and abnormal bilateral frontal gyration	Total absence
4	M	7 years	At birth	Hypotonia and feeding problems	1650	Generalized weakness with axial and proximal predominance and improvement in motor function	Sat unsupported	No	No cognitive delay and no seizures	White matter changes and abnormal occipital gyration	Total absence
5	M	7 years	At birth	Hypotonia	1156	Muscular weakness with facial and bulbar paresis and slight improvement in motor function	Sat unsupported	Knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
6	F	17 years	At birth	Hypotonia and arthrogryposis	nd	Generalized weakness with axial predominance	Good cephalic control and sat unsupported	Knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
7	M	13 years	2 months	Generalized hypotonia	840	Proximal weakness with mild progression, facial and bulbar paresis, severe scoliosis (>9 years), and nocturnal ventilation (>3 years)	Cephalic control and sat unsupported	Generalized	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
8	M	4 years	At birth	Hypotonia and neonatal asphyxia	3782	Muscular weakness with axial and proximal predominance	Cephalic control and moves hands towards and manipulates objects (not against gravity)	Bilateral *talipes equinus*	No apparent cognitive delay and no seizures	nd	Total absence

Table 1. Continued

Patient	Sex	Age	Age of onset	Clinical presentation	Highest CPK (U/l)	Pattern/ progression of weakness	Best motor achievement	Contractures	Central nervous system involvement/ seizures	Magnetic resonance imaging	Laminin-α2 in muscle[a]
9	M	11 years	At birth	Hypotonia and feeding problems	nd	Muscular weakness with axial and proximal predominance and scoliosis	Cephalic control, trunk control with brace, and handwrites	Elbows and knees	No cognitive delay and no seizures	nd[c]	Total absence
10	M	7 years	At birth	Hypotonia and poor spontaneous movements	2697	Muscular weakness with axial and proximal predominance	Sat unsupported	Elbows and knees	No cognitive delay and no seizures	nd	Total absence
11	M	24 years	3 years	Spastic paraparesis	838	Slowly progressive spastic paraparesis	Spastic gait	No	Slight cognitive delay and no seizures	White matter changes and no gyral abnormalities	Partial absence
12	M	3 years	At birth	Hypotonia and mild neonatal distress	1085	Generalized axial weakness and respiratory distress episodes	Sat unsupported	No	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
13	M	Died at 9 months	At birth	Hypotonia, hyporeflexia and bilateral *talipes equinus*	nd	Generalized axial hypotonia, *pectus excavatum*, and respiratory distress episodes	No motor milestones achieved	No	No apparent cognitive delay and no seizures	nd	Total absence
14	F	2 years	At birth	Hypotonia and feeding and respiratory problems	1999	Muscular weakness with axial and proximal predominance	Sat unsupported	No	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	nd
15	F	27 years	At birth	Hypotonia and arthrogryposis	nd	Proximal weakness and scoliosis	Sat unsupported	Elbows and knees	No cognitive delay and seizures (generalized epilepsy since age 2 years)	White matter changes and abnormal bilateral frontal gyration	Total absence
16	F	4 years	*In utero*	Hypotonia	4460	Muscular weakness with facial diparesis, scoliosis, severe muscle atrophy, and under BIPAP	Plays with hands	Generalized	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
17	M	2 years	At birth	Hypotonia and bilateral *talipes equinus*	3866	Muscular weakness with facial diparesis	Sat unsupported	No	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
18	F	7 years	At birth	Generalized hypotonia and areflexia	5080	Severe and generalized weakness, hypotonia, scoliosis, *equinus varus* feet, and under BIPAP	No trunk or cephalic control	Generalized	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence

Table 1. Continued

Patient	Sex	Age	Age of onset	Clinical presentation	Highest CPK (U/l)	Pattern/ progression of weakness	Best motor achievement	Contractures	Central nervous system involvement/ seizures	Magnetic resonance imaging	Laminin-α2 in muscle[a]
19	M	20 years	At birth	Generalized hypotonia and areflexia	3264	Muscular weakness with axial and proximal predominance and scoliosis	Independent gait	Elbows and ankles	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	nd
20	F	18 years[b]	2 years	Generalized hypotonia and areflexia	593	Generalized weakness and no scoliosis	Independent gait	No	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Partial absence
21	F	4 years	At birth	Hypotonia and hydronephrosis (left kidney) grade III–IV	6987	Scoliosis and plagiocephalus	Assisted trunk control	No	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
22	M	9 months	At birth	Hypotonia, poor spontaneous movements and feeding problems	4706	Muscular weakness with proximal predominance and slight improvement in motor function	Incomplete cephalic control	Elbows, knees and ankles	No apparent cognitive delay and no seizures	No changes[d]	Total absence
23	F	2 years	At birth	Hypotonia and feeding and respiratory problems	1700	Muscular weakness with axial and proximal predominance	Sat unsupported	Elbows and knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
24	M	3 years	At birth	Hypotonia and feeding problems	1770	Muscular weakness with proximal predominance and hip congenital luxation	Cephalic control and assisted trunk control	Knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
25	F	3 months	*In utero*	Generalized hypotonia with proximal predominance and feeding problems	5530	Muscular weakness with axial and proximal predominance	No cephalic control	Knees and ankles	No apparent cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
26	M	1 year	*In utero*	Hypotonia and arthrogryposis (hands and feet)	5615	Severe muscular weakness with proximal predominance and facial diparesis	Sat unsupported	Knees	No apparent cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence

BIPAP, bi-level positive airway pressure; CPK, creatine phosphokinase; F, female; M, male; nd, not done.
[a]Immunohistochemical staining with NCL-merosin monoclonal antibody (Novocastra), recognizing the 300-kDa amino-terminus fragment.
[b]Lost to follow-up (patient 3 at age 4 years and patient 20 at age 16 years).
[c]White matter changes observed on computerized tomography scan.
[d]Tested in the neonatal period.

Genomic DNA analysis

Genomic DNA (gDNA) was extracted from peripheral blood by the salting-out method (15). All 65 exons of *LAMA2* were amplified by PCR using intronic M13-tailed primers. Amplicons were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH) and sequenced with M13 universal primers and BigDye™ Terminator Cycle Sequencing Kit V1.1 (Applied Biosystems, Foster City, CA). Products were resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Mutation analysis was aided by SEQSCAPE V2.5 software (Applied Biosystems) using the complementary DNA (cDNA) reference sequence NM_000426.3. Population screening was carried out in 150 anonymized control samples by single-stranded conformation analysis (SSCA), direct sequencing or fragment size analysis.

Bioinformatics

The effect of splicing mutations was predicted with the aid of the GENSCAN program (http://genes.mit.edu/GENSCAN.html) using segments of the *LAMA2* genomic reference sequence NC_000006:129246035–129879404. The scores of normal and mutated splice sites were calculated according to Shapiro and Senapathy (16). For mutations predictably inducing amino acid alterations, phylogenetic conservation analysis was performed with laminin-α2 protein sequences from several organisms (Data S1, supporting information online), aligned using the software CLUSTALX version 1.83 (17).

cDNA analysis

Total RNA was extracted from muscle or skin biopsies using the Versagene RNA Purification kit (Gentra, Minneapolis, MN). Reverse transcription–PCR (RT-PCR) was performed using Superscript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) and previously described primer sets (5). For the gross deletion, the specific primers designed to amplify exons 54–58 were c.54F – 5′GGTGTTACCAAAGGATGTTCCC3′ and c.58R – 5′CAGCATTTTTGAAGGACACAGG3′. Products were sequenced as described above.

Haplotyping

Typing was performed using short tandem repeats (STRs) flanking the *LAMA2* locus: D6S1715, D6S407, D6S1620, D6S1705 and D6S1572; and intragenic single nucleotide polymorphisms (SNPs): c.3174+38A>G, c.5466G>A, c.5502A>G, c.5727-24_5727-21delinsACTG, c.6237G>A, c.6707+37T>C, c.7760T>C, c.7830C>G, c.7845G>A and c.*190_192dupATA.

Southern blot analysis

gDNA from patients 1 and 12 was digested with *Acc*I (New England Biolabs, Beverly, MA), electrophoresed on a 0.8% agarose gel and transferred to a GeneScreen Plus® membrane (Perkin Elmer, Waltham, MA). This was hybridized with a cDNA probe recognizing exons 54–58 labelled with fluorescein (Gene Images Random Prime Labelling Module, GE Life Sciences, Piscataway, NJ). After antibody incubation, the membrane was washed at 60°C once in 1× SSC/0.1% SDS and twice in 0.5× SSC/0.1% SDS for 15 min each and developed using CDP-Star detection reagent (GE Life Sciences).

Long-range and deletion-specific PCR

Exon 56 deletion breakpoints were determined by amplification of gDNA using the BIO-X-ACT™ Long DNA Polymerase kit (Bioline, Taunton, MA) and the following primers complementary to exons 55 and 57: c.55F – 5′CTAGGAGAAAACGAAGGCAGAC3′ and c.57R – 5′TCAACTGTCAGGTTTTGCATG3′. Resolved PCR fragments were purified using the MinElute PCR Purification Kit (Qiagen, Germantown, MD) and sequenced with the internal primer g.INT55F–5′CTCTACAAGCCAGCAATTCCAC3′. A rapid deletion-specific PCR was developed using the following primers: g.INT55-F2 – 5′ATCAGCTGGAGAACAGAGAGGC3′ and g.INT57-R – 5′-GTTTCAGTGGCTGATTCTTAGAGTTTC3′. Because this fragment only amplifies in deletion-positive individuals, it was multiplexed with an internal control (*DYSF* exon 20).

Results

Patients

Table 1 summarizes the clinical, neuroradiological and neuropathological data. Except for patients 11 and 20, all had symptoms since infancy. Patient 11 had a peculiar clinical picture with progressive spastic paraparesis followed later by a slowly progressive neuropathy. Pregnancy and delivery had been uneventful. Nerve conduction studies (at the age of 14 years) revealed delayed F waves. While myopathy was not clinically evident,

variation in fibre size was observed in the muscle biopsy. Patient 20 also had a milder phenotype with myopathy and clinically a mild neuropathy. MRI, performed in 22 patients, revealed white matter changes in all but one patient (tested in the neonatal period where these changes are not always visible). In another patient, this was observed in the computerized tomography scan. In three patients, cerebral changes included abnormal gyration. Seizures were reported only in the oldest patient. Twenty-two patients presented total absence of laminin-α2 staining in muscle, two patients presented partial absence and in a further two patients, this was not determined.

Identification of *LAMA2* gene mutations

Causative mutations were identified in all 26 patients (Table 2). Only one mutant allele was identified in patients 19 and 24; the mother of patient 24 carried the mutation, while the parents of patient 19 were not tested.

A total of 18 different mutations were identified, 14 of which have not been reported previously. Distribution by type was as follows: four nonsense mutations (22%), four duplications (22%), five deletions (28%), three splicing mutations (17%) and two missense mutations (11%). The two most frequent mutations were c.3085C>T (11 disease

Table 2. Mutations identified in the *LAMA2* gene

Patient	Mutations[a,b]	Gene location	Effect on mRNA	Predicted polypeptide change[c]	Reference
1 and 15	c.[7750-1713_7899-2153del]+ [7750-1713_7899-2153del]	Introns 55–56	Frameshift	p.Ala2584HisfsX8	This report
2 and 10	c.[3976C>T]+[3976C>T]	Exon 27	PTC	p.Arg1326X	(13)
3, 13, and 18	c.[3085C>T]+[3085C>T]	Exon 22	PTC	p.Arg1029X	(13)
4	c.[4739dupG]+ [7490_7493dupAAGA]	Exon 33 Exon 54	Frameshift Frameshift	p.Leu1581ProfsX5 p.Asp2498GlufsX4	This report This report
5	c.[1854_1861dupACGTGTTC]+ [1854_1861dupACGTGTTC]	Exon 13	Frameshift	p.Leu621HisfsX7	(13)
6	c.[8244+1G>A]+ [7750-1713_7899-2153del]	Intron 58 Intron 55–56	Splicing Frameshift	p.Pro2693ValfsX12 p.Ala2584HisfsX8	This report This report
7	c.[363C>A]+ [7750-1713_7899-2153del]	Exon 3 Intron 55–56	PTC Frameshift	p.Tyr121X p.Ala2584HisfsX8	This report This report
8	c.[4318C>T]+ [4739dupG]	Exon 30 Exon 33	PTC Frameshift	p.Gln1440X p.Leu1581ProfsX5	This report This report
9, 14, and 23	c.[3085C>T]+ [7750-1713_7899-2153del]	Exon 22 Intron 55–56	PTC Frameshift	p.Arg1029X p.Ala2584HisfsX8	(13) This report
11	c.[1854_1861dupACGTGTTC]+ [3832G>T]	Exon 13 Exon 26	Frameshift Missense	p.Leu621HisfsX7 p.Gly1278Cys	(13) This report
12	c.[1854_1861dupACGTGTTC]+ [7750-1713_7899-2153del]	Exon 13 Intron 55–56	Frameshift Frameshift	p.Leu621HisfsX7 p.Ala2584HisfsX8	(13) This report
16	c.[3085C>T]+ [5234+1G>A]	Exon 22 Intron 36	PTC Splicing	p.Arg1029X p.Val1765SerfsX21	(13) This report
17	c.[3976C>T]+ [8776_8792del]	Exon 27 Exon 62	PTC Frameshift	p.Arg1326X p.Thr2926TrpfsX14	(13) This report
19	c.[1798_1800delGGA]+ [=]	Exon 13 ?	Codon del ?	p.Gly600del ?	This report ?
20	c.[8613dupC]+ [412T>C]	Exon 61 Exon 4	Frameshift Missense	p.Ser2872HisfsX34 p.Tyr138His	This report This report
21	c.[2049_2050delAG]+ [6993-2A>C]	Exon 14 Intron 49	Frameshift Splicing?	p.Arg683SerfsX20 ?	(11) This report
22 and 25	c.[5234+1G>A]+[5234+1G>A]	Intron 36	Splicing	p.Val1765SerfsX21	This report
24	c.[3085C>T]+ [=]	Exon 22 ?	PTC ?	p.Arg1029X ?	(13) ?
26	c.[8443_8450delACAGTTCA]+ [8443_8450delACAGTTCA]	Exon 60	Frameshift	p.Thr2815AlafsX11	This report

cDNA, complementary DNA; del, deletion; PTC, premature termination codon.
[a]Mutations described according to Human Genome Variation Society nomenclature (23).
[b]cDNA reference sequence with accession number NM_000426.3.
[c]Deduced from the changes detected at the genomic or cDNA level.

alleles) and c.7750-1713_7899-2153del (10 disease alleles) together accounting for 42% of the mutant alleles (21/50).

The in-frame deletion c.1798_1800delGGA (p.Gly600del) and the missense variants c.412T>C (p.Tyr138His) and c.3832G>T (p.Gly1278Cys) were not detected in 300 normal alleles. Additionally, protein alignments indicated that the missense mutations affected highly conserved amino acids (Data S1, supporting information online). The single residue deletion (p.Gly600del), although coinciding with a known missense polymorphism in humans and between species (p.Gly600Arg), may have a detrimental effect on protein folding and/or function. The GENSCAN program predicted that the splice mutations c.5234+1G>A and c.8244+1G>A promote skipping of exons 36 and 58, respectively, whereas c.6993-2A>C should disrupt the acceptor splice site with subsequent use of a cryptic splice site located 7 bp into exon 50. Transcript analysis in patients 6 and 16 confirmed the *in silico* prediction for the former mutations, showing full skipping of the neighbouring preceding exons (data not shown). However, no muscle or fibroblast specimens from patient 21 were available for cDNA analysis to confirm the effect of c.6993-2A>C.

A total of 55 polymorphisms were also identified in the course of this study, 22 of which had not been reported previously (Data S2, supporting information online).

All the variants detected in this study were submitted to the *LAMA2* LSDB (http://www.lovd.nl/LAMA2).

Characterization of a new gross deletion

A large genomic deletion was first suspected in patient 1 when exon 56 failed to amplify. Further evidence was provided by haplotype analysis in patient 12, seen to be hemizygous for three informative SNPs in exon 56 (Fig. 1a). cDNA analysis in patients 1 and 12 revealed the presence of a smaller fragment missing the entire exon 56 (Fig. 1b). Southern blotting and hybridization indicated that the genomic deletion was approximately 5 kb long (Fig. 2a). This was confirmed by long-range PCR (Fig. 2b), which was used to further delineate the deletion endpoints. Sequencing revealed the absence of exon 56 plus a significant part of the flanking introns (4987 bp) characterized as c.7750-1713_7899-2153del (Fig. 2c). If translated, this frame shift predictably originates a truncated polypeptide (p.Ala2584HisfsX8).

The deletion-specific PCR that was developed enabled rapid screening of additional individuals. In all, 8 patients presented the c.7750-1713_7899-2153del mutation (2 homozygous and 6 heterozygous), and 10 family members were found to be carriers (data not shown).

Discussion

We describe the nature and frequency of *LAMA2* mutations in a group of 26 CMD patients. In this relatively large cohort, the high mutation detection rate of 96% (50/52 disease alleles) was achieved by direct gDNA sequencing or by combining this with RT-PCR analysis in cases where

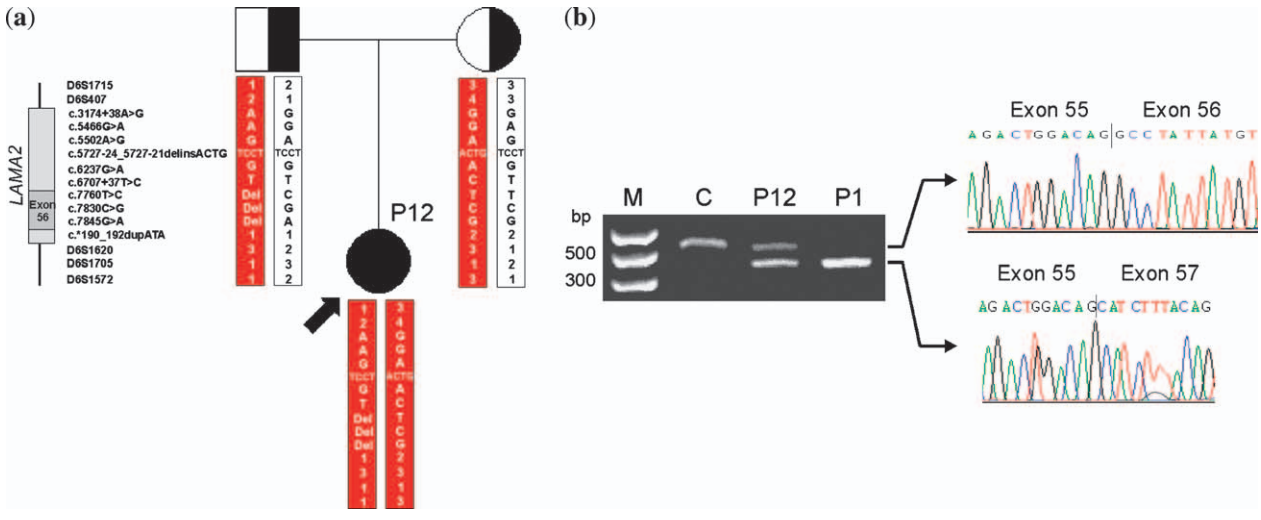

Fig. 1. (**a**) Pedigree of family/patient 12 showing analysis with microsatellite markers around the *LAMA2* locus and intragenic single nucleotide polymorphisms. Haplotyping suggested the presence of a heterozygous deletion of exon 56. (**b**) Complementary DNA analysis of *LAMA2* transcripts by Reverse transcription–polymerase chain reaction amplification of exons 54–58. Results revealed a smaller fragment missing the entire exon 56 in patients 1 and 12. C, control; M, molecular weight marker; P, patient.

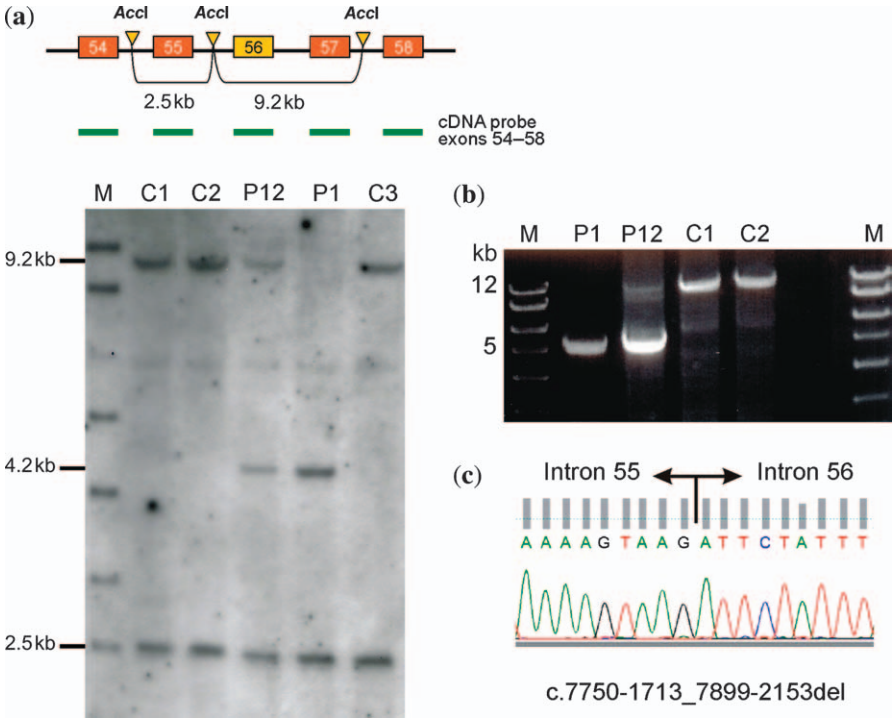

Fig. 2. (**a**) The size of the genomic deletion was determined by Southern blotting and hybridization using a complementary DNA probe encompassing exons 54–58. (**b**) Long-range PCR amplification of the genomic region between exons 55 and 56. (**c**) The resulting PCR fragments were sequenced revealing the deletion of exon 56 and part of the flanking intronic sequences (4987 bp). C, control; M, molecular weight marker; P, patient; PCR, polymerase chain reaction.

only one mutation had been detected. In patients 19 and 24, no adequate specimens were available for mRNA studies, and these were the only two cases where a single causal mutation was identified.

Similar previous studies, in a significant number of patients, have reported lower mutation detection rates ranging from about 60% to 80% (11, 12, 14). Because *LAMA2* is a large gene, most groups applied indirect mutation scanning techniques such as SSCA or denaturing high performance liquid chromatography combined with RT-PCR. However, these approaches may not detect all the mutations that are possibly present. Pegoraro et al. (11) also described the use of the protein truncation test (PTT) applied to *LAMA2* mutation detection. Because the majority of the mutations described in this gene cause premature translation termination, the use of this technique allowed the identification of 80% of the mutations in their cohort. Incomplete sensitivity was attributed to failure in the amplification of the mutated allele, the presence of mutations in the primer binding site or the fact that PTT is limited to the detection of nonsense mutations and mutations that alter the reading frame – small in-frame deletions/duplications or missense mutations – could be missed (11).

Almost 30% of the mutations described to date in the *LAMA2* gene are of the nonsense type. In our group of patients, these were also found to be the most frequent, comprising 18 of the 50 disease alleles (36%); however, c.3085C>T (p.Arg1029X) alone accounted for 11 of these.

A new 5-kb genomic deletion, encompassing exon 56, was also seen to be very frequent in this cohort (20% mutated alleles). To our knowledge, this is the first fully characterized genomic deletion described in *LAMA2*. A previous report documents an exon 56 deletion detected by RT-PCR (12); however, it remains uncertain that the genomic defect is the same. Although this mutation was detected in Portuguese and Spanish patients, no common haplotypes were found using STRs flanking the *LAMA2* locus (data not shown). These preliminary results point towards either a single ancient mutational event that has suffered genetic drift or a recurrent event that has occurred on different genetic backgrounds.

Quantitative assays such as multiple ligation-dependent probe amplification should improve mutation detection rates, enabling the identification of other gross rearrangements, which are probably underestimated in the *LAMA2* gene. Such changes could account for a reasonable number of MDC1A alleles and should therefore be

considered in the diagnostic setting, especially in patients with a single mutation identified by routine screening methods. An important implication in the failure to detect gross heterozygous deletions is that the identification of a single variant sequence may be erroneously interpreted as a homozygous mutation if masked by a coincident exonic deletion. Moreover, the absence of the variant in one of the parents may be mistaken as evidence of a *de novo* occurrence.

As expected, given the patients' inclusion criteria for the molecular study, the majority of mutations in our series were predictably truncating (94% of the mutated alleles). In line with previous observations (11), strict genotype/phenotype correlations were difficult to establish in such severely affected patients. Nevertheless, patients 11 and 20, who were compound heterozygous for a truncating and a missense mutation, presented partial deficiency of laminin-α2 in muscle. The clinical phenotype of these patients was also clearly milder with a later age of onset (2–3 years) when compared with the other patients (birth to 4 months). They achieved the ability to walk unsupported, which is rarely observed in MDC1A. Patient 19 has also kept independent ambulation till the age of 17 years; in this case, a single in-frame deletion was detected, but laminin-α2 expression in muscle has not been accessed. These patients may belong to a clinical subform of CMD with primary partial laminin-α2 deficiency, as has been suggested previously (18).

Our results have enabled the reliable determination of carrier status in additional family members and a more accurate prenatal diagnosis in several pregnancies. The importance of extensive molecular characterization is increasingly recognized also in light of the new therapeutic strategies that are currently being developed, such as the use of antisense oligonucleotides (19) and drug-induced stop codon read-through in MDC1A patients (20). To this end, mutations need to be contextualized. In those inducing premature termination codons, the extent of nonsense-mediated mRNA decay needs to be assessed by real-time RT-PCR (20, 21, 22) because mRNA stability influences the efficacy of transcript rescue. It is thus foreseeable that future demand on diagnosis will include qualitative and quantitative transcript analysis to identify the cases amenable to the different targeted therapies.

Supporting information

Data S1. Partial laminin-α2 protein sequences alignment.
Data S2. New polymorphisms detected in *LAMA2*.
Supporting information are available as part of the online article at http://www.blackwell-synergy.com

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PAPER VI

Reviewing large LAMA2 deletions and duplications in congenital muscular dystrophy patients.

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Research Report

Reviewing Large LAMA2 Deletions and Duplications in Congenital Muscular Dystrophy Patients

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Abstract.

Background: Congenital muscular dystrophy (CMD) type 1A (MDC1A) is caused by recessive mutations in laminin-α2 (*LAMA2*) gene. Laminin-211, a heterotrimeric glycoprotein that contains the α2 chain, is crucial for muscle stability establishing a bond between the sarcolemma and the extracellular matrix. More than 215 mutations are listed in the *locus* specific database (LSDB) for *LAMA2* gene (May 2014).

Objective: A limited number of large deletions/duplications have been reported in *LAMA2*. Our main objective was the identification of additional large rearrangements in *LAMA2* found in CMD patients and a systematic review of cases in the literature and LSDB.

Methods: In four of the fifty-two patients studied over the last 10 years, only one heterozygous mutation was identified, after sequencing and screening for a frequent *LAMA2* deletion. Initial screening of large mutations was performed by multiplex ligation-dependent probe application (MLPA). Further characterization implied several techniques: long-range PCR, cDNA and Southern-blot analysis.

Results: Three novel large deletions in *LAMA2* and the first pathogenic large duplication were successfully identified, allowing a definitive molecular diagnosis, carrier screening and prenatal diagnosis. A total of fifteen deletions and two duplications previously reported were also reviewed. Two possible mutational "hotspots" for deletions may exist, the first encompassing exons 3 and 4 and second in the 3' region (exons 56 to 65) of *LAMA2*.

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Conclusions: Our findings show that this type of mutation is fairly frequent (18.4% of mutated alleles) and is underestimated in the literature. It is important to include the screening of large deletions/duplications as part of the genetic diagnosis strategy.

Keywords: LAMA2, congenital muscular dystrophy, large deletion, large duplication, review

INTRODUCTION

LAMA2-related dystrophy (LAMA2-RD) collectively gathers two distinct clinical entities: the classical phenotype with congenital onset known as MDC1A, and a milder limb-girdle type muscular dystrophy with onset during childhood (late-onset or "ambulant" LAMA2-RD) [reviewed by 1, 2]. As foreseeable by this designation, these entities are caused by recessive mutations in *LAMA2* gene located on chromosome 6q22-23 and spanning 65 exons [3, 4]. This gene codes for the α2 chain of laminin-211, an extracellular glycoprotein expressed in the basal membrane of striated muscles, peripheral nerves, brain and trophoblast [5–7]. The interaction of laminin-211 with cell-surface receptors such as α-dystroglycan and integrin (mainly α7β1 in adult skeletal muscle) explains its major relevance in the overall extracellular architecture, integrity and cell adhesion [reviewed by 8].

MDC1A represents the most frequent form of CMD in western countries, accounting for 30 to 50% of cases [9]. Typical clinical features includes severe hypotonia associated with muscle weakness manifesting at birth or during early infancy, proximal joint contractures, elevated creatine kinase (CK) levels, cerebral white matter abnormalities and delayed motor milestones with affected children usually not achieving independent ambulation [10–12]. Feeding problems and respiratory insufficiency are commonly reported complications often requiring gastrostomy and/or artificial ventilator support [13, 14].

Other features such as cardiac involvement, a sensory and motor demyelinating neuropathy, epilepsy and mental retardation have been also documented in some forms of LAMA2-RD [15–19].

An important diagnostic aspect is that skeletal muscle biopsies from these patients have changes in laminin-α2 expression detected by immunohistochemical (IHC) analysis [10]. However, there is a recent report of a muscular dystrophy patient with apparently normal laminin-α2 IHC expression and having mutations in *LAMA2* gene [20].

Milder LAMA2-RD cases have been reported in the past few years expanding the phenotypic spectrum of the disease [21]. These patients have slower disease progression and acquire independent locomotion, and are usually associated with a partial expression of laminin-α2 [22–26].

More than 375 distinct sequence variants (215 of them with known clinical relevance) have been reported in the LSBD for *LAMA2* gene (http://www.dmd.nl/LAMA2, data accessed May 2014). Pathogenic changes include small deletions/insertions (34.9%), nonsense mutations (25.1%), changes affecting splicing (25.6%) and also missense substitutions (12.1%). In spite of the relevant amount of mutational data available there is still a limited number (2.3%) of large deletions and duplications reported in this gene. The initial suspicion of a large *LAMA2* deletion (which was predicted to include exons 23 to 56) was identified by protein truncation test in the work of Pegoraro and collaborators [27]. The first fully characterized large deletion in *LAMA2*, corresponds to an out-of-frame deletion of exon 56 (c.7750-1713_7899-2154del), which has been proven to be one of the most frequent pathogenic variants detected in Portuguese MDC1A patients [28].

One of the main objectives of this work was to describe additional novel pathogenic deletions and duplications associated with the *LAMA2* gene, identified in our cohort of CMD patients. Moreover, a systematic review of all cases with large deletions/duplications reported in the literature and mutation databases is presented. Our findings showed that this type of mutation is fairly frequent and is underestimated in the literature, reinforcing the importance to screen large deletions/duplications in *LAMA2* gene as part of the genetic diagnosis strategy.

MATERIAL AND METHODS

Patients

Over the last 10 years (2004-2014) our group performed genetic studies in 94 CMD patients. Mutations in genes related with CMD were identified in 68% ($n = 64$) of these patients. The majority of these patients have *LAMA2* mutations ($n = 52$) and were referred for molecular studies due to changes in muscle laminin-α2 detected by IHC analysis and/or compatible white matter anomalies detected by magnetic resonance imaging (MRI). In four patients of this cohort only

Table 1
Clinical data of CMD patients with novel large deletions and duplications in *LAMA2*

Patient	Sex	Age*	Age of onset	Clinical presentation	Highest CK (IU/l)	Pattern/ progression of weakness	Best motor achievement	Contractures	Central nervous system involvement/ seizures	Magnetic resonance imaging	Laminin-α2 in muscle
P1 (P19 in [28])	M	20 yr	At birth	Generalized hypotonia and areflexia	3264	Muscular weakness with axial and proximal predominance and scoliosis	Independent ambulation	Elbows and ankles	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	nd
P2 (P24 in [28])	M	3 yr	At birth	Hypotonia and feeding problems	1770	Muscular weakness with proximal predominance and hip congenital luxation	Cephalic control and assisted trunk control	Knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
P3	M	2 yr	4 mo	Hypotonia and muscular weakness	7644	Muscular weakness with axial and proximal UL predominance	Cephalic control and assisted trunk control	Knees, ankles and rigid spine	No cognitive delay and no seizures	White matter changes	nd
P4	M	8 mo	At birth	Generalized hypotonia	4400	Muscular weakness with proximal UL predominance	Cephalic and trunk control	Discrete equinus	No cognitive delay and no seizures	nd	Partial deficiency

CK-creatine phosphokinase; M- male; mo- months; nd- not determined; P- patient; UL- upper limbs; yr- years; *- age at last clinical follow-up.

one heterozygous mutation was detected upon *LAMA2* genomic sequencing (described in Table 1). In these patients we conducted screening of large deletions and duplications in the *LAMA2* gene. This research was approved by the ethics committee from Hospital Centre of Porto (CHP).

LAMA2 gene analysis

Genomic DNA (gDNA) was obtained from peripheral blood using the salting-out method [29]. *LAMA*2 gene sequencing was done according to [28], which comprised all coding and adjacent intronic sequences of *LAMA*2. Variants were described according to the Human Genome Variation Society (HGVS) guidelines for mutation nomenclature (version 2.0) [30] and using the cDNA reference sequence with accession number NM_000426.3.

MLPA analysis

Screening for deletions and duplications in *LAMA2* gene was performed by multiplex ligation-dependent probe application (MLPA) technique using two sets of probe mixes (P391-A1 and P392-A1) from MRC-Holland (Amsterdam, the Netherlands). These probe mixes contain one probe for each exon of the gene with the exception of exons 18, 44 and 48. Two probes are present for exon 1, 2, 4 and 65 and three probes for exon 56. Also, probemix P391 contains 9 reference probes and P392 contains 8 reference probes detecting different genomic regions. For the MLPA procedure, 150 ng gDNA was used for each patient and normal control samples. Amplification products were subsequently separated by capillary electrophoresis on an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, CA). Data analysis was conducted using GeneMarker Software V1.5 (SoftGenetics LLC, State College, PA). Population normalization method was selected and data was plotted using probe ratio.

Southern blot

gDNA samples from patient P3, respective parents and normal controls were digested with *BglI* (NewEngland Biolabs, Beverly, MA), resolved on a 0.7% agarose gel and vacuum transferred to a GeneScreen Plus membrane (Perkin Elmer, Waltham, MA) using a saline method. A cDNA probe recognizing exons 2–4 was prepared using digoxigenin (DIG) DNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA) and incubated overnight using the Easy Hyb Buffer (Roche Applied Science). The membrane was washed at 60°C in 1xSSC (Saline-Sodium Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) (Sigma-Aldrich, St. Louis, MO) and twice in 0.5xSSC/0.1% SDS for 15 min each. Subsequently, the membrane was prepared with DIG Wash and Block Buffer Set (Roche Applied Science), incubated with Anti-DIG-AP conjugate (Roche Applied Science), and the DIG-labeled probe detected with ready-to-use CDP-Star (Roche Applied Science).

LAMA2 cDNA analysis

cDNA studies were carried out in patient P2. Total RNA was extracted from patient and control muscle biopsy samples using the PerfectPure RNA Fibrous Tissue kit (5 PRIME, Germany) and converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). *LAMA2* transcripts were subjected to PCR amplification using specific primers for the region corresponding to exons 27–32 (27F- 5′AAATTTCATGCGACAAAGCAGG; 32R-5′GCTTGCAGCCGTCACACTTC). Resulting PCR products were purified and sequenced as described before.

Long-range PCR

The deletion breakpoints encompassing exon 3 (patient P3) and also exon 17 (patient P1) were determined by amplification of gDNA using the BIO-X-ACT Long DNA Polymerase kit (Bioline, Taunton, MA). Specific primers were designed for each case; complementary to intron 2 and exon 4, for the deletion of exon 3 (2i-F:5′ACAAAGCCTGATGGAGG GAAAC; 4-R:5′AAAGCGTTAGGCACTCCGTGTC) and complementary to regions in exons 16 and 18, for the deletion of exon 17 (16F:5′-TTGGTCATG CGGAGTCCTG; 18R:5′ TGGCACGTTGGGC-TAAAGC). Resolved PCR fragments were purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA), and subsequently sequenced.

RESULTS

Novel large deletions and duplications

We previously reported a large deletion encompassing exon 56 of the *LAMA2* gene which is relatively frequent in our laboratory patient cohort (present in 23% of our cases) [28]. This initial study suggested that it is clinically relevant to screen this type of mutations in CMD patients, and indirectly drove the development of a MLPA commercial kit for *LAMA2* gene (P391-A1

and P392-A1 from MRC-Holland). As part of this work we initially assessed the effectiveness of the MLPA kit using previously genotyped DNA samples from our patient cohort. Patients presenting homozygous and heterozygous deletions encompassing *LAMA2* exon 56 were tested. MLPA technique successfully detected this mutation in homo- and heterozygous states (Supplementary data I).

Four patients are presented in detail in this work; P1 and P2 were previously described in the literature as P19 and P24, respectively in [28] while two additional patients (P3 and P4) were more recently referred for *LAMA2* gene analysis. All of these patients had incomplete molecular characterization: only one heterozygous mutation was detected by genomic sequencing and thus the molecular defect in the other *LAMA2* allele remained unknown. MLPA technique was performed in DNA samples from these patients to tentatively identify the second pathogenic mutation in *LAMA2*.

Patient P1 presents a congenital muscular dystrophy (neonatal onset) and remained ambulatory until the age of 17 years. In this patient a heterozygous codon deletion (c.1798_1800del, p.Gly600del) was initially detected in *LAMA2*. MLPA analysis for this patient revealed reduced hybridization of one probe corresponding to exon 17 (Fig. 1A), compatible with the presence of a heterozygous deletion involving this exon. By reviewing genomic sequencing data we excluded a potential sequence change which could compromise the affinity of the MLPA probe. A long-range PCR experiment was performed using primers designed to bind regions presumably not involved in the deletion (exons 16 and 18). Upon amplification, two PCR products were detected in the patient, whereas the experimental control had a single band (data not shown). Sequencing across the deletion breakpoint revealed that part of intron 16 is joined to intron 17, corresponding to the loss of ~5.3Kb that spans exon 17 (Fig. 1B). This novel large mutation, c.2322+259_2450+2037del, predictably causes frame-shifting. DNA samples from the patient's parents were unavailable for study.

Patient P2 was referred for molecular study at 3 years of age having a typical MDC1A phenotype with absence of laminin-α2 in IHC analysis of muscle. The heterozygous nonsense mutation c.3085C>T (p.Arg1029*) was the only pathogenic sequence change detected by sequencing the *LAMA2* gene. A large heterozygous duplication encompassing exons 28 and 29 was identified in patient P2 by MLPA (Fig. 2A). cDNA studies performed in the

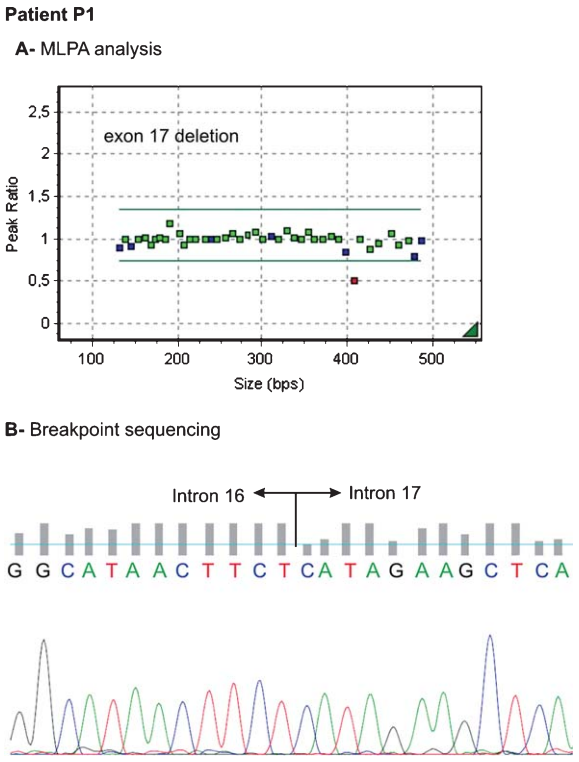

Fig. 1. Results obtained for patient P1. (A) MLPA technique showing reduction of one probe (0.5 peak ratio) corresponding to exon 17 of the *LAMA2* gene. (B) Sequencing electropherogram of a fragment (obtained from long-range PCR) that enabled the identification of the deletion breakpoint, corresponding to the loss of ~5.3 Kb.

patient revealed a normal transcript together with other abnormal PCR products (Fig. 2B). These include one out-of-frame transcript resulting from the contiguous duplication of exons 28 and 29 (Fig. 2C).

Patient P3 with an MDC1A phenotype has a novel nonsense mutation in exon 4 (c.497G>A, p.Trp166*) detected in a heterozygous and apparently homozygous state, depending on the primer-pair used to study this region (Fig. 3A). These ambiguous results led us to suspect a possible deletion comprising at least part of intron 3. The application of MLPA confirmed this assumption, since a reduced amplification signal was observed for the exon 3-specific probe (Fig. 3B). For further characterization, and since no RNA was available for study, we performed Southern-blotting and hybridization using a cDNA probe that recognizes exons 1 to 4. This experiment suggested that the genomic deletion originates a new fragment of approximately 6 Kb in the patient, that is absent in the control (Fig. 3C). To delineate the deletion endpoints several primers were tested to perform a deletion-specific PCR. A 42931 bp deletion combined

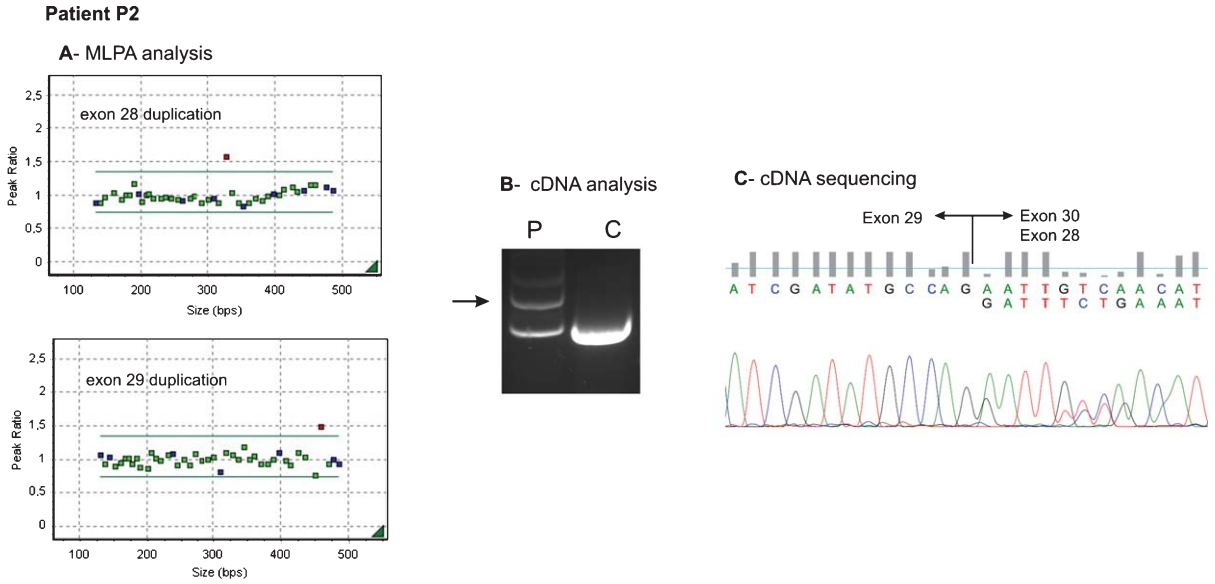

Fig. 2. Duplication identified in patient P2. (A) Increased signal for MLPA probes (peak ratios around 1.5) recognizing exon 28 (upper panel) and exon 29 (lower panel). (B) cDNA analysis of *LAMA2* transcripts revealed the presence of abnormal PCR products in the patient that were not detected in the control. (C) Sequencing electropherogram of the cDNA PCR product (indicated by arrow) reveals the adjacent duplication of exons 28 and 29. C- control; P- patient.

with the insertion of three nucleotides was identified, annotated as c.284-4685_397-146delinsATA (Fig. 3D). Compound heterozygosity for these two mutations was confirmed by the analysis of patient's parents.

Lastly, patient P4 has also an MDC1A phenotype but partial laminin-α2 absence in muscle. Besides one heterozygous 8 bp duplication in exon 13 (c.1854_1861dup) previously reported in the literature [9], we were able to identify a large heterozygous deletion involving exons 3 to 10 by MLPA (Fig. 4). This deletion is predicated to be out-of-frame. A more extensive characterization was not possible since no RNA sample was available for study. Compound heterozygosity was verified since each parent carried a different mutation.

Novel point mutations in LAMA2

Fifty-two patients with *LAMA2* mutations have been characterized until now: 26 of which were previously reported in 2008, and another two more recently in a publication describing their atypical phenotype associated with novel missense mutations [19]. In all cases mutations have been identified in both disease alleles. A list of 10 novel mutations is shown in supplementary data II. These include four nonsense mutations (c.3520C>T, c.5263A>T, c.6501C>G e c.6979G>T), four changes affecting splice sites (c.396+1G>T, c.2450+4A>G, c.6708-1G>T and c.8988+1G>A), one single nucleotide duplication (c.2350dupT) and a missense mutation (c.3235T>G, p.Cys1079Gly).

Reviewing large deletions and duplications in LAMA2

As part of this work we reviewed all large deletions and duplications reported in the literature or in the *locus* specific database for the *LAMA2* gene (http://www.lovd.nl/LAMA2, information last accessed in May 2014). In addition to the mutations presented in this publication, 12 different large deletions and a single duplication were previously described (Table 2).

LAMA2 large deletions ($n = 15$, reported in 35 patients) are apparently dispersed throughout the gene, but two possible mutational "hotspots" may exist: i) one includes exons 3 and 4 (5 different deletions) and ii) in the 3' region of the gene (from exons 56 to 65). The largest *LAMA2* deletion, encompassing exons 23 to 56, was detected by the protein truncation test [27] and comprehends more than half of the gene's coding regions. At least six different deletions affect single exons and were confirmed by a second technique in order to exclude false positive results. The majority of deletions are predicted to cause frameshifting (out-of-frame deletions). Still, only two cases were further characterized at the cDNA level which limits the accuracy of this data. Considering deletions

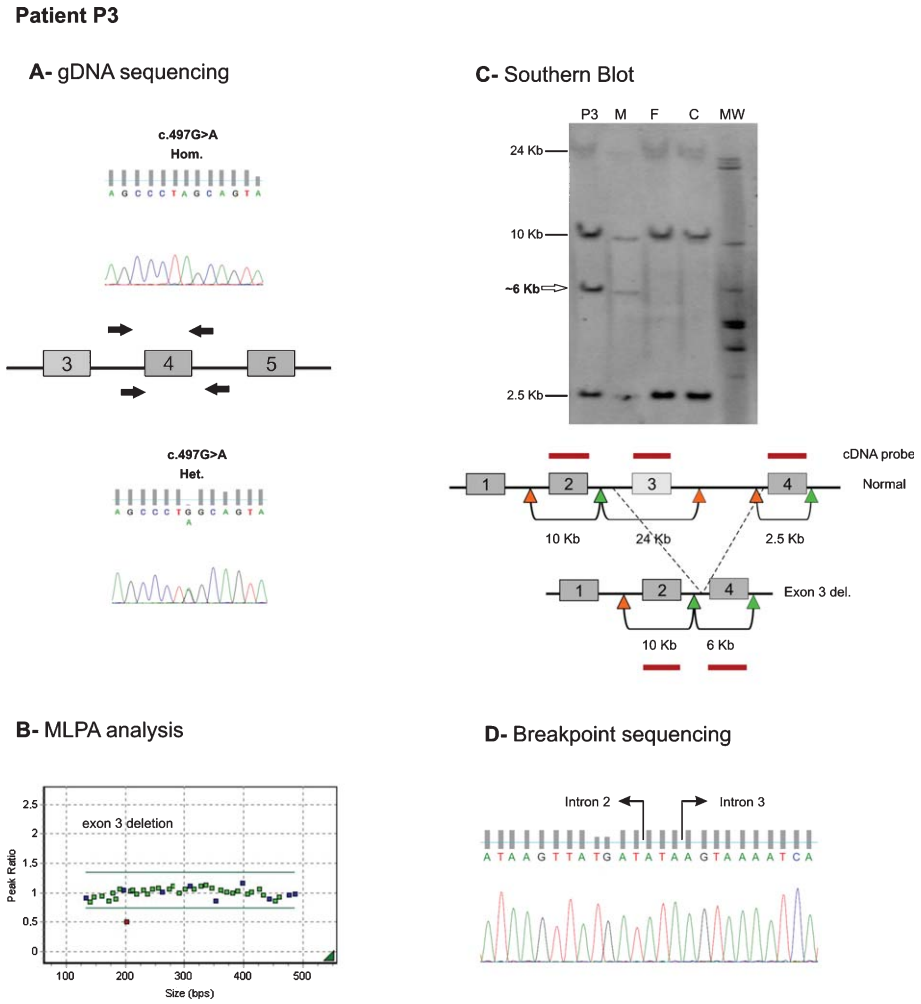

Fig. 3. Characterization of a heterozygous deletion in patient P3. (A) The initial suspicion of a heterozygous deletion encompassing intron 3 derived from the genomic sequencing data. The nonsense mutation c.497G>A located in exon 4 was detected both in heterozygosity and homozygosity depending upon the primers used. (B) Confirmation by MLPA, with reduced amplification signal for the exon 3 probe. (C) Southern-blot followed by hybridization using a cDNA probe that recognizes exons 1 to 4, revealing a 6 Kb fragment in the patient and in his mother, but not in the father nor the control. (D) Deletion breakpoint identified by deletion-specific PCR followed by sequencing. Sequencing electropherogram revealed a 42.9 Kb deletion combined with the insertion of three nucleotides. C- control; F- father; M- mother; MW- molecular weight marker; P3- patient 3.

predicted to be in-frame, all except one [31] were reported in combination with a second truncating mutation (causing a frame-shift or a nonsense mutation). All of these patients have typical MDC1A phenotypes, except the patient with the deletion of exons 41 to 48, which remains ambulant at the age of 10 years (patient #6, reported by [32]). Six patients have been described with large homozygous deletions which may be explained by a higher frequency of a particular mutation within the population (deletion of exon 56 in Portugal and exon 4 deletion in Chinese patients) or due to consanguinity in individual sporadic cases. Patients with homozygous deletions are usually detected by genomic sequencing, since the affected regions will fail to amplify during PCR. Until now, no patients have been reported with compound heterozygosity between two different large rearrangements.

LAMA2 duplications are even rarer mutational events; besides our report of a novel duplication encompassing exons 28 and 29 detected in patient P2, only one other heterozygous in-frame duplication involving exons 5 to 12 has been documented in a patient presenting muscular dystrophy [31]. However, these authors did not identify a second mutation in the patient, which might have explained an autosomal recessive LAMA2-RD.

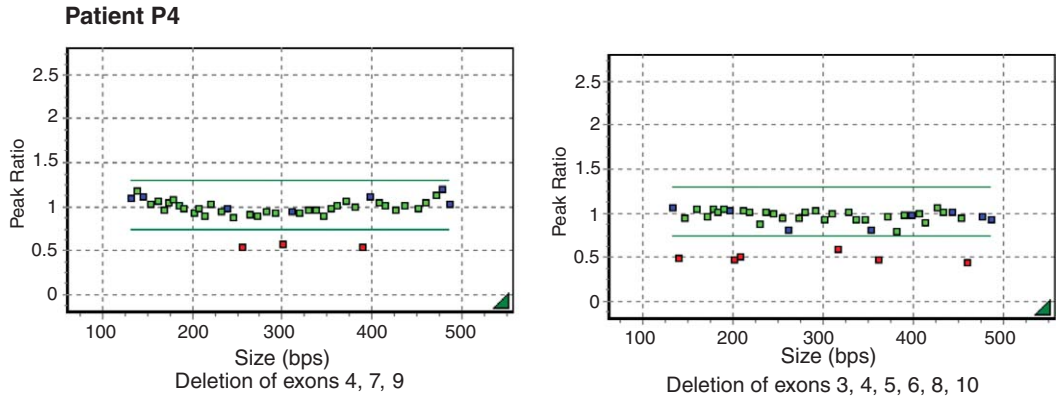

Fig. 4. MLPA results for patient 4. Several *LAMA2* probes with decreased peak ratios (0.5), corresponding to exons 3 to 10, compatible with a large heterozygous deletion.

The frequency of large deletions and duplications in *LAMA2* may be estimated based on the two largest patient cohorts reported and that employed quantitative techniques: 18/104 alleles from our patient cohort of 52 patients and 17/86 alleles from the recent work of Xiong and collaborators that studied 43 patients [32]. The overall frequency of these mutations is thus around 18.4% (35/190 alleles).

DISCUSSION

This work describes the detailed genetic characterization of four patients with compatible features with a MDC1A phenotype, but with only one heterozygous pathogenic sequence variant detected upon complete *LAMA2* sequencing. Three novel large deletions in the *LAMA2* gene and the first pathogenic large duplication were successfully identified in this group of patients, allowing a definitive molecular diagnosis, carrier screening and prenatal diagnosis. Characterization of these mutations implied the use of a variety of techniques such as long-range PCR, cDNA and Southern-blot analysis. These methods are not generally used in the routine genetic diagnosis of this disease, but are essential to obtain accurate genotype-phenotype correlations.

Up to now reports of large deletions and duplications in *LAMA2* are very rare; only three publications have referred this type of mutation [28, 31, 32]. The work of the Italian group included a more heterogeneous patient cohort and a broader technical approach. An array-based comparative genomic hybridization (array-CGH) developed to screen genes implicated in neuromuscular diseases, enabled the identification of several novel copy number variants (CNVs) including two present in the *LAMA2* gene [31]. Based on the data from a total of 95 fully genotyped LAMA2-RD patients, from two large cohorts, we estimate that the frequency of large deletions and duplications in *LAMA2* may be as high as 18.4%. Considering this relatively high frequency, it is important to include screening techniques such as MLPA or array-CGH in the molecular diagnostic work-up. Here, laboratories should consider the variety of equipments required, running costs and sensitivity of these two approaches to screen this type of rearrangement. The presence of a single heterozygous large deletion or duplication, especially when in-frame, should be carefully evaluated. It is conceivable that the presence of a non-pathogenic CNV in a CMD patient may not necessarily explain the clinical phenotype.

Readers should also be aware that genomic sequencing is the technique with the highest sensitivity to screen for *LAMA2* mutations (>80%), especially in CMD cases with laminin-α2 deficiency. Our current strategy for *LAMA2* genetic analysis is sub-divided in three tiers: i) the first level comprising a selected number of exons (namely: 3, 13, 22, 27, 33, 36, 54, 58, and 61) corresponding to the genomic regions where the majority of point mutations in our population are located, together with the screening of exon 56 deletion; ii) the second tier includes the remaining *LAMA2* exons; and finally iii) MLPA analysis (two panels). Until now, seventeen patients have been analyzed in this manner. In 35% of patients both mutated alleles were identified using tier 1, and in 82% at least one heterozygous mutation was detected. We consider feasible in our population to screen these *LAMA2* regions in patients with compatible features of CMD (such as white matter changes in brain MRI), even before performing a muscle biopsy.

Table 2
Review of large deletions and duplications reported in the *LAMA2* gene

Affected gene regions (exons)	Nr. of affected exons	Mutation description		Impact on reading frame (prediction)	Nr. of patients reported	Zygosity // other mutation type	Phenotype	References
		gDNA	RNA					
Deletions								
2–3	2	c.113-?_396+?del	r.(del)	OF	2	het. ($n=2$) // splice-site mutation	MDC1A; MDC1A (ambulant at 5yr)	[32]
3	1	c.284-4685_397-146delinsATA	r.(del)	OF	1	het. // nonsense mutation	MDC1A	P3, this paper
3–4	2	c.284-?_639+?del	r.(del)	OF	1	hom.	MDC1A	[32]
3–10	8	c.284-?_1467+?del	r.(del)	OF	1	het. // 8 bp OF duplication	MDC1A	P4, this paper
4	1	c.397-?_639+?del	r.397_639del	IF	5	hom. ($n=2$), het. ($n=3$) // several	MDC1A	[32]
5	1	c.640-?_819+?del	r.(del)	IF	2	het. ($n=2$) // 2 bp OF deletion	MDC1A	[32]
10–12	3	c.1307-?_1782+?del	r.(del)	OF	1	het. // 4 bp OF duplication	MDC1A	[32]
13–37	15	c.1783-19594_5445+1681del	r.(del)	IF	1	het. // unknown	myopathy	[31] Db
17	1	c.2322+259_2450+2037del	r.(del)	OF	1	het. // 3 bp IF deletion	MDC1A (ambulant at 17yr)	P1, this paper P19, [28]
23–56	34	c.(?_3175)_(7898_?)del	r.(del)	OF	1	het. // 1 bp OF deletion	MDC1A	[27]
41–48	8	c.5866-?_6867+?del	r.(del)	IF	1	het. // 4 bp OF duplication	MDC1A (ambulant at 10yr)	[32]
56	1	c.7750-1713_7899-2154del	r.7750_7898del	OF	14	hom. ($n=2$), het. ($n=12$) // several	MDC1A; LAMA2-related MD	[28] Db
57–3UTR	9	c.7899-?_(*219_?)del	r.(?)	?	2	hom. ($n=2$)	MDC1A	Db
59–63	5	c.8245-?_8988+?del	r.(del)	IF	1	het. // nonsense mutation	MDC1A	[32]
63	1	c.8858-?_8988+?del	r.(del)	OF	1	het. // nonsense mutation	MDC1A	[32]
Duplications								
5–12	8	c.640-?_1782+?dup	r.(dup)	IF	1	het. // unknown	muscular dystrophy	[31] Db
28–29	2	c.4059-?_4311+?dup	r.4059_4311dup	OF	1	het. // nonsense mutation	MDC1A	P2, this paper P24, [28]

Mutations described according to HGVS nomenclature using cDNA reference sequence with accession number NM_000426.3. bp- base pairs; Db- *locus*-specific mutation database for *LAMA2* gene (http://www.lovd.nl/LAMA2); het.- heterozygous; hom.- homozygous; IF- in-frame; OF- out-of-frame; MD- muscular dystrophy; Nr.- number; yr- years.

Large deletions and duplications detected in CMD patients are not confined to *LAMA2* gene; in fact we have recently reported a patient with a Fukuyama CMD caused by a multi-exonic duplication in *FKTN* (fukutin) [33]. There are additional reports of other pathogenic CNVs in CMD genes, such as: *ISPD* (isoprenoid synthase domain containing) [34], *LARGE* (like-glycosyltransferase) [35, 36] and *POMGNT1* [protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-)] [37].

New mutation screening methods are currently being developed based on next-generation sequencing (NGS) technology, which will contribute to establish the genetic causes of hereditary myopathies that remain unsolved. However, prior to its application, it is important to exclude large deletions and duplications as a cause of these diseases. Bioinformatic pipelines for NGS usually do not incorporate algorithms that enable their automatic detection, but we have previously shown that, when properly applied, this technology can help delineate large genomic rearrangements [38].

In summary, we have reassessed the impact of large deletions and duplications in LAMA2-RD and emphasize the importance of including screening for these rearrangements as part of the diagnostic strategy, especially in patients where a single heterozygous mutation has been detected.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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PAPER VII

Atypical phenotype in two patients with LAMA2 mutations.

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Case report

Atypical phenotype in two patients with *LAMA2* mutations

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Abstract

Congenital muscular dystrophy type 1A is caused by mutations in the *LAMA2* gene, which encodes the α2-chain of laminin. We report two patients with partial laminin-α2 deficiency and atypical phenotypes, one with almost exclusive central nervous system involvement (cognitive impairment and refractory epilepsy) and the second with marked cardiac dysfunction, rigid spine syndrome and limb-girdle weakness. Patients underwent clinical, histopathological, imaging and genetic studies. Both cases have two heterozygous *LAMA2* variants sharing a potentially pathogenic missense mutation c.2461A>C (p.Thr821Pro) located in exon 18. Brain MRI was instrumental for the diagnosis, since muscular examination and motor achievements were normal in the first patient and there was a severe cardiac involvement in the second. The clinical phenotype of the patients is markedly different which could in part be explained by the different combination of mutations types (two missense versus a missense and a truncating mutation).

Keywords: Laminin α2-chain; Merosin; Cardiomyopathy; Epilepsy; Congenital muscular dystrophy 1A

1. Introduction

Mutations in *LAMA2*, encoding the α2-subunit of the extracellular matrix protein laminin, are normally associated with muscular dystrophy and white matter abnormalities of the central nervous system (CNS). Classical congenital muscular dystrophy type 1A (MDC1A) is characterized by severe generalized muscle weakness, joint contractures and peripheral neuropathy [1]. Primary partial laminin-α2 deficiency is associated with a less severe phenotype and with clinical and genetic heterogeneity [2–4].

In cardiac muscle, through its interactions with the α2-chain of laminin 211, α-dystroglycan makes an important connection to the extracellular matrix, forming a link between the sarcolemma and the

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extracellular matrix [5]. Symptomatic and subclinical cardiac involvement has been previously reported, notably in typical cases with absent laminin-α2 staining [4,6,7]. Regarding partial defects, cardiac involvement was documented only in one patient [8].

Previous studies indicate that laminin deficiency delays oligodendrocyte maturation through dysregulation of critical developmental signaling pathways [9]. Laminin-α2 binds to β1-integrin and is distributed punctately on cortical neuronal processes [10]. Cerebral white-matter changes are typical; cortical dysplasia and polymicrogyria are seldom seen. Most patients are cognitively normal, but epilepsy and mental retardation have been described [11].

We report two patients with atypical phenotypes for primary partial laminin-α2 deficiency. The first case is remarkable for the predominant CNS involvement with normal muscular strength and the second had an unusually severe cardiac involvement.

2. Case reports

2.1. Patient 1

This patient is the second child of healthy unrelated parents and he was born after an uneventful pregnancy. Macrocephaly (3SD) was detected in the first year of life. Refractory epilepsy associated with progressive cognitive regression started by the age of 6, with atypical absences, myoclonic and atonic seizures. Physical exam revealed macrocephaly and bilateral divergent strabismus. Motor complaints were never reported. He had normal muscular strength, tone and deep tendon reflexes. His gait was normal, with preserved coordination, and he was able to run, climb stairs and ride his bike. Brain magnetic resonance imaging (MRI) showed an area of agyria in the occipital cortex. In T2-weighted images, extensive white matter abnormalities, swelling and widening of gyri, mainly frontal, were detected (Fig. 1A and B). Serial electroencephalograms (EEG) revealed bilateral multifocal paroxysmal activity, suggestive of symptomatic epilepsy (Fig. 1C). *LAMA2* gene study was performed after extensive metabolic and genetic workup, given the occipital agyria and typical white matter abnormalities [12]. The highest creatine kinase (CK) value detected was 1589 UI/L. Electromyography (EMG), nerve conduction study and echocardiogram were unremarkable.

Epilepsy was initially controlled, but rapidly became refractory, with progressive cognitive impairment and

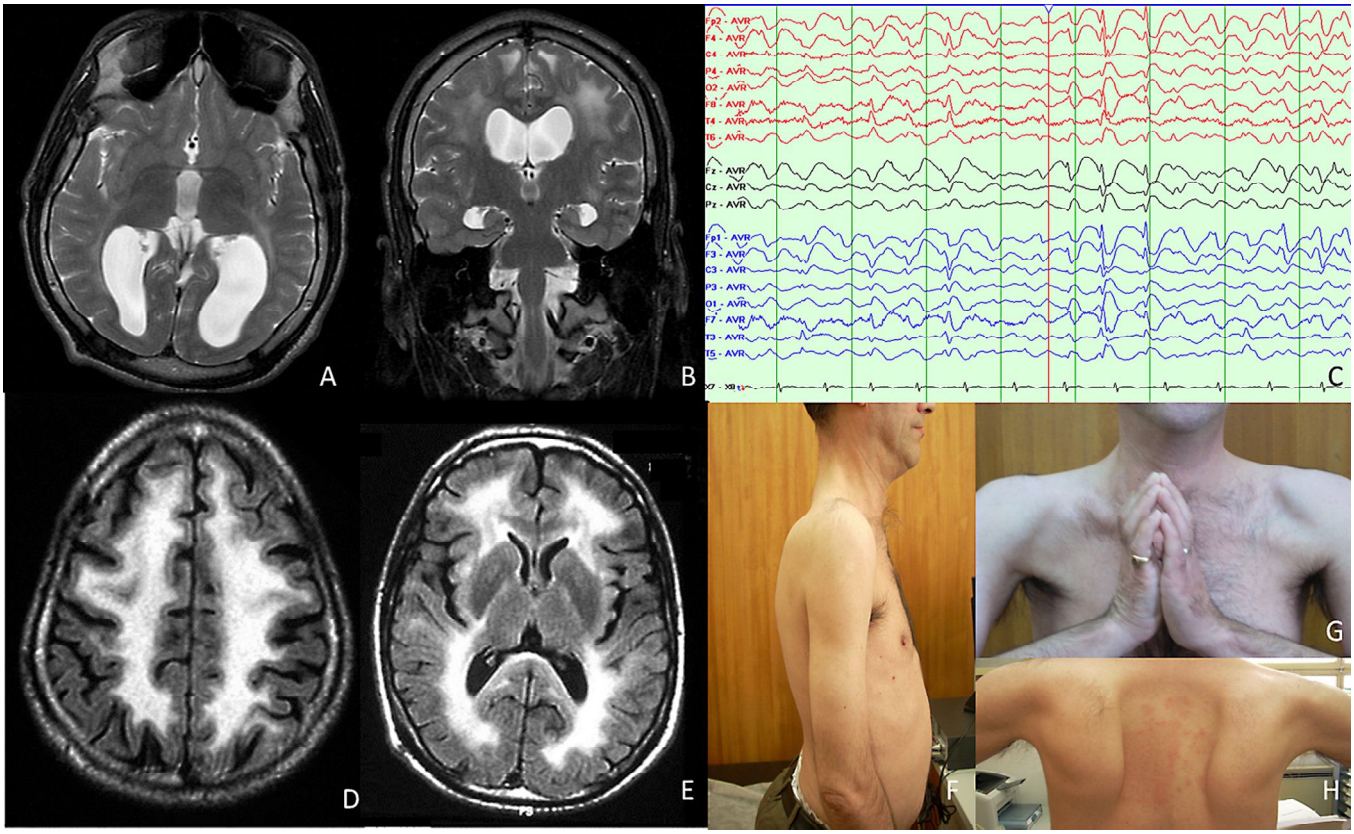

Fig. 1. T2-weighted axial (A) and coronal (B) brain MRI of patient 1 at age 15; note the occipital agyria and white matter hyperintensity; (C) EEG of patient 1: diffuse slowing of background activity; bilateral paroxysms, stronger in the anterior regions. (D and E) Axial FLAIR T2-weighted brain MRI of patient 2 with extensive white matter hyperintensities; (F) lumbar hyperlordosis in patient 2; (G) tendinous retractions in patient 2; (H) scapular winging in patient 2.

loss of expressive language. Motor function remains normal at age 21. Rufinamide significantly reduced the severity and frequency of atonic seizures.

2.2. Patient 2

This 55 year-old male patient was a single child of a healthy, non-consanguineous couple without family history of neuromuscular diseases. He presented delayed developmental motor milestones: he started walking after the age of 2 and always had difficulty running. By the age of 47 he sought medical attention, complaining of slowly progressive proximal muscle weakness and frequent falls, starting in his early forties. His neurological exam was notable for shoulder and pelvic girdle weakness, weak knee flexion and extension, and positive Gowers' maneuver; muscular atrophy with scapular winging (Fig. 1H); lumbar hyperlordosis (Fig. 1F) and dorsolumbar scoliosis. A rigid spine syndrome was noted as well as multiple tendinous retractions (Fig. 1G). Brain MRI showed typical white matter abnormalities (Fig. 1D and E). CK values were 351 UI/L. EMG was consistent with a myopathic process. Echocardiogram showed impaired left ventricular contractility and mitral valve prolapse. ECG Holter was normal. A slowly but progressive worsening of his motor function was noted over the years; he remains ambulant with bilateral support. Cardiac function has clearly declined and he developed dilated cardiomyopathy with dysfunction of the left ventricle contractility (shortening fraction 18%), marked dilatation of the left ventricle and congestive heart failure NYHA class II–III.

3. Muscle histology

Specimens were obtained by open left deltoid muscle biopsy, frozen and stained using standard techniques. Additionally, 6 μm sections were immunolabeled with antibodies targeting the 300 kDa N-terminal fragment of laminin-α2 (NCL-merosin, Novocastra). Histological analysis in both cases revealed unspecific signs of muscular dystrophy (Fig. 2A–D). Immunohistochemistry labeling for dystrophin, sarcoglycans and dysferlin was normal, but a partial and irregular labeling for laminin-α2 was documented in both specimens (Fig. 2E–G).

4. Molecular studies

4.1. Genomic DNA analysis

LAMA2 gene sequencing was performed according to Oliveira et al. [2], where all coding and adjacent intronic sequences were analyzed. Sequence variants were described according to the Human Genome Variation Society guidelines for mutation nomenclature (version 2.0) [13] and the reference sequence with accession NM_000426.3. Two heterozygous missense mutations were identified in patient 1, namely c.812C>T (p.Thr271Ile) in exon 5 and c.2461A>C (p.Thr821Pro) in exon 18 (Fig. 3). Family studies confirmed that these were allelic in the patient. Heterozygosity was also found in patient 2, who presented the missense mutation

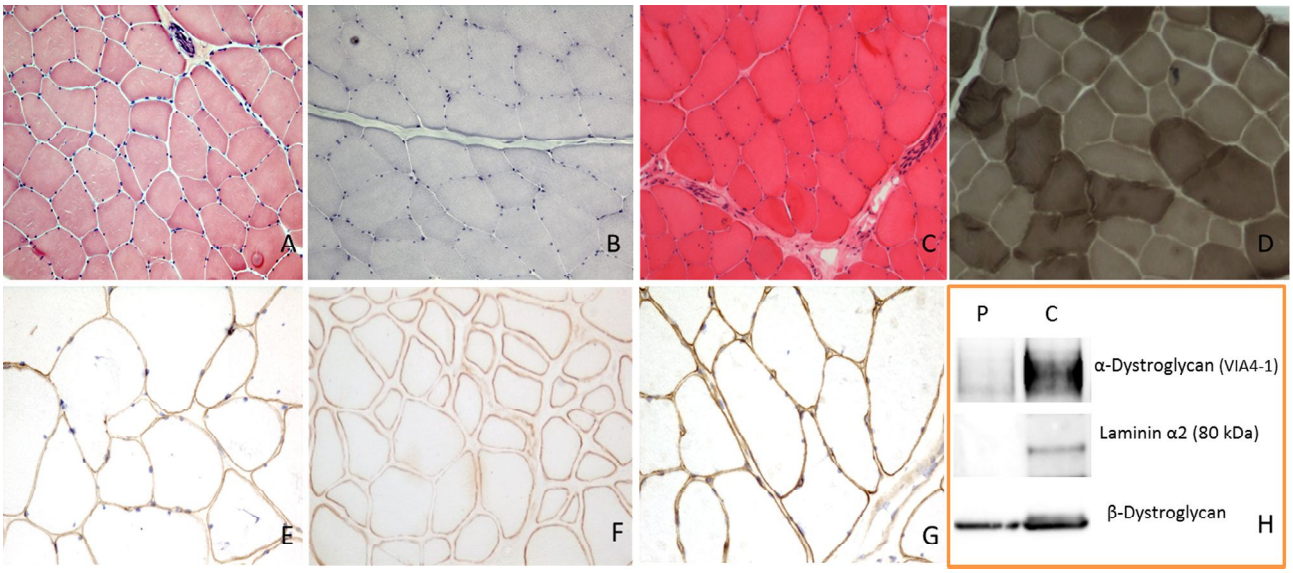

Fig. 2. Histologic and molecular studies performed in both patients. (A) Patient 1, hematoxylin and eosin staining (10×) discloses a mild increase in the variability of fiber diameter; (B) patient 1, gomori trichrome staining (×10); (C) patient 2, hematoxylin and eosin (×10), revealing increased variability of fiber diameter with round atrophic fibers, dispersed in the fascicles or in groups in the same area and frequent central nuclei; (D) patient 2, ATPase reaction (pH 9.4; ×10). Type 1 fibers predominated; (E) partial and irregular laminin-α2 labeling in patient 1 muscle specimen (×20); (F) partial and irregular laminin-α2 labeling in patient 2 muscle specimen (×20); (G) normal control for laminin-α2 labeling. (H) Immunoblotting analysis of patient 1 muscular sample by comparison to a control. P = patient; C = control muscle.

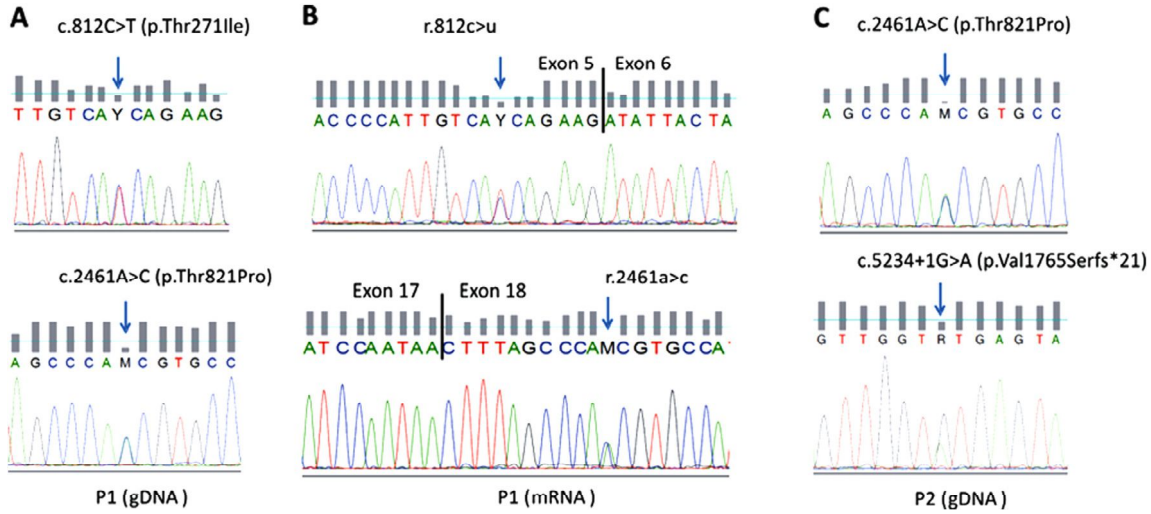

Fig. 3. Genetic studies performed in both patients. (A) gDNA sequencing results showing the two novel *LAMA2* mutations (arrows indicate changes) detected in heterozygosity in patient 1 (P1). (B) mRNA studies in patient 1, excluded possible splicing defects in the vicinity of the mutations. (C) Heterozygous mutations identified at the gDNA level in patient 2 (P2); the first in common with patient 1 and the second found to be a previously reported splicing mutation in intron 36 (c.5234+1G>A).

c.2461A>C (p.Thr821Pro), in common with patient 1, and the splicing mutation c.5234+1G>A (p.Val1765Serfs*21) known to cause skipping of exon 36 [2].

Population screening for these missense variants was carried out by direct sequencing of exon 5 in the case of c.812C>T and by high resolution melting curve analysis (hrMCA) of exon 18 in the case of c.2461A>C. Neither variants were detected in 300 control alleles, corroborating their pathogenic nature.

4.2. cDNA studies

Knowing that variants that are predictably missense can actually induce splicing defects [14], and since both variants are located in the vicinity of splice sites, we evaluated their possible effects on pre-mRNA processing. Transcript analysis was performed in a cryopreserved muscle specimen from patient 1, using primers designed specifically for the regions of interest. Results showed that neither appeared to have an effect on mRNA splicing (no exon skipping or cryptic splice site activation) and that bi-allelic expression was evidenced with both mutations detected in heterozygosity (Fig. 3).

4.3. Western Blotting

Laminin-α2, α-dystroglycan and β-dystroglycan expression in a muscle sample from patient 1 was assessed by Western Blotting (WB) analysis using monoclonal antibodies against the 80 kDa C-terminal segment of laminin-α2 (MAB1922, Chemicon, Millipore, Temecula, CA), a glycosylated epitope of α-dystroglycan (VIA4-1, Upstate, Millipore, Temecula, CA) and β-dystroglycan (NCL-b-DG, Novocastra, Leica Microsystems, Newcastle Upon Tyne, UK). The latter was used as an internal control. The WB procedure was adapted from the protocol reported by Anderson et al. [15]. For the purpose of densitometry, protein loading was normalized with the myosin band in the post-blotted gel. Immunoblot analysis revealed complete absence of merosin with the anti-80 kDa antibody. A 75% reduction was detected in α-dystroglycan expression by comparison with the control (Fig. 2, H), possibly due to technical issues related with the heterogeneous glycosylation pattern of the epitope recognized by the VIA4-1 antibody.

4.4. Bioinformatic analysis of missense variants

Besides the in vitro experiments described above we also assessed the pathogenicity of the missense variants using a diversity of bioinformatic tools and databases. As depicted in table S1 in supplementary data, all algorithms consistently suggested a deleterious effect for these mutations. In addition, the mutations c.2461A>C and c.812C>T were not present in SNP databanks including dbSNP, 1000 Genomes and Exome Variant Server.

5. Discussion

We report two patients with partial laminin-α2 deficiency and atypical phenotypes. The first patient had epileptic encephalopathy with normal muscular examination. The second case had a limb-girdle muscular dystrophy phenotype with unusually severe cardiac involvement. Brain imaging was instrumental for diagnosis. Two missense mutations were found in patient 1, located in the N-terminus of laminin-α2; p.Thr271Ile in domain LN (laminin N-terminal) and p.Thr821Pro in domain LEb). Regarding patient 2, two heterozygous mutations were detected: the same missense mutation c.2461A>C

(p.Thr821Pro) in exon 18 and the splicing mutation c.5234+1G>A (p.Val1765Serfs*21) in intron 36, which has been previously described [2]. The clinical phenotype of both patients is markedly different which could only in part be explained by the different allelic combination of mutations detected in each case. In the Leiden Muscular Dystrophy Pages (http://www.dmd.nl/LAMA2), we have found an entry for the c.2461A>C variant, but reported as being of unknown pathogenicity. Interestingly, this mutation was reported in this database in together with the c.5234+1G>A mutation, the same combination as in patient 2. However, the phenotypes of both patients seem to differ significantly (seizures, abnormal white matter, and cervical spine fusion as indicated in the Leiden database, without mention of a cardiac phenotype).

Reports of MDC1A (MIM: 607855) patients with severe cognitive impairment are very scarce and the severity of CNS involvement combined with absent muscular complaints, as described here for patient 1, is a new form of presentation of this disorder. In previous series of patients [7] there was no apparent correlation between mental retardation and severity of weakness, suggesting that different mechanisms contribute to muscular and CNS involvement. Rufinamide was shown to be effective in epileptic encephalopathies other than Lennox–Gastaut syndrome, particularly in patients with drop-attacks and (bi)frontal spike-wave discharges, matching our patient's clinical features [16].

Cardiac involvement in laminin-α2 deficiency has been largely underreported. One study specifically addressed the cardiac involvement in children with laminin-α2 deficiency [6]. A cohort of 16 children with congenital muscular dystrophy was studied (6 with MDC1A) where two children with significant left ventricular dysfunction had complete laminin-α2 deficiency. In another series of 51 patients with MDC1A [4], 15 had cardiac assessment, of whom 5, with complete laminin-α2 deficiency, had cardiac abnormalities. Documentation on cardiac status was unavailable for the remaining patients. In the largest bibliographical review (248 published patients with abnormal immunohistochemical staining for laminin-α2) cardiac function was described in 20 [7]. Cardiac dysfunction was reported in 7 patients (7/20, 35%), of whom 4 were asymptomatic. Cardiac abnormalities varied, including right bundle branch block, dilated cardiomyopathy and "borderline changes in cardiac function".

To the best of our knowledge, only one case of partial defect of laminin-α2 with cardiac involvement was previously reported [8]. Long-term evaluation led to a diagnosis of dilated cardiomyopathy with ventricular arrhythmias, requiring implantation of an intracardiac defibrillator. In this patient, *LAMA2* gene analysis revealed two different mutations, a missense mutation in exon 29 (c.4405T>C, p.Cys1469Arg) and a nonsense mutation in exon 31 (c.4645C>T, p.Arg1549*).

We want to highlight the need for cardiac status assessment in this disorder, given the potential of severe cardiac involvement, even in patients with residual expression of the laminin-α2 chain.

This report widens the clinical presentation associated with genetic defects in *LAMA2*. These cases are probably under-diagnosed and seldom reported in the literature, especially when only subtle changes in laminin-α2 chain are detected in IHC studies. Considering the interplay role of laminin-α2 in the basal membrane and extracellular matrix, it is conceivable that mutations in this protein may only affect the interaction with other proteins maintaining at least partial interaction with α-dystroglycan, and leading to other phenotypes not directly related with muscle weakness.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nmd.2014.01.004.

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PAPER VIII

A Portuguese case of Fukuyama congenital muscular dystrophy caused by a multi-exonic duplication in the fukutin gene.

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Case report

A Portuguese case of Fukuyama congenital muscular dystrophy caused by a multi-exonic duplication in the fukutin gene

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Abstract

Fukuyama congenital muscular dystrophy (FCMD) is one of the most common autosomal recessive diseases among the Japanese population, due to a founder mutation of the fukutin gene (*FKTN*). Mutations in *FKTN* are now being described in an increasing number of non-Japanese patients. We report a Portuguese child with FCMD. The diagnosis was supported by clinical, histological, magnetic resonance imaging (MRI) and genetic studies. Genetic analysis of *FKTN* by Multiplex Ligation Probe Amplification (MLPA) revealed a homozygous duplication from exon 4 to exon 7. This in-frame duplication was confirmed by cDNA analysis. To our knowledge this is the first report of a FCMD case caused by an intragenic gross exonic duplication in the *FKTN* gene. This report widens the clinical and mutational spectrum in FCMD and corroborates the importance of screening for large deletions and duplications in CMD patients.

Keywords: Duplication; Fukutin; FKTN; Fukuyama congenital muscular; Dystrophy; α-Dystroglycanopathies

1. Introduction

Congenital muscular dystrophies (CMDs) with hypoglycosylation of α-dystroglycan (α-DG) are clinically and genetically heterogeneous disorders often involving brain, eyes, and muscle [1]. Causal mutations have been identified in eight glycosyltransferase genes: protein-*O*-mannosyl transferase 1 (*POMT1*) [2], protein-*O*-mannosyl transferase 2 (*POMT2*) [3], protein-*O*-mannose1,2-*N*-acetylglucosaminyltransferase 1 (*POMGNT1*) [4], fukutin (*FKTN*) [5], fukutin-related protein (*FKRP*) [6], like-glycosyltransferase (*LARGE*) [7], isoprenoid synthase domain-containing protein (*ISPD*) [8,9] glycosyltransferase-like domain containing 2 (*GTDC2*) [10]. Mutations in each of these genes have been associated with a wide variety of phenotypes ranging from the severe Walker–Warburg syndrome (WWS) to milder variants of limb girdle muscle dystrophy.

Fukuyama congenital muscular dystrophy (FCMD), is the second most common form of muscular dystrophy in the Japanese population, after Duchenne muscular dystrophy, with a carrier frequency of one in 88 [11], an estimated incidence of 1 in 10,000 births [12] and is one of the most common autosomal recessive disorders in

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Japan. The high incidence is related to a founder mutation located in the 3'UTR of *FKTN* [5]. Seventy-five percent of Japanese patients are homozygous for this ancestral mutation [13]. Clinically, the classical Japanese FCMD is characterized by an early onset with hypotonia, symmetric generalized muscle weakness and structural brain malformations and mental retardation. Affected individuals have contractures of the hips, knees, and interphalangeal joints. Later features include myopathic facial appearance, pseudohypertrophy of the calves and forearms, convulsions (febrile or afebrile), ophthalmologic abnormalities including visual impairment and retinal dysplasia, and progressive cardiac involvement in individuals in the second decade of life. A static course is frequent until early childhood followed by extensive muscle wasting, most prominent proximally, and later progression of joint contractures. Most patients are never able to walk independently [11,14]. The most common brain malformations in FCMD include cobblestone lissencephaly of the cerebrum and cerebellum, but also posterior fossa malformations (cerebellar polymicrogyria, vermis hypoplasy and cysts) may be present [15].

Non-Japanese patients with *FKTN* mutations have been described for the first time in 2003 by Silan et al. [16] and de Bernabe et al. [17]. These two reports described Turkish infants homozygous for frameshift mutations associated with a severe CMD phenotype matching the established criteria for WWS. In fact, besides WWS, non-Japanese *FKTN* mutations can cause a wide clinical spectrum including FCMD, muscle-eye-brain disease (MEB, OMIM#253800), a congenital form without mental retardation (MDDGB4, OMIM#613152), a milder limb-girdle form (MDDGC4, OMIM#611588) also designated LGMD2M, and dilated cardiomyopathy (CMD1X, OMIM#611615) with mild or no limb-girdle muscle involvement. In particular, Godfrey et al. [18] defined FCMD/MEB as congenital onset muscular dystrophy with fronto-parietal pachygyria, cerebellar dysplasia and frequent flattening of the pons and brainstem. Eye abnormalities are often seen, and rare patients may acquire the ability to walk or to learn a few words.

We report a CMD patient with central nervous system (CNS) changes and no ocular involvement, where the genetic study revealed a new, large, in-frame duplication in the *FKTN* gene.

2. Case report

A seventeen-month-old Portuguese female child was referred for evaluation due to psychomotor retardation and hypotonia, after having been treated for hip dislocation in our hospital. She was the first child of young, healthy and non-consanguineous parents and no significant family history was registered. She was born at term and pregnancy and delivery were unremarkable.

The physical examination showed head circumference normal for the age, facial diplegia with an open mouth and drooling but without major dimorphisms, strabismus or ophtalmoparesis. The patient had axial weakness with poor head control and was unable to sit without support; a symmetric flaccid tetraparesis predominately proximal and brachial; contractures of hips, knees, ankles and interphalangeal joints of the hands; a rigid spine and absent tendon reflexes. She evidenced a mental disability with a developmental quotient of 50. At the present age (3 and a half years) she is able to sit without support and has some antigravity movements of the four limbs, but she cannot stand independently or hold any objects (Fig. 1A). For the time being ophthalmologic and cardiac examinations remain normal.

The investigation revealed elevation of creatine kinase (CK) values [6777U/l(17mo)/5084U/l(19mo)]. Muscular histology revealed small round muscle fibers of variable size along with necrotic and regenerative changes. The hematoxylin staining showed end-stage muscular dystrophy with atrophic fibers and replacement with connective tissue (Fig. 1B). Immunohistochemical analysis revealed major loss of α-dystroglycan (Fig. 1C) as compared to control (Fig. 1D).

Brain magnetic resonance imaging (MRI) at the age of 20 months illustrated an extensive bilateral frontal, temporal and parietal dysplasia, with preservation of posterior temporal and occipital regions; significant anomalies of the supratentorial white matter; cystic degeneration of white matter and multiple cerebellar cysts (Fig. 2A–D).

Molecular analysis was initially performed for *LAMA2*, *FKRP, POMT1* and *POMT2* genes and no mutations were found by direct genomic sequencing. Considering the presence of cobblestone lissencephaly with cerebellar cysts and muscular dystrophy, the diagnosis of FCMD was considered and a genetic test for the *FKTN* gene was performed. All 11 exons and flanking intronic regions were analyzed by direct sequencing (reference sequences with accession number NM_001079802.1). Only three previously known homozygous polymorphisms were detected: c.608G>A, c.1026C>A and c.1044+44A>G. In order to identify potential large deletions or duplications in *FKTN*, a quantitative study was performed using the Multiplex Ligation Probe Amplification (MLPA) technique. We used the commercial kit from MRC-Holland that includes probes for intron 1 and exons 4, 6, 7, 8, and 11 of *FKTN*. This analysis revealed that the patient has a duplication involving exons 4 to 7 of *FKTN* (Fig. 3A). Results also show that this mutation is present in a homozygous state, considering the probe ratios (~2.0) of the duplicated regions and that both parents are carriers of the same duplication (Fig. 3B). In order to determine the impact of this mutation at the cDNA level, expression studies were conducted using mRNA obtained from a cryopreserved muscle specimen of the patient. Amplification and sequencing of *FKTN* transcripts

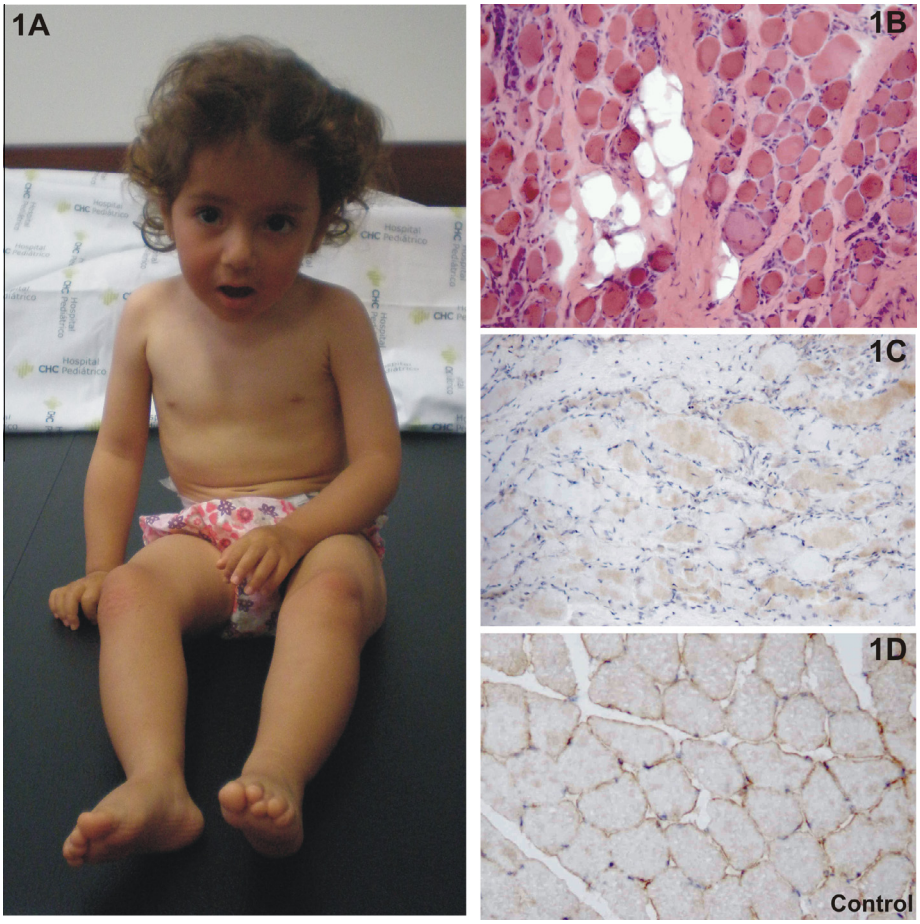

Fig. 1. Patient at the age of 3 years with evidence of facial diplegia, tetraparesis and contractures (A); Histological analysis of muscle biopsy with hematoxylin and eosin staining (B); Imunohystochemistry analysis of α-dystroglycan, markedly reduced in the patient (C) as compared to control (D). All muscle biopsy images with 200× magnification.

revealed that the sequence corresponding to exons 4–7 is also duplicated at the mRNA level (Fig. 3C and D), resulting in an in-frame duplication (r.106_780dup).

3. Discussion

In this report we describe a Portuguese patient who congregates the main clinical characteristics of the classical Japanese phenotype, associated with all-embracing central nervous system (CNS) anomalies described in FCMD, but also including the posterior fossa malformations reported mostly in non-Japanese patients.

From the clinical point of view the follow-up period is still short, but nevertheless it is important to highlight the absence of eye structural malformations or cardiac involvement. There are three other reports of non-Japanese FCMD with brain defects and no eye involvement. Godfrey et al. [18] identified one Caucasian FCMD/MEB patient (P27) with a homozygous missense mutation (p.Trp305Cys) in *FKTN*. MRI showed cerebellar cysts, white matter abnormalities and hydrocephalus, and there were no eye abnormalities in this patient. Vuillaumier-Barrot et al. [19] reported two Portuguese sisters with compound heterozygosity for two missense mutations in *FKTN* (p.Ala170Glu/p.Tyr371Cys). Both girls had a severe phenotype, with congenital muscular dystrophy, joint contracture, respiratory insufficiency, and mental retardation, but no eye involvement. Finally, Xiong et al. [20] reported a Chinese FCMD patient with cerebral and cerebellar gyrus abnormalities with white matter signal intensity changes and no eye anomalies. Genetic analysis of *FKTN* revealed one allele with the Japanese founder 3'UTR retrotransposal insertion and the other allele with a previously-known nonsense mutation (p.Arg47∗).

The CNS migration disturbances displayed in the MRI are consistent with cobblestone lissencephaly, according to literature [15,21]. The pathomechanism of CNS lesions in FCMD and functions of fukutin are still poorly elucidated. It is not entirely clear, but it is known that fukutin is involved in basement membrane formation via the glycosylation of α-DG. Defects in the fukutin gene result in hypoglycosylation of α-DG causing the muscular, CNS and eye lesions of FCMD. In these patients, hypoglycosylation of α-DG was observed in the sarcolemma of striated muscle and in the glia limitans of the CNS, where the basement membrane is formed [22–24]. The CNS lesions of FCMD mainly result from

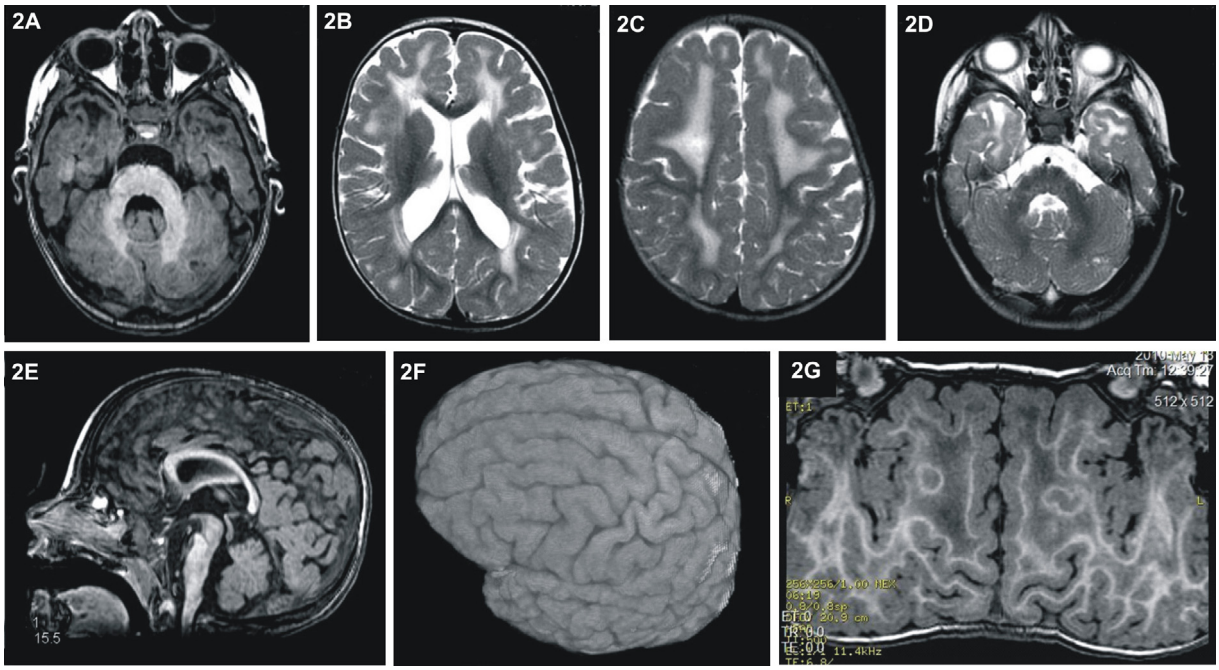

Fig. 2. Central nervous system MRI. Axial T1- cerebellar microcysts suggesting cerebellar dysplasia (A); Axial T2-diffuse white matter hypersignal, diffuse polimycrogyria with anterior predominance (B, C); Axial T2-cortical cerebellar microcysts (D); Sagital T1-moderate protuberance (E); Reconstrution 3D, curv – anterior cobblestone; white matter fronto-parietal and temporal hyposignal, preserving the subcortical fibers (F, G).

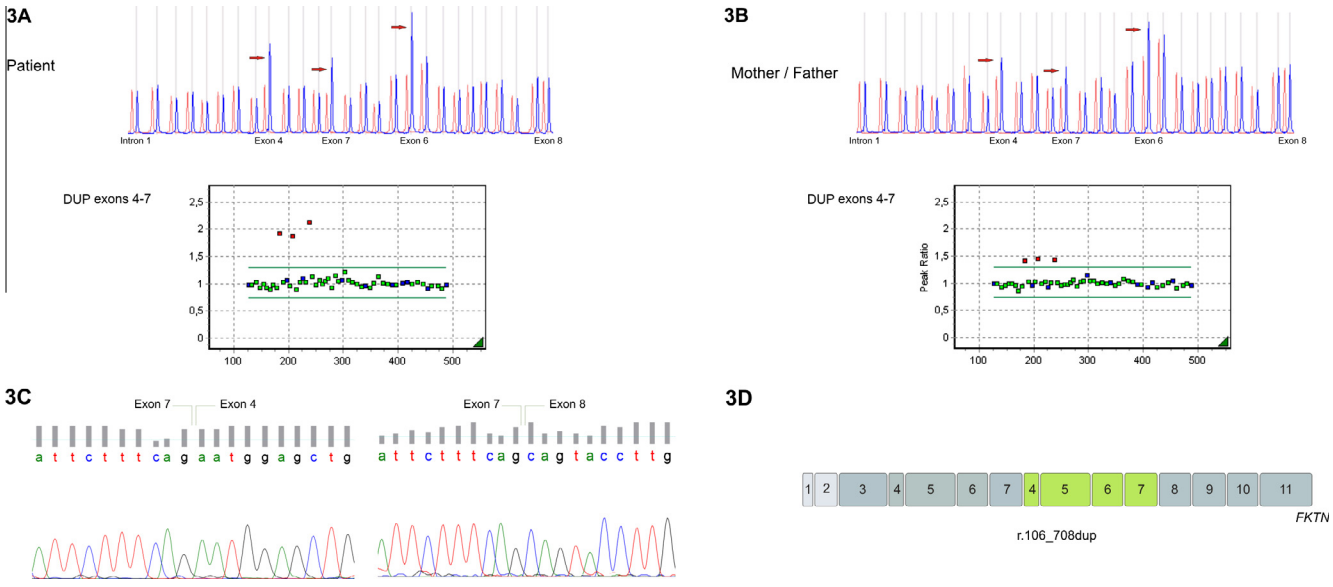

Fig. 3. *FKTN* duplication of exons 4–7 detected in homozygosity in the patient (A) and in heterozygosity in the parents (B); Characterization of the mutation at the mRNA level showing the duplicated region (C); Schematic representation of the mutated transcript r.106_780dup (D).

disruptions of the glia limitans during the fetal period, which is evoked by a fragile basement membrane because of the hypoglycosylation of α-DG. During the fetal period immature neurons over-migrate through the disruptions and believed to result in cobblestone lissencephaly. Because the glia limitans is formed by astrocytic endfeet and covered with the basement membrane, the role of fukutin in astrocytes appears to be important in the pathogenesis of the CNS lesions [22,24].

As seen in previously published cases, point mutations located in potentially important domains of fukutin, or null mutations, generate a severe WWS phenotype [11]. On the other hand, other mutations may lead to the development of a less severe phenotype. It is thus likely that the complete loss of fukutin activity leads to a WWS phenotype [25], whereas mutations that produce a protein with partial activity result in a less severe phenotype such as FCMD, the limb-girdle form MDDGC4, or even

milder phenotypes such as a cardiomyopathy with minimal skeletal muscle weakness [14].

To date, a total of 39 distinct mutations have been described in the *FTKN* gene. These changes are displayed in the public locus-specific database developed for this gene (*FKTN*-LOVD) [26]. The majority are single nucleotide substitutions ($n = 26$, 66.7%) followed by small deletions ($n = 6$, 15.4%), insertions ($n = 2$, 5.1%) and insertions/deletions ($n = 2$, 5.1%). The only gross rearrangements previously described in *FKTN* are a 3602 bp insertion (Japanese founder mutation) and a 473 bp deletion, both located in the 3'UTR of the gene, as well as an insertion of a 1.2 kb L1 element within intron 7 [25,27,28]. This work describing the first multi-exonic duplication in *FKTN* widens its mutation spectrum and provides justification for the routine screening of large deletions and duplications using quantitative techniques such as MLPA. However, a current limitation is that the commercially available MLPA kit for *FKTN* does not include probes for all the exonic regions of the gene.

We have shown that the large homozygous duplication that encompasses exons 4–7 of *FKTN* gene is conservative in terms of the reading frame at the mRNA level (r.106_780dup). Presumably, the resulting larger mutated polypeptide (p.36_260dup, an additional 225 aminoacids) maintains at least partial enzymatic activity.

This case report highlights the need to consider the diagnosis of FCMD in CMD patients with compatible clinic and imaging, as it augments the spectrum of genetic aetiologies of this complex clinical entity.

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5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This work focuses on one of the largest cohorts of Portuguese patients presenting the principal forms of muscular dystrophy, namely D/BMD, LGMD and CMD. Referrals were from the entire Country, over a period of almost 25 years, thereby providing a realistic epidemiological picture in terms of proportions of the three different forms and their sub-types, as well as the occurrence of private mutations and founder effects.

A total of 615 unrelated probands/families were studied, with successful molecular characterization achieved in 546 cases (89%). In the three groups of patients, positivity rates were 284 D/BMD (91%), 195 LGMD (76%) and 67 CMD (~75%). These figures need to be weighted in light of two main and opposing stringencies; firstly, patients were pre-selected based on a set of generally well established criteria for each of the three forms of muscular dystrophy, but on the other hand not all known candidate genes for LGMD and CMD were screened in the patients that remained uncharacterized.

There are several major conclusions to be drawn from this work, some of which have repercussion on the algorithms for clinical and molecular diagnostic approaches to be applied in Portugal. It is evident that the frequency of the different forms of muscular dystrophy varies between populations, and that this is primarily attributable to founder effects that occur with the different mutated genes. Such information is useful in the diagnostic setting, where pre-screening tests are set up, especially for the larger genes. Not surprisingly, the dystrophinopathies are an exception, admittedly because the *DMD* gene is highly mutable and thus even the proportion of mutation types within this gene are globally comparable.

Perhaps the most transversal observation is the corroboration that the hitherto recognized clinical and genetic frontiers between congenital forms and childhood or even adult forms, is dissipating, as are the frontiers between some myopathies and neuropathies. This is highlighted in our report on two patients with *LAMA2* mutations and partial laminin-α2 deficiency in muscle; the first had marked CNS involvement, with macrocephaly detected in the first year of life, whereas the second had essentially an LGMD phenotype and sought medical attention only in his forties.

With the clinical and genetic continuum not only within subtypes, as was already known, but most significantly now also evidenced amongst the different forms of muscular dystrophy and between the different groups of neuromuscular disorders, the classification of the diseases has been confounded. This also adds further difficulty to the prioritization of genetic testing based on clinical presentation, often even with the aid of certain features observed in muscle histology. Achieving an accurate genetic diagnosis has thus become increasingly challenging.

The considerably high positivity rates in this study were achieved not only as a result of patient selection but most importantly also because of multiple genes being screened in many patients, in a step-wise manner. This costly and laborious screening for numerous and often large genes will be circumvented in the near future by rapidly evolving molecular technologies. In particular, massive parallel sequencing, also referred to as *next generation sequencing*, should soon substitute the gene-by-gene approach, setting a new paradigm in the diagnostic pathway to differential diagnosis. Targeted resequencing using NGS platforms, currently applied essentially in a research setting, appears to effectively improve clinical and diagnostic investigations in neuromuscular disorders, This has been shown using a large panel targeting 267 known genes [250] and smaller panels for specific groups of disorders such as the LGMDs, targeting over 20 candidate genes (http://www.centogene.com; https://www.preventiongenetics.com), or the panel that was developed by ourselves, designed to target 20 genes known to be involved in congenital myopathies [in preparation].

Targeted resequencing also has applicability in the screening of single, very large genes such as *DMD* [251] - in this case it is particularly useful in patients with compatible clinical presentation and absence of dystrophin in their muscle biopsy, yet who remain genetically uncharacterized using conventional molecular techniques. At least some of the 19 uncharacterized patients in our cohort would benefit from this NGS approach, which can readily detect mid-intronic mutations or lesions in the regulatory sequences.

Besides the gene panels to circumvent the gene-by-gene approach, NGS technology may be used to screen all exonic regions of the human genome (whole exome sequencing - WES) or even the entire genome itself (whole/individual genome sequencing). This will enable the identification of new genes involved in the diseases [252]. Given the ever-growing evidence of clinical and genetic overlap, this will also identify additional target genes which have hitherto been associated with other disease subtypes. Indeed, in a recent sequel to the work presented here, we have successfully applied WES in the differential diagnosis of a patient where three decades of follow-up and the conventional sequencing of eight candidate genes had failed to provide a diagnosis [253]. The identification of this rare form of CMD (21 patients reported in the literature) now justifies revisiting undiagnosed patients with a similar phenotype and muscle pathology.

Notwithstanding, NGS remains a screening technology and results need to be confirmed by conventional sequencing. Its introduction in the routine diagnostic workflow still faces some challenges, particularly considering the large amount of data that is generated and that requires filtering. This is true even in the analysis of single large genes with a high mutation rate and polymorphic content, as occurs in *DMD*. NGS is therefore not a first tier

choice as yet, although it is foreseeable that this may eventually change over time, once enough information on all variants has been collated in the respective locus-specific databases.

Although these technical advances will transform the diagnostic pathway for the muscular dystrophies, muscle biopsies should continue to assume an important role for some time yet. It is evident from this work and from previous studies [254], that gDNA-based mutation interpretation may fail to predict the correct mutation outcome, even with the aid of bioinformatic algorithms. Thus, with the exception of variants that have been previously described as being causative, in many cases transcript analysis, using muscle-derived cDNA will still remain the ultimate tool for confirmation of cause-effect for most detected variants.

Beyond the purpose of prognosis and adequate clinical management, muscle transcript analysis becomes increasingly important in light of the recent and promising mutation-based therapeutic approaches, many of which target the RNA products. Both nonsense mutation read-through and exon skipping induced by antisense oligonucleotides (AONs) are yielding promising results [255-260]. Tests on muscle biopsy remain crucial also for evaluating outcome measures of such therapies during clinical trials.

Still with respect to these emergent therapies, the detailed genotyping of patients takes on particular relevance, where it is indispensable for adequate candidate selection. The development of a National D/BMD Patient Registry, which is linked to the TREAT-NMD Global Database, constitutes an opportunity for our patients to enrol in clinical trials which are overseen by international authorities in standards of care. It is envisaged, near-future, that similar registries will be set up for the other forms of muscular dystrophy.

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7. APPENDICES

7.1 APPENDIX I: Gene Tables

7.1.1 Gene table of the Limb-girdle Muscular Dystrophies

Gene symbol	Locus	Gene size	Nº of exons	Product	Location / function
DMD	Xp21.2	2.3 Mb	79	Dystrophin	Sarcolemma / structural, component of DGC
EMD	Xq28	2,1 kb	6	Emerin	Inner nuclear membrane / structural, interacts with lamins and chromatin
FHL1	Xq26.3	3,6 kb	6	Four-and-a-half LIM domains 1	Sarcomere & sarcolemma / structural…
LMNA	1q21.2	24 kb	12	Lamin A/C	Inner nuclear membrane / Intermediate filament family
SYNE1	6q25	550 kb	147	Synaptic nuclear envelope protein 1 (nesprin 1)	Nuclear membrane / links nucleo-skeleton & inner nuclear membrane to outer nuclear membrane & cytoskeleton
SYNE2	14q23.2	370 kb	115	Synaptic nuclear envelope protein 2 (nesprin 2)	Nuclear membrane / links nucleo-skeleton & inner nuclear membrane to outer nuclear membrane & cytoskeleton
TMEM43	3p25.1	19 kb	12	Transmembrane protein 43	Inner nuclear membrane
TOR1AIP1	1q25.2		11	Torsin A interacting protein 1	Inner nuclear membrane / binds type A & B lamins
PTRF	17q22	12 kb	2	Polymerase I and transcript release factor (cavin)	Sarcolemma / caveola formation & sequestration of mobile caveolin into immobile caveolae
MYOT	5q31.2	18 kb	10	Myotilin (Titin immunoglobulin domain protein - TTID)	Sarcomere & sarcolemma / Z-disc; interacts with α-actinin, actin, γ-filamin
CAV3	3p25.3	12 kb	2	Caveolin-3	Integral caveolar membrane / scaffolding protein
DNAJB6	7q36.3		10	HSP-40 homologue, subfamily B, number 6	Component of caveolae / chaperone; M-band > Z-band; prevents irreversible aggregation of client proteins
DES	2q35	8,4 kb	9	Desmin	Nucleus & cytoplasm / muscle-specific intermediate filament, spans from sarcolemma to sarcomere to nucleus.
TNPO3	7q32.1		23	Transportin 3	Nucleus / importin; transports serine- & arginine-rich proteins into nucleus
HNRPDL	4q21	5,6 kb	9	Heterogeneous nuclear ribonucleoprotein D-like	Nucleus / RNA processing; splicing of specific exons in pre-mRNA
CAPN3	15q15.1	53 kb	26	Calpain-3	Nucleus & cytosol / associates with titin C-terminus; calcium-activated muscle-specific non-lysosomal protease
DYSF	2p13	223 kb	57	Dysferlin	Sarcolemma / binds to CAV3 & CAPN3; calcium-mediated membrane resealing.
SGCG	13q12	100 kb	8	Gamma sarcoglycan	Sub-sarcolemma / component of DGC; binds moderately to β-sarcoglycan
SGCA	17q21	17 kb	10	Alpha sarcoglycan	Sarcolemma / component of DGC; binds moderately to β- & δ-sarcoglycans
SGCB	4q12	14 kb	6	Beta sarcoglycan	Sub-sarcolemma / component of DGC; binds strongly to δ- & moderately to γ-sarcoglycan; extracellular domain binds to β-dystroglycan
SGCD	5q33	433 kb	9	Delta-sarcoglycan	Sub-sarcolemma / component of DGC; binds strongly to β- & γ- sarcoglycans
TCAP	17q12	1,2 kb	2	Telethonin (titin-cap protein)	Sarcomeric Z-disc / links Z-disc proteins to titin; substrate of serine kinase domain of titin

TRIM32	9q33.2	14 kb	3	Tripartite motif-containing 32	Cytoplasmic & nuclear bodies / E3-ubiquitin ligase
FKRP	19q13.3	16 kb	4	Fukutin-related protein	Golgi / glycosyltransferases; co-localizes with α-mannosidase II; associated with dystroglycan glycosylation
TTN	2q24	294 kb	363	Titin	Sarcomere / structural, giant continuous filament spanning sarcomeric Z-disc to M-band
POMT1	9q34.1	20 kb	20	Protein-O-mannosyltransferase 1	ER membrane / associated with dystroglycan glycosylation
ANO5	11p14	89 kb	22	Anoctamin-5	Integral membrane glycoprotein / calcium-activated chloride channel
FKTN	9q31	100 kb	10	Fukutin	Cis-Golgi compartment / associated with dystroglycan glycosylation
POMT2	14q24.3	46 kb	21	Protein-O-mannosyltransferase 2	ER membrane / associated with dystroglycan glycosylation
POMGNT1	1p32		22	O-linked mannose β1,2-N-acetylglucosaminyltransferase	Transmembrane in Golgi / O-mannosyl glycosylation, required for binding between α-dystroglycan & laminin; possible role in neuronal migration
PLEC1	8q24	32 kb	32	Plectin 1	Stress sites; sarcolemma & Z-disks / intermediate filament binding protein; interacts with DYS & β-dystroglycan
TRAPPC11	4q35.1	54,3 kb	30	Trafficking protein particle complex, subunit 11	Ubiquitous / ER to Golgi vesicle trafficking
GMPPB	3p21.3	2,5 kb	10	GDP-mannose-1-phosphate guanyltransferase beta	/ required for GDP-mannose formation; associated with dystroglycan glycosylation
DAG1	3p21		3	Dystroglycan-1 (cleaved to α- & β-dystroglycan)	Sarcolemma / component(s) of DGC; binds to extracellular laminin
DPM3	1q22		2	Dolichyl-phosphate mannosyltransferase 3	ER membrane / binds DPM1; glycosyl donor for all mannosylation reactions on luminal side of ER
ISPD	7p21.2	334 kb	10	Isoprenoid synthase domain containing	/ associated with dystroglycan glycosylation
GAA	17q25.3		20	Alpha-1,4-glucosidase	/ associated with glycogen storage
LIMS2	2q14		7	Lim and senescent cell antigen-like domains 2	Z-disk / focal adhesion protein; associated with integrin signaling

Data collated from the following sources: http://www.genecards.org/; http://neuromuscular.wustl.edu/; http://www.omim.org/

7.1.2 Gene table of the Congenital Muscular Dystrophies

Gene symbol	Locus	Gene size	Nº of exons	Product	Location
LAMA2	6q22.33	260 kb	64	Laminin α-2 chain of merosin	Extracellular matrix / heterotrimeric glycoprotein; binds to α-dystroglycan in basal lamina
COL6A1	21q22.3		37	Alpha-1 chain of type VI collagen	Extracellular matrix / microfibrillar network of monomers assembled with COL6A1, COL6A2 & COL6A3; anchors basement membranes by interacting with extracellular matrix components
COL6A2	21q22.3	36 kb	30	Alpha-2 chain of type VI collagen	Extracellular matrix / (as above)
COL6A3	2q37.3		44	Alpha-3 chain of type VI collagen	Extracellular matrix / (as above)
SEPN1	1p36.11	18,5 kb	13	Selenoprotein N,1	ER membrane / catalyze oxidation-reduction reactions; involved in cell proliferation & regeneration processes
FHL1	Xq26.3	3,6 kb	6	Four-and-a-half LIM domains 1	Sarcomere & sarcolemma
ITGA7	12q13.2		32	Integrin α-7 (muscle-specific integrin subunit)	External sarcolemma / α7β1 integrin is primary integrin in muscle; laminin receptor; links cytoskeleton to extracellular matrix; involved in myoblast migration
DNM2	19p13.2		22	Dynamin 2	Actin and microtubule networks
TCAP	17q12	1,2 kb	2	Telethonin (titin-cap protein)	Sarcomeric Z-disc / links Z-disc proteins to titin; substrate of serine kinase domain of titin
LMNA	1q21.2	24 kb	12	Lamin A/C	Inner nuclear membrane / Intermediate filament family
FKTN	9q31	100 kb	10	Fukutin	Cis-Golgi compartment / associated with dystroglycan glycosylation
POMT1	9q34.1	20 kb	20	Protein-O-mannosyltransferase 1	ER membrane / associated with dystroglycan glycosylation
POMT2	14q24.3	46 kb	21	Protein-O-mannosyltransferase 2	ER membrane / Dystroglycan glycosylation
FKRP	19q13.3	16 kb	4	Fukutin-related protein	Golgi / glycosyltransferases; co-localizes with α-mannosidase II; associated with dystroglycan glycosylation
POMGNT1	1p32		22	O-linked mannose β1,2-N-acetylglucosaminyltransferase	Transmembrane in Golgi / O-mannosyl glycosylation, required for binding between α-dystroglycan & laminin; possible role in neuronal migration
ISPD	7p21.2	334 kb	10	Isoprenoid synthase domain containing	/ associated with dystroglycan glycosylation
GTDC2	3p22.1		1	Glycosyltransferase-like domain-containing 2	/ contains uncharacterized glycosyltransferase domain; possible role in α-dystroglycan glycosylation
B3GNT1	11q13.2		2	Beta-1,3-N-acetylglucosaminyltransferase 1	Transmembrane in Golgi / forms complex with LARGE
GMPPB	3p21.3	2,5 kb	10	GDP-mannose-1-phosphate guanyltransferase beta	/ required for GDP-mannose formation; associated with dystroglycan glycosylation
LARGE	22q12.3	664 kb	16	Acetylglucosaminyltransferase-like protein	Golgi / associated with dystroglycan glycosylation
DPM1	20q13.1		10	Dolichyl-phosphate mannosyltransferase 1	
DPM2	9q34.13		4	Dolichyl-phosphate mannosyltransferase 2	ER membrane, luminal side
ALG13	Xq23		33	UDP-N-acetylglucosaminyltransferase	ER membrane
B3GALNT2	1q42.3		12	Beta-1,3-N-acetylgalactosaminyltransferase 2	ER membrane / involved in glycosylation - completes trisaccharide GalNAc-b1,3-GlcNAc-b1,4-Man

TMEM5	12q14.2		8	Transmembrane protein 5	/ exostosin family domain; possible role in α-dystroglycan glycosylation
POMK	8p11.21		5	Protein-O-mannose kinase	
CHKB	22q13.3		11	Choline kinase beta	Sarcolemma & mitochondrial membrane / catalyzes phosphorylation of choline by ATP - 1st step in phosphatidylcholine biosynthesis
ACTA1	1q42.1		7	Alpha actin, skeletal muscle 1	Sarcomere
SYNE1	6q25	550 kb	147	Synaptic nuclear envelope protein 1 (nesprin 1)	Nuclear membrane / links nucleo-skeleton & inner nuclear membrane to outer nuclear membrane & cytoskeleton
DAG1	3p21		3	Dystroglycan-1 (cleaved to α- & β-dystroglycan)	Sarcolemma / component(s) of DGC; binds to extracellular laminin

Data collated from the following sources: http://www.genecards.org/; http://neuromuscular.wustl.edu/; http://www.omim.org/

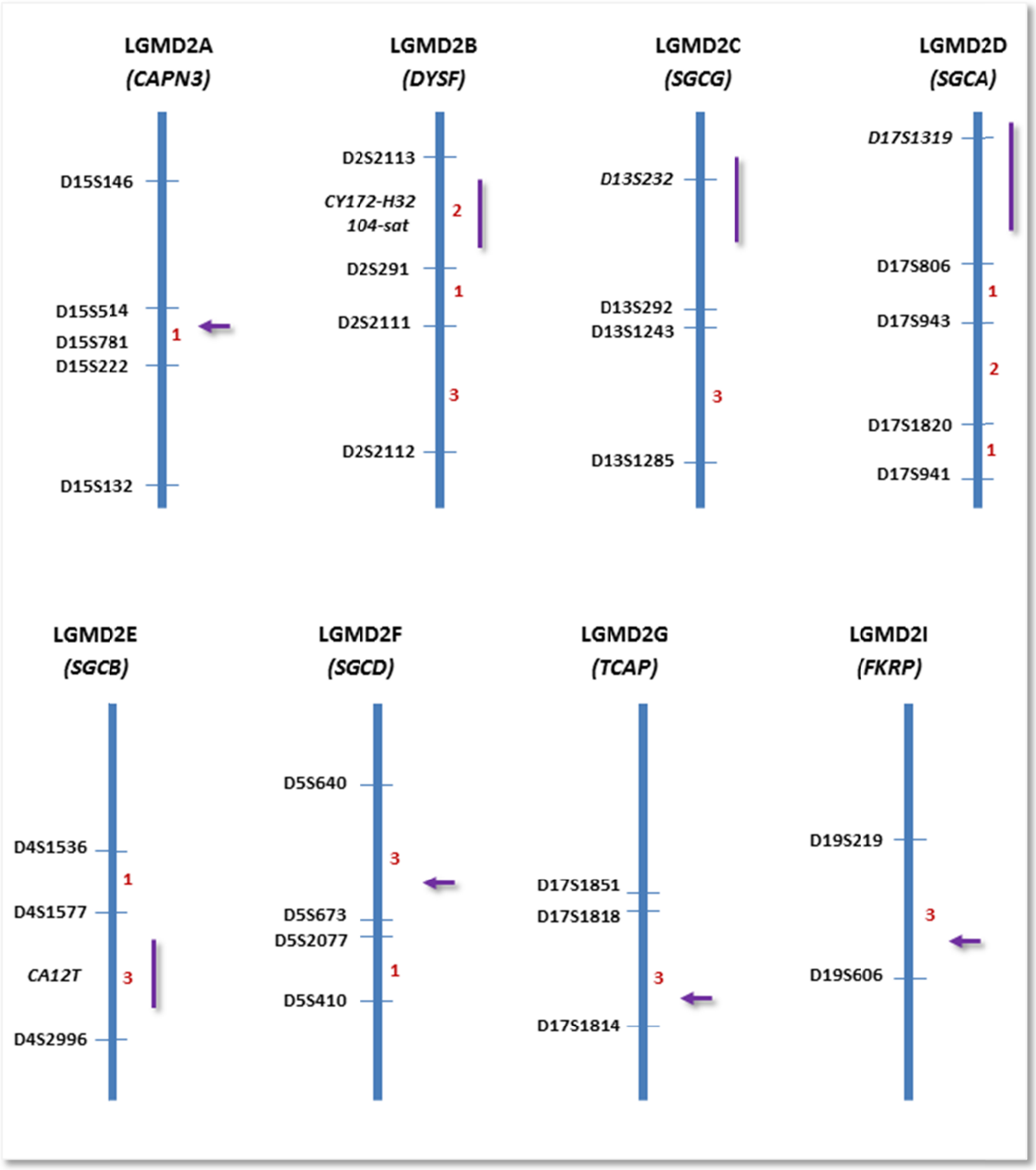
7.2 APPENDIX II: Linkage Analysis

7.2.1 Polymorphic markers linked to *DMD* and the most common LGMD genes

Gene	Marker	PIC
CAPN3	D15S146	0,68
	D15S514	0,75
	D15S781	0,61
	D15S222	0,74
	D15S132	0,74
DYSF	D2S2113	0,83
	D2S291	0,73
	D2S2111	?
	D2S2112	?
	CY172-H32	0.72
	104-sat	0,7
SGCG	D13S232	0,76
	D13S1243	0,78
	D13S292	0,66
	D13S1285	0,76
SGCA	D17S1319	0,76
	D17S943	0,73
	D17S1820	0,82
	D17S806	0,89
	D17S941	0,68
SGCB	D4S1536	0,62
	D4S1577	0,52
	CA12T	0,7
	D4S2996	0,8

Gene	Marker	PIC
SGCD	D5S410	0,79
	D5S640	0,78
	D5S2077	0,74
	D5S673	O,81
DMD	5'DYS III	0,6
	5'DYS I	0,6
	5'DYS II	0,8
	5-'5n3	0,8
	DMDSTR07A	0,7
	DMDSTR07B	0,4
	5'-7n4	0,5
	STR-44	0,9
	P20-CA	0,8
	STR-45	0,9
	STR-49	0,9
	STR-50	0,7
	J66	0,6
	MP1P	0,2
	3'19-n8	0,6
TCAP	D17S1851	0,82
	D17S1818	0,84
	D17S1814	0,79
FKRP	D19S219	0,77
	D19S606	0,81

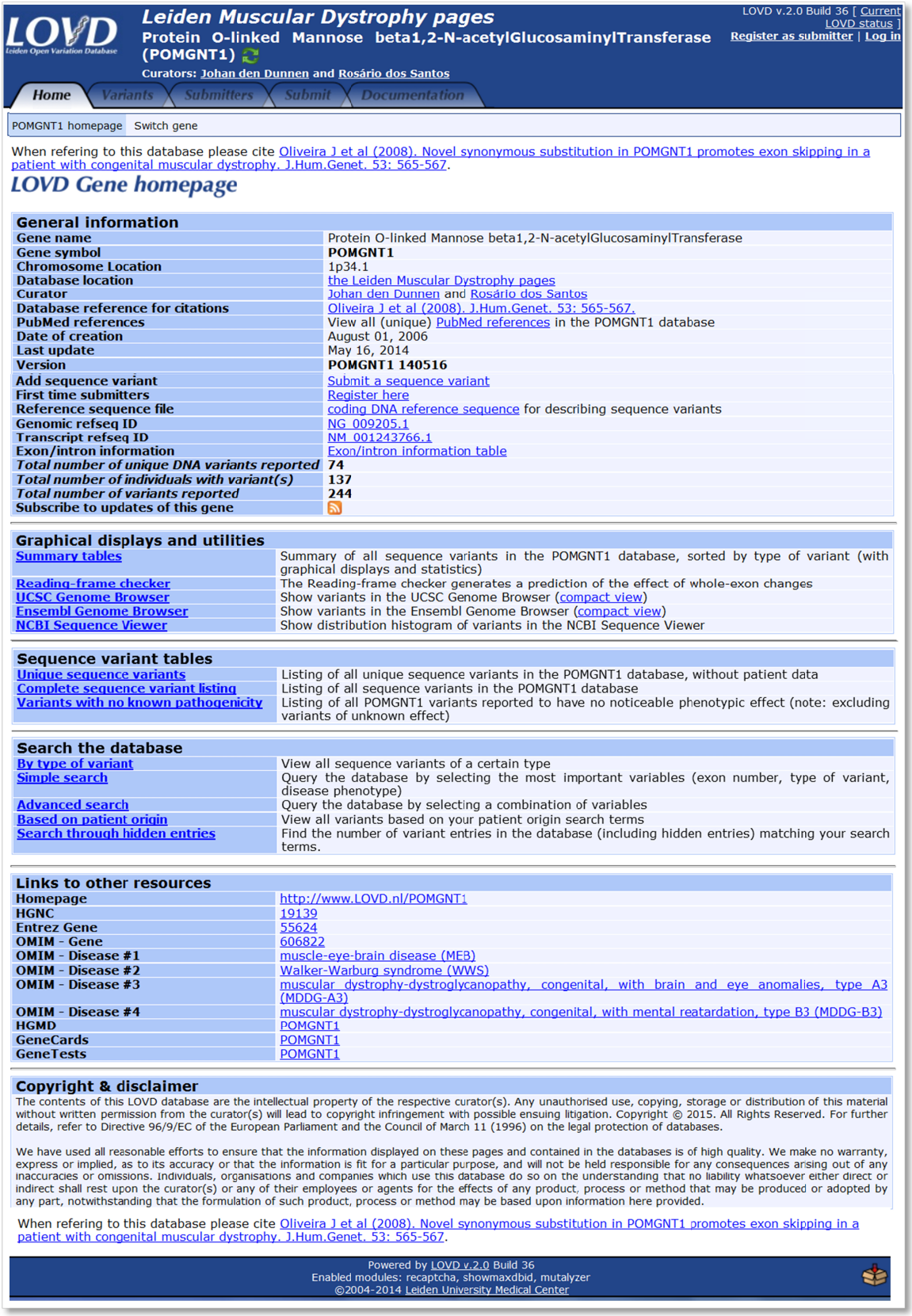
7.2.2 Map of polymorphic markers linked to the most common LGMD genes



LEGEND: Genetic distance between markers (cM) is shown in red. Purple arrows and bars indicate gene *loci*.

7.3 APPENDIX III: LOVD locus specific databases_Example

7.3.1 Screen print of *POMGNT1* LSDB homepage



LOVD v.2.0 Build 36 [Current LOVD status]
Register as submitter | Log in

Leiden Muscular Dystrophy pages
Protein O-linked Mannose beta1,2-N-acetylGlucosaminylTransferase (POMGNT1)
Curators: Johan den Dunnen and Rosário dos Santos

Home | Variants | Submitters | Submit | Documentation

POMGNT1 homepage | Switch gene

When refering to this database please cite Oliveira J et al (2008). Novel synonymous substitution in POMGNT1 promotes exon skipping in a patient with congenital muscular dystrophy. J.Hum.Genet. 53: 565-567.

LOVD Gene homepage

General information	
Gene name	Protein O-linked Mannose beta1,2-N-acetylGlucosaminylTransferase
Gene symbol	**POMGNT1**
Chromosome Location	1p34.1
Database location	the Leiden Muscular Dystrophy pages
Curator	Johan den Dunnen and Rosário dos Santos
Database reference for citations	Oliveira J et al (2008). J.Hum.Genet. 53: 565-567.
PubMed references	View all (unique) PubMed references in the POMGNT1 database
Date of creation	August 01, 2006
Last update	May 16, 2014
Version	**POMGNT1 140516**
Add sequence variant	Submit a sequence variant
First time submitters	Register here
Reference sequence file	coding DNA reference sequence for describing sequence variants
Genomic refseq ID	NG_009205.1
Transcript refseq ID	NM_001243766.1
Exon/intron information	Exon/intron information table
Total number of unique DNA variants reported	**74**
Total number of individuals with variant(s)	**137**
Total number of variants reported	**244**
Subscribe to updates of this gene	

Graphical displays and utilities	
Summary tables	Summary of all sequence variants in the POMGNT1 database, sorted by type of variant (with graphical displays and statistics)
Reading-frame checker	The Reading-frame checker generates a prediction of the effect of whole-exon changes
UCSC Genome Browser	Show variants in the UCSC Genome Browser (compact view)
Ensembl Genome Browser	Show variants in the Ensembl Genome Browser (compact view)
NCBI Sequence Viewer	Show distribution histogram of variants in the NCBI Sequence Viewer

Sequence variant tables	
Unique sequence variants	Listing of all unique sequence variants in the POMGNT1 database, without patient data
Complete sequence variant listing	Listing of all sequence variants in the POMGNT1 database
Variants with no known pathogenicity	Listing of all POMGNT1 variants reported to have no noticeable phenotypic effect (note: excluding variants of unknown effect)

Search the database	
By type of variant	View all sequence variants of a certain type
Simple search	Query the database by selecting the most important variables (exon number, type of variant, disease phenotype)
Advanced search	Query the database by selecting a combination of variables
Based on patient origin	View all variants based on your patient origin search terms
Search through hidden entries	Find the number of variant entries in the database (including hidden entries) matching your search terms.

Links to other resources	
Homepage	http://www.LOVD.nl/POMGNT1
HGNC	19139
Entrez Gene	55624
OMIM - Gene	606822
OMIM - Disease #1	muscle-eye-brain disease (MEB)
OMIM - Disease #2	Walker-Warburg syndrome (WWS)
OMIM - Disease #3	muscular dystrophy-dystroglycanopathy, congenital, with brain and eye anomalies, type A3 (MDDG-A3)
OMIM - Disease #4	muscular dystrophy-dystroglycanopathy, congenital, with mental reatardation, type B3 (MDDG-B3)
HGMD	POMGNT1
GeneCards	POMGNT1
GeneTests	POMGNT1

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When refering to this database please cite Oliveira J et al (2008). Novel synonymous substitution in POMGNT1 promotes exon skipping in a patient with congenital muscular dystrophy. J.Hum.Genet. 53: 565-567.

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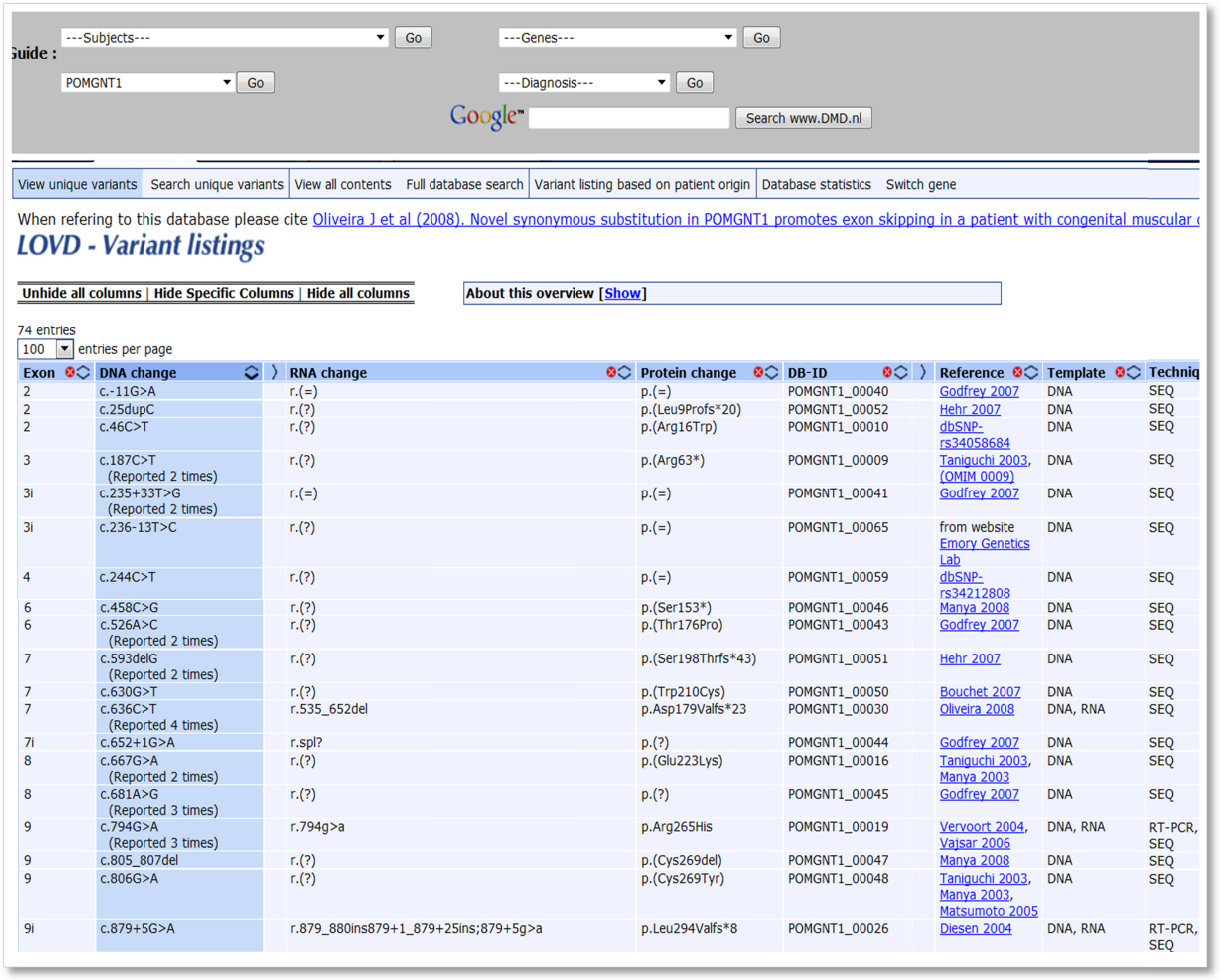
7.3.2 Screen print of *POMGNT1* LSDB "Unique sequence variants" tab

View of partial list of parameters annotated for each variant.



Guide : ---Subjects--- Go ---Genes--- Go

POMGNT1 Go ---Diagnosis--- Go

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View unique variants | Search unique variants | View all contents | Full database search | Variant listing based on patient origin | Database statistics | Switch gene

When refering to this database please cite Oliveira J et al (2008). Novel synonymous substitution in POMGNT1 promotes exon skipping in a patient with congenital muscular

LOVD - Variant listings

Unhide all columns | Hide Specific Columns | Hide all columns

About this overview [Show]

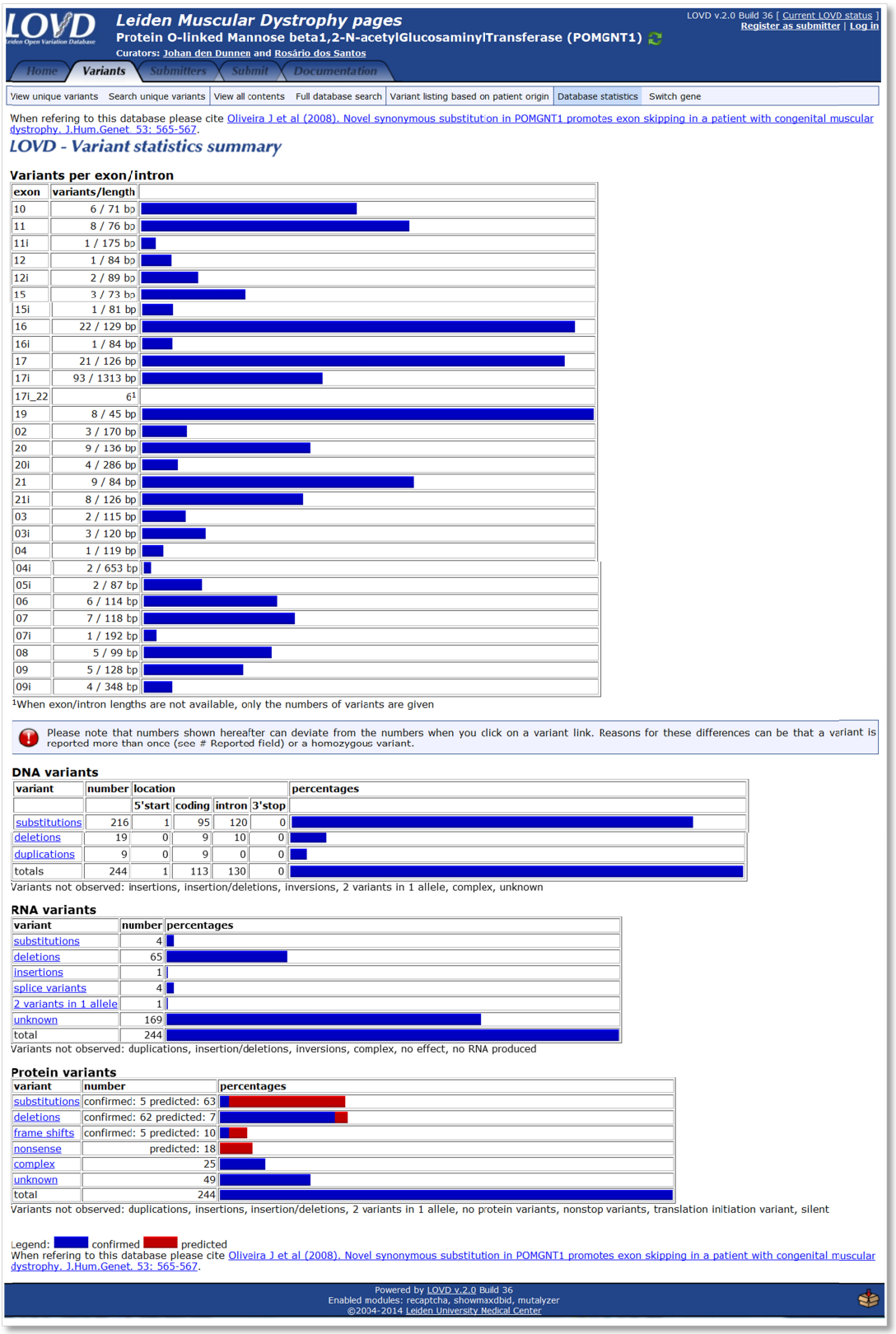
74 entries

100 entries per page

Exon	DNA change	RNA change	Protein change	DB-ID	Reference	Template	Techniq
2	c.-11G>A	r.(=)	p.(=)	POMGNT1_00040	Godfrey 2007	DNA	SEQ
2	c.25dupC	r.(?)	p.(Leu9Profs*20)	POMGNT1_00052	Hehr 2007	DNA	SEQ
2	c.46C>T	r.(?)	p.(Arg16Trp)	POMGNT1_00010	dbSNP-rs34058684	DNA	SEQ
3	c.187C>T (Reported 2 times)	r.(?)	p.(Arg63*)	POMGNT1_00009	Taniguchi 2003, (OMIM 0009)	DNA	SEQ
3i	c.235+33T>G (Reported 2 times)	r.(=)	p.(=)	POMGNT1_00041	Godfrey 2007	DNA	SEQ
3i	c.236-13T>C	r.(?)	p.(=)	POMGNT1_00065	from website Emory Genetics Lab	DNA	SEQ
4	c.244C>T	r.(?)	p.(=)	POMGNT1_00059	dbSNP-rs34212808	DNA	SEQ
6	c.458C>G	r.(?)	p.(Ser153*)	POMGNT1_00046	Manya 2008	DNA	SEQ
6	c.526A>C (Reported 2 times)	r.(?)	p.(Thr176Pro)	POMGNT1_00043	Godfrey 2007	DNA	SEQ
7	c.593delG (Reported 2 times)	r.(?)	p.(Ser198Thrfs*43)	POMGNT1_00051	Hehr 2007	DNA	SEQ
7	c.630G>T	r.(?)	p.(Trp210Cys)	POMGNT1_00050	Bouchet 2007	DNA	SEQ
7	c.636C>T (Reported 4 times)	r.535_652del	p.Asp179Valfs*23	POMGNT1_00030	Oliveira 2008	DNA, RNA	SEQ
7i	c.652+1G>A	r.spl?	p.(?)	POMGNT1_00044	Godfrey 2007	DNA	SEQ
8	c.667G>A (Reported 2 times)	r.(?)	p.(Glu223Lys)	POMGNT1_00016	Taniguchi 2003, Manya 2003	DNA	SEQ
8	c.681A>G (Reported 3 times)	r.(?)	p.(?)	POMGNT1_00045	Godfrey 2007	DNA	SEQ
9	c.794G>A (Reported 3 times)	r.794g>a	p.Arg265His	POMGNT1_00019	Vervoort 2004, Vajsar 2005	DNA, RNA	RT-PCR, SEQ
9	c.805_807del	r.(?)	p.(Cys269del)	POMGNT1_00047	Manya 2008	DNA	SEQ
9	c.806G>A	r.(?)	p.(Cys269Tyr)	POMGNT1_00048	Taniguchi 2003, Manya 2003, Matsumoto 2005	DNA	SEQ
9i	c.879+5G>A	r.879_880ins879+1_879+25ins;879+5g>a	p.Leu294Valfs*8	POMGNT1_00026	Diesen 2004	DNA, RNA	RT-PCR, SEQ

7.3.3 Screen print of *POMGNT1* LSDB "Summary tables" tab

Visual translation of database statistics.



LOVD Leiden Muscular Dystrophy pages

Protein O-linked Mannose beta1,2-N-acetylGlucosaminylTransferase (POMGNT1)

Curators: Johan den Dunnen and Rosário dos Santos

LOVD v.2.0 Build 36 [Current LOVD status]
Register as submitter | Log in

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When refering to this database please cite Oliveira J et al (2008). Novel synonymous substitution in POMGNT1 promotes exon skipping in a patient with congenital muscular dystrophy. J.Hum.Genet. 53: 565-567.

LOVD - Variant statistics summary

Variants per exon/intron

exon	variants/length
10	6 / 71 bp
11	8 / 76 bp
11i	1 / 175 bp
12	1 / 84 bp
12i	2 / 89 bp
15	3 / 73 bp
15i	1 / 81 bp
16	22 / 129 bp
16i	1 / 84 bp
17	21 / 126 bp
17i	93 / 1313 bp
17i_22	6[1]
19	8 / 45 bp
02	3 / 170 bp
20	9 / 136 bp
20i	4 / 286 bp
21	9 / 84 bp
21i	8 / 126 bp
03	2 / 115 bp
03i	3 / 120 bp
04	1 / 119 bp
04i	2 / 653 bp
05i	2 / 87 bp
06	6 / 114 bp
07	7 / 118 bp
07i	1 / 192 bp
08	5 / 99 bp
09	5 / 128 bp
09i	4 / 348 bp

[1]When exon/intron lengths are not available, only the numbers of variants are given

Please note that numbers shown hereafter can deviate from the numbers when you click on a variant link. Reasons for these differences can be that a variant is reported more than once (see # Reported field) or a homozygous variant.

DNA variants

variant	number	location				percentages
		5'start	coding	intron	3'stop	
substitutions	216	1	95	120	0	
deletions	19	0	9	10	0	
duplications	9	0	9	0	0	
totals	244	1	113	130	0	

Variants not observed: insertions, insertion/deletions, inversions, 2 variants in 1 allele, complex, unknown

RNA variants

variant	number	percentages
substitutions	4	
deletions	65	
insertions	1	
splice variants	4	
2 variants in 1 allele	1	
unknown	169	
total	244	

Variants not observed: duplications, insertion/deletions, inversions, complex, no effect, no RNA produced

Protein variants

variant	number	percentages
substitutions	confirmed: 5 predicted: 63	
deletions	confirmed: 62 predicted: 7	
frame shifts	confirmed: 5 predicted: 10	
nonsense	predicted: 18	
complex	25	
unknown	49	
total	244	

Variants not observed: duplications, insertions, insertion/deletions, 2 variants in 1 allele, no protein variants, nonstop variants, translation initiation variant, silent

Legend: confirmed predicted

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