

# Diagnostic approach to the congenital muscular dystrophies <sup>☆</sup>

Carsten G. Bönnemann<sup>a,\*</sup>, Ching H. Wang<sup>b</sup>, Susana Quijano-Roy<sup>c</sup>, Nicolas Deconinck<sup>d</sup>, Enrico Bertini<sup>e</sup>, Ana Ferreira<sup>f</sup>, Francesco Muntoni<sup>g</sup>, Caroline Sewry<sup>g</sup>, Christophe Bérout<sup>h</sup>, Katherine D. Mathews<sup>i</sup>, Steven A. Moore<sup>i</sup>, Jonathan Bellini<sup>j</sup>, Anne Rutkowski<sup>k</sup>, Kathryn N. North<sup>l</sup>, and Members of the International Standard of Care Committee for Congenital Muscular Dystrophies

<sup>a</sup> National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, United States

<sup>b</sup> Driscoll Children's Hospital, Corpus Christi, TX, United States

<sup>c</sup> Hôpital Raymond Poincaré, Garches, and UFR des sciences de la santé Simone Veil (UVSQ), France

<sup>d</sup> Hôpital Universitaire des Enfants Reine Fabiola, Brussels and Ghent University Hospital, Ghent, Belgium

<sup>e</sup> Bambino Gesù Children's Research Hospital, Rome, Italy

<sup>f</sup> UMR787 INSERM/UPMC and Reference Center for Neuromuscular Disorders, Groupe Hospitalier Pitié-Salpêtrière, Paris, France

<sup>g</sup> Dubowitz Neuromuscular Centre, UCL Institute of Child Health, London, United Kingdom

<sup>h</sup> INSERM U827, Laboratoire de Génétique Moléculaire, Montpellier, France

<sup>i</sup> University of Iowa, Iowa City, IA, United States

<sup>j</sup> Stanford University School of Medicine, Stanford, CA, United States

<sup>k</sup> Kaiser SCPMB, Los Angeles, CA, United States

<sup>l</sup> Murdoch Childrens Research Institute, Melbourne, Victoria, Australia

Received 4 November 2013; received in revised form 23 December 2013; accepted 31 December 2013

## Abstract

Congenital muscular dystrophies (CMDs) are early onset disorders of muscle with histological features suggesting a dystrophic process. The congenital muscular dystrophies as a group encompass great clinical and genetic heterogeneity so that achieving an accurate genetic diagnosis has become increasingly challenging, even in the age of next generation sequencing. In this document we review the diagnostic features, differential diagnostic considerations and available diagnostic tools for the various CMD subtypes and provide a systematic guide to the use of these resources for achieving an accurate molecular diagnosis. An International Committee on the Standard of Care for Congenital Muscular Dystrophies composed of experts on various aspects relevant to the CMDs performed a review of the available literature as well as of the unpublished expertise represented by the members of the committee and their contacts. This process was refined by two rounds of online surveys and followed by a three-day meeting at which the conclusions were presented and further refined. The combined consensus summarized in this document allows the physician to recognize the presence of a CMD in a child with weakness based on history, clinical examination, muscle biopsy results, and imaging. It will be helpful in suspecting a specific CMD subtype in order to prioritize testing to arrive at a final genetic diagnosis.

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**Keywords:** Congenital muscular dystrophy; Collagen VI; Laminin alpha2; Alpha-dystroglycan; SEPNI; Lamin A/C; RYR1; Diagnostic guideline

<sup>☆</sup> See table of abbreviations at end of paper.

\* Corresponding author. Address: Neuromuscular and Neurogenetic Disorders of Childhood Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 35 Convent Drive, Building 35, Room 2A-116, Bethesda, MD, United States. Tel.: +1 301 594 5496; fax: +1 301 480 3365.

E-mail address: [carsten.bonnemann@nih.gov](mailto:carsten.bonnemann@nih.gov) (C.G. Bönnemann).

## 1. Introduction

The congenital muscular dystrophies (CMDs) and the congenital myopathies (non-dystrophic myopathies with characteristic histological and histochemical findings) constitute the two most important groups of congenital onset muscle disease. The CMDs are defined as early onset muscle disorders in which the muscle biopsy is compatible with the presence of a dystrophic process (even if not fully developed) without histological evidence of another neuromuscular disease [1,2]. However, it has become clear that there is overlap between the CMDs and the congenital myopathies on the clinical, morphological and genetic level. For example, mutations

in RYR1 and SEPN1 can cause both core disorders (belonging to the congenital myopathies) as well as CMD-like presentations. The clinical as well as genetic complexity of the disorders subsumed under the CMDs has resulted in different genetic as well as clinical classification schemes [3–5]. Also, the genetic nomenclature used is not always consistent. For instance MDC1A (muscular dystrophy, congenital, type 1A) refers to disease caused by mutations in LAMA2, but this nomenclature system has not been systematically carried forward for all CMDs. Table 1 lists most currently used names and symbols for reference. Gene symbols in this review are not italicized. We have used the gene or protein name annotated by “–related dystrophy (–RD)”

Table 1  
Brief CMD classification overview (underlined: abbreviated nomenclature used in this paper).

| Subtype and alternate nomenclatures<br>Associated Genes  | Associated phenotypic spectrum  |
|--|---|
| Collagen VI related dystrophies ( <u>COL6-RD</u> )<br>COL6A1, COL6A2, COL6A3   | <ul style="list-style-type: none"> <li>■ Ullrich congenital muscular dystrophy (UCMD) – severe nonambulant and transient ambulant</li> <li>■ Intermediate phenotype</li> <li>■ Bethlem myopathy (BM, milder disease course)</li> </ul>  |
| Laminin $\alpha$ 2 related dystrophy ( <u>LAMA2-RD</u> , includes MDC1A, Merosin deficient CMD, LAMA2-CMD)<br>LAMA2  | <ul style="list-style-type: none"> <li>■ Non-ambulant LAMA2-RD</li> <li>■ Ambulant LAMA2-RD</li> <li>■ Non-ambulant typically correlates with absent laminin <math>\alpha</math>2 staining on muscle biopsy and ambulant with partial deficiency (with exceptions)</li> </ul>   |
| $\alpha$ Dystroglycan related dystrophy ( <u><math>\alpha</math>DG-RD</u> , also alpha dystroglycanopathy, $\alpha$ DGpathy)<br>FKRP, FKTN, POMT1, POMT2, POMGnT1, LARGE, ISPD, GTDC2, DAG1, TMEM5, B3GALNT2, B3GNT1, GMPPB, SGK196 (DPM1, DPM2, DPM3, DOLK) | <ul style="list-style-type: none"> <li>■ Walker–Warburg syndrome</li> <li>■ Muscle–eye–brain disease; Fukuyama CMD; Fukuyama-like CMD</li> <li>■ CMD with cerebellar involvement; cerebellar abnormalities may include cysts, hypoplasia, and dysplasia</li> <li>■ CMD with mental retardation and a structurally normal brain on imaging; this category includes patients with isolated microcephaly or minor white matter changes evident on MRI</li> <li>■ CMD with no mental retardation; no evidence of abnormal cognitive development</li> <li>■ Limb-girdle muscular dystrophy (LGMD) with mental retardation (milder weakness, maybe later onset) and a structurally normal brain on imaging</li> <li>■ LGMD without mental retardation (milder weakness, maybe later onset)</li> </ul> |
| SEPN1 related myopathy ( <u>SEPN1-RM</u> , also rigid spine CMD, RSMD1)<br>SEPN1   | <ul style="list-style-type: none"> <li>■ Consistent rigid spine early respiratory failure phenotype</li> <li>■ despite variable histological presentations as multimicore disease, desmin positive Mallory body inclusions, congenital fiber-type disproportion, mild CMD, or nonspecific myopathy</li> </ul>   |
| RYR1 related myopathy ( <u>RYR1-RM</u> , includes RYR1-CMD)<br>RYR1  | <ul style="list-style-type: none"> <li>■ RYR1 related myopathies (RYR1-RM) include central core, multi-mini-core, centronuclear and nonspecific pathologies, which can assume CMD like characteristics</li> <li>■ Clinically significant for early scoliosis and absent or limited ambulation</li> </ul>  |
| LMNA related dystrophy ( <u>LMNA-RD</u> , includes LMNA-CMD, L-CMD, and Emery Dreifuss)<br>LMNA  | <ul style="list-style-type: none"> <li>■ CMD presentation: Dropped head syndrome, axial and scapuloperoneal involvement, absent or early loss of ambulation</li> <li>■ Milder presentations fuse with early-onset Emery–Dreifuss muscular dystrophy</li> </ul>  |
| CMD without genetic diagnosis  | <ul style="list-style-type: none"> <li>■ Congenital onset weakness with CMD compatible histology and variable clinical features, without confirmed genetic diagnosis, despite testing for currently known genes</li> </ul>  |

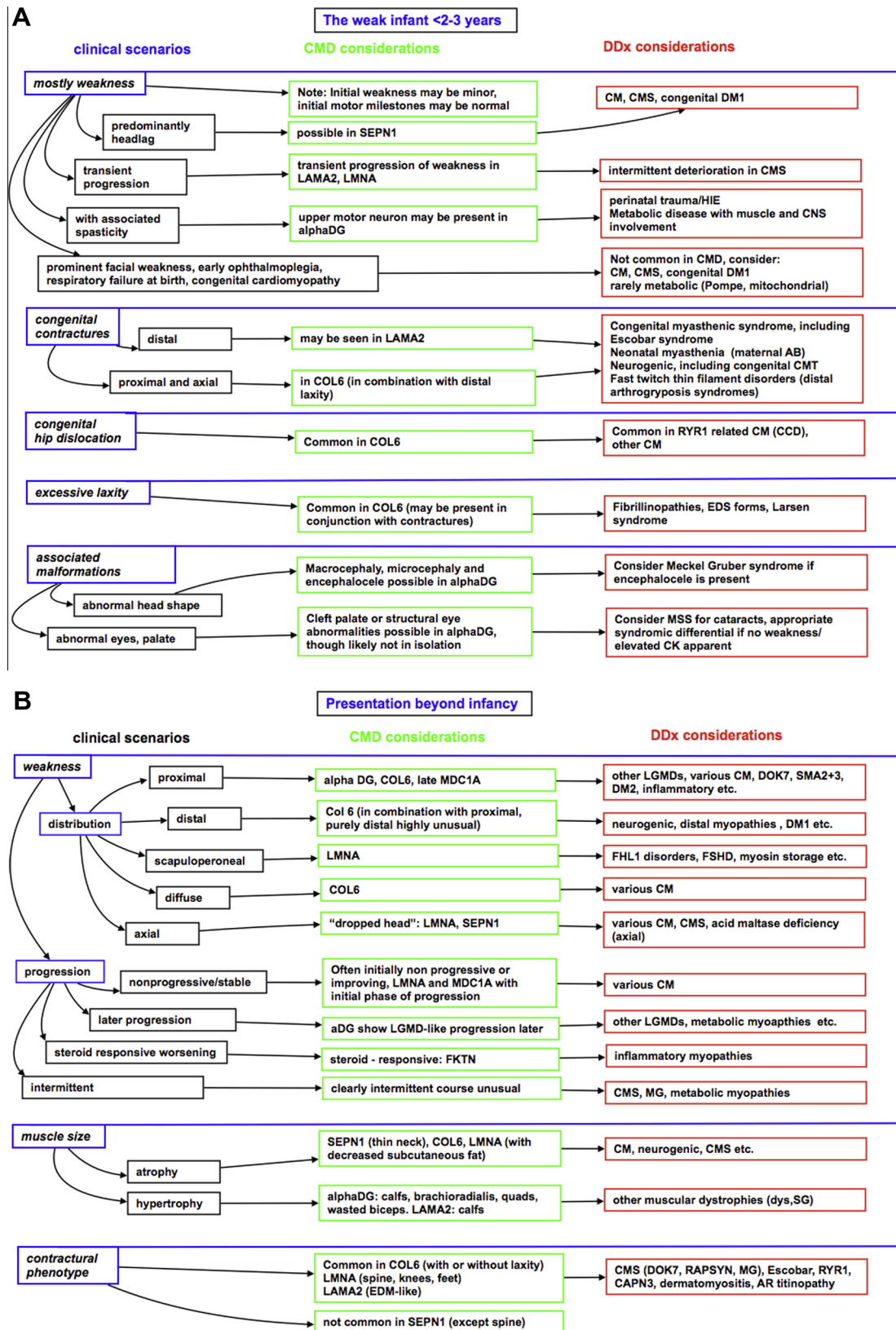


Fig. 1. A–D: Differential diagnostic considerations for various clinical findings in infancy (A) and beyond infancy (B and C), as well as for various laboratory findings that may be available at the outset of the diagnostic encounter (D). *Note:* The most important tools in the clinical differential diagnosis are: EMG/NCV to diagnose neurogenic involvement, muscle biopsy, and selective biochemical and genetic testing. The differential diagnostic considerations are not exhaustive but highlight a few of the more relevant conditions to consider with a given clinical picture. To save space we are only using the gene/protein symbols to indicate specific diagnosis.

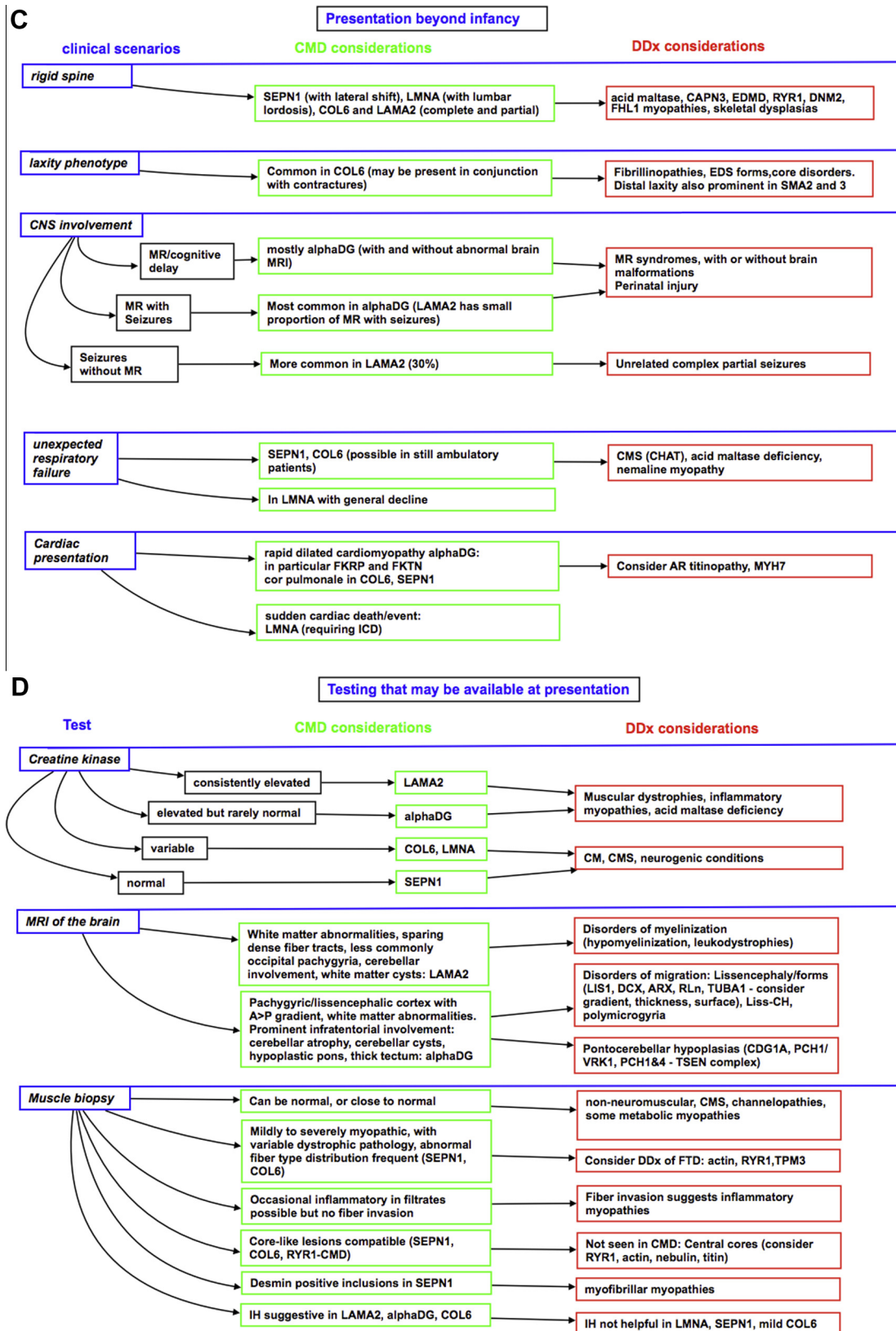


Fig 1. (continued)

or “-related myopathy (-RM)” for several of the CMD phenotypic classes to reflect the type of pathology that is more typically encountered in a biopsy for the subtype

while allowing for a broad phenotypic and histopathological spectrum associated with the respective primary gene. If we are referring specifically to the



congenital onset dystrophy without including later onset presentations we use “-related CMD”. Myopathy here is meant to reflect a pathology without clear evidence of degeneration and regeneration in the majority of cases, although such features may be evident in some cases. The conditions designated as “related myopathy” are also those that may have presentations as more typical congenital myopathies.

The incidence and prevalence of CMD in various populations is not sufficiently known and may have been underestimated in early published CMD surveys owing to more limited diagnostic means available. Point prevalence in early studies ranges from 0.68 to 2.5 per 100,000 [6–9]. The relative frequency of individual types also varies in different populations. In Japan, the most commonly diagnosed CMD subtype is Fukuyama CMD caused by a founder mutation in the fukutin gene, followed by COL6-RD [10], while fukutin mutations are very rare in other populations [11,12].

Individual CMD forms are rare so that only highly specialized centers have the combined diagnostic experience and technology to cover all subtypes. It thus frequently falls on the primary pediatric, neuromuscular provider or pathologist caring for a patient with suspected CMD to coordinate and interpret data and results from different disciplines and laboratories in an effort to achieve a diagnosis for an individual patient. Establishing a molecular diagnosis however is of importance for genetic and prenatal counseling, prognosis and anticipatory management, and also for future stratification for clinical trials and treatment approaches that are specific for an individual subtype or even are mutation-specific. In an effort to arrive at consensus guidelines for achieving a specific genetic diagnosis in an individual patient, an international group involving the majority of experts in the field have participated in working groups and meetings to summarize currently available data and literature, unpublished experience, and individual expertise to develop a rational and comprehensive approach to the specific diagnosis of the heterogeneous disorders currently subsumed under CMD.

## 2. General clinical findings in the congenital muscular dystrophies

(See diagnostic schematics “On Presentation, Fig. 1A–C”.)

*Initial presentation and its differential diagnosis:* When presenting in infancy there are certain clinical signs that point towards or are compatible with a CMD diagnosis, while other presenting features make a diagnosis of CMD much less likely (specific clinical findings will be discussed under diagnostic aspects of the CMD subtypes below). The most important differential diagnostic considerations for the hypotonic and weak infant outside of CMD (and excluding systemic metabolic and acquired conditions) are the congenital myopathies, congenital myasthenic

syndromes, congenital metabolic myopathies, very early SMA and amongst the non-neuromuscular genetic conditions in particular Prader–Willi syndrome. Fig. 1A contains a diagnostic schematic for infants <2–3 years starting with clinical findings and linking them to the diagnostic subtype considerations within the CMD and also to differential diagnostic considerations outside of CMD.

*Presentation at an older age and its differential diagnosis:* It is not infrequent that a patient may present for diagnosis at an older age either because a definitive CMD diagnosis has not yet been established despite congenital onset or because symptom onset or symptom recognition had been delayed. Several clinical clues help to arrive at a clinical and finally a molecular diagnosis. Fig. 1 B and C cover clinical findings in the older child and adult, similarly starting from clinical observations such as the distribution of weakness and linking them to diagnostic subtype considerations as well as to differential diagnostic considerations outside of CMD.

*Initial testing available:* CK levels can be normal in SEPNI-RM and is often normal or only mildly elevated in COL6-RD, however, it is consistently elevated in LAMA2-RD and elevated most of the time (but not in 100% of patients) in  $\alpha$ DG-RD. Brain MRI can help support the clinical diagnosis in the  $\alpha$ DG-RD and LAMA2-RD (see below). While EMG/nerve conduction testing findings are not diagnostic in CMD they often show myopathic features (in LAMA2-RD they commonly also show peripheral motor and sensory neuropathy of lower extremities [13–15]). A typical decremental response on repetitive stimulation is not compatible with a CMD diagnosis and should suggest a congenital myasthenic syndrome.

## 3. Diagnostic aspects of specific subtypes

### 3.1. LAMA2-CMD-RD (Laminin $\alpha$ 2 related CMD, Merosin deficient CMD, MDC1A)

*Diagnostic considerations:* Laminin  $\alpha$ 2 related CMD is caused by mutations in the LAMA2 gene, encoding the  $\alpha$ 2 heavy chain of the laminin 211 isoform ( $\alpha$  2/ $\beta$ 1/ $\gamma$ 1), also known as merosin [16–19]. In the genetic nomenclature, this CMD subtype is also referred to as MDC1A. Complete absence of laminin  $\alpha$ 2 staining on muscle (or skin biopsy) is more common and in general associated with a more severe non-ambulatory phenotype compared to a partial laminin  $\alpha$ 2 deficiency [20]. Patients (Fig. 2G, F) with complete laminin  $\alpha$ 2 deficiency present at birth with significant hypotonia and weakness of the extremities, which may worsen in the first few weeks of life in some infants. There may be contractures in the hands and feet at birth (arthrogryposis). In patients with complete deficiency the degree of muscle weakness usually precludes independent ambulation, although patients may get to a



Fig. 2. A: Hand of a patient with COL6-RD. Note the significant hyperlaxity even in the most distal interphalangeal joints. B: Foot of an infant with COL6-RD. Note the ability to dorsiflex the foot back to the shin, the soft palmar skin, the pes planus (loss of arch) and the prominent calcaneus. C: Patient with COL6-RD. Note flexible fingers and round face with facial erythema. He also has contractures in the elbows and knees. D: Patient with LMNA-CMD. Note the dropped head, hyperlordosis and adducted foot indicative of peroneal weakness, and overall thinness. E: Patient with SEPNI-RM, note atrophy of inner thigh muscles and lateral deviation of spine (status after surgical rod placement). F: Twins with LAMA2-CMD. Note hypotonic posture with splayed legs (“frog leg” posture), weak arms, flexed fingers and foot contractures. G: Patient with LAMA2-CMD. Note facial weakness and foot contracture. She has no antigravity strength in the upper extremity. H: Patient with  $\alpha$ DG-RD (POMT1). Note weak sitting posture, hypotonic lower face with open mouth characteristic of congenital myopathic disorders. I: Same patient with  $\alpha$ DG-RD (POMT1) at an older age, note calf and quadriceps hypertrophy and mild forearm hypertrophy.

standing position and rarely achieve independent ambulation (2/33 patients in one series) [20]. Partial laminin  $\alpha$ 2 deficiency due to mutations in LAMA2 tends to present with milder and more variable phenotypes, including LGMD-like proximal weakness, and an Emery–Dreifuss like contracture phenotype, although manifestations may also be as severe as in the complete deficient patient [10,20–23]. In LAMA2-RD, particularly in the first 2 years of life, the CK is typically elevated more than five times normal. Typical

findings on brain MRI (Fig. 3A, B) include high signal in the white matter on T2 weighted and FLAIR images and are seen in all patients but are most obvious in patients greater than 6 months of age. The internal capsule, corpus callosum, and other dense fiber tracts are usually spared, but there may be subcortical cyst formation. White matter changes once evident do not require further imaging. White matter abnormalities on MRI are also seen in patients with incomplete deficiency, while patients with very late adulthood onset

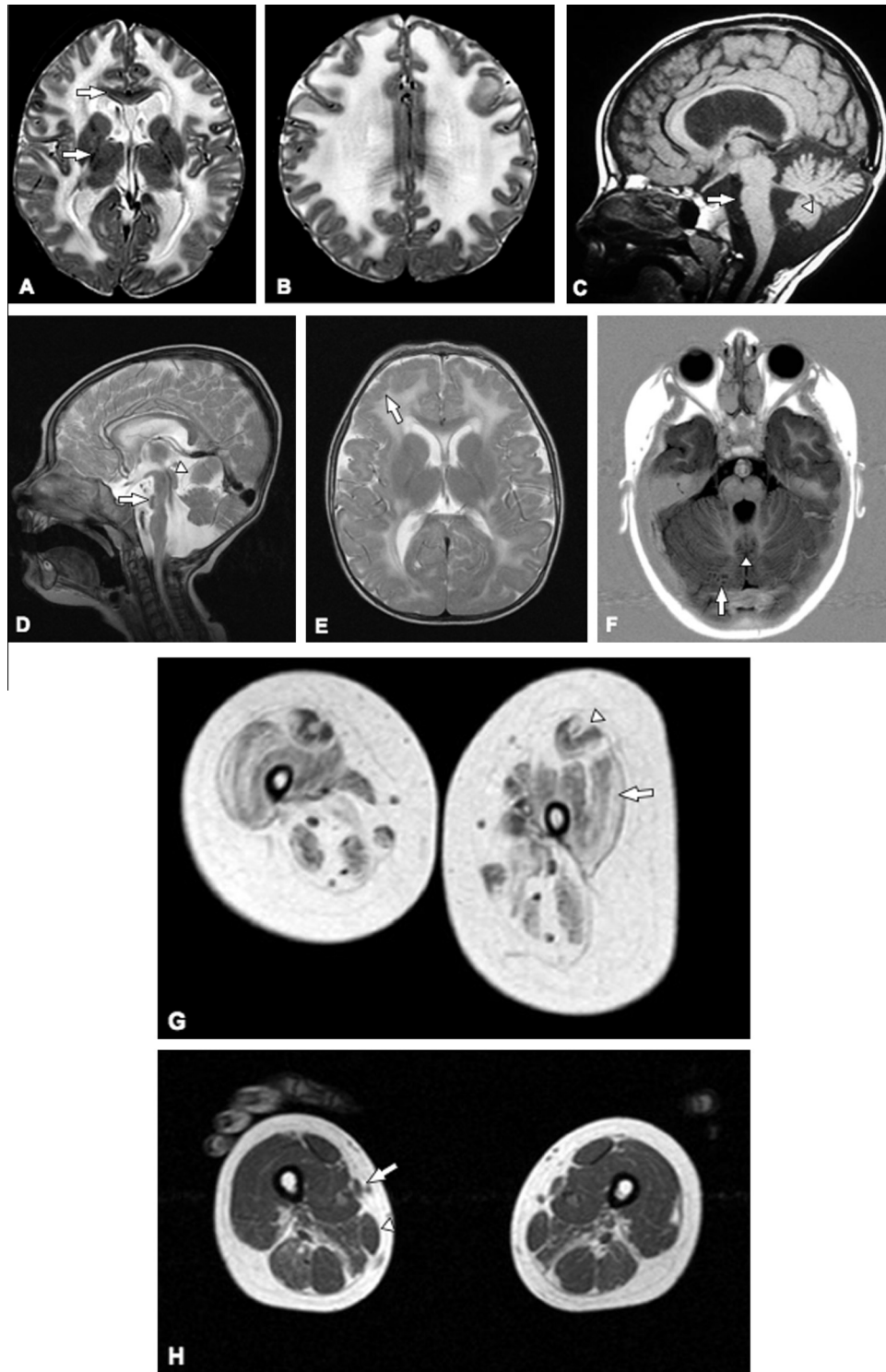


Fig. 3. A and B: T2-weighted brain MR images in LAMA2-CMD. Note extensive signal abnormalities of the cerebral white matter while the corpus callosum and the internal capsule are spared (arrows). C: T1 weighted brain MRI in  $\alpha$ DG-RD (POMT2). Note thinning of the corpus callosum, the relatively flat pons (arrow) and atrophic and dysplastic cerebellar vermis (arrow head). D and E: T2-weighted MR images in  $\alpha$ DG-RD. Note thin corpus callosum, extremely small pons, relatively thick tectum (arrow head), and small and dysplastic cerebellar vermis on the sagittal cut (D). Frontal polymicrogyria (arrow) and abnormal white matter signal is evident on the axial cut (E). F: T1-weighted MR images in  $\alpha$ DG-RD. Note abnormal configuration of the pons and corticospinal tracts and dysplastic cerebellum with cerebellar cysts (arrow) and small vermis (arrow head)). G: T1-TSE weighted thigh MR images in a COL6-RD, a patient with typical phenotypic UCMD presentation. Note in particular the striated aspect of vastus lateralis caused by outer rim of increased signal (arrow) and increased signal around the central fascia of the rectus femoris (arrow head) (courtesy of Dr. R Carlier). H: T1-TSE weighted thigh MR images in SEPNI-RM. Note selective involvement of sartorius (arrow), biceps femoris and adductor magnus and sparing of the gracilis (arrow head).

my have normal brain MRI. A smaller percentage (about 5%) of patients shows more obvious brain structural abnormalities, including a particular occipital cortical dysgenesis with a subcortical band of heterotopia and cerebellar hypoplasia [24]. Seizures occur in about 30% of all patients with LAMA2-RD, including in those with no obvious evidence for a cortical malformation on imaging.

*Selected genotype–phenotype correlations:* Most mutations resulting in typical complete laminin  $\alpha 2$  deficiency are functionally null mutations leading to the absence of the laminin  $\alpha 2$  protein on immunostaining and a more severe non-ambulatory phenotype. 55% of mutations in one series were located in exons 14, 25, 26, 27 [20]. Compound heterozygosity for a null mutation and an in-frame deletion or exon skipping mutations may lead to a milder phenotype with partial deficiency of laminin  $\alpha 2$  [10]. In contrast, in-frame deletions affecting the N-terminal G-domain, critical for binding of laminin isoforms to  $\alpha$ -dystroglycan and various integrins, affect the function of this molecule profoundly, leading to a severe phenotype, even though laminin  $\alpha 2$  may be partially present in the basement membrane by immunohistological exam [25]. Rare homozygous missense mutations have been associated with laminin  $\alpha 2$  deficiency [20]. Affected siblings may demonstrate intra-familial variability for onset and severity of clinical manifestations and degree of laminin  $\alpha 2$  deficiency noted on muscle biopsy immunostaining.

### 3.2. Alpha dystroglycan related dystrophies ( $\alpha$ DG-RD)

*Diagnostic considerations:* The  $\alpha$ DG-RDs, are characterized by reduced O-mannosyl and LARGE-dependent glycosylation of  $\alpha$ -dystroglycan, a sarcolemmal membrane structural protein. This is the result of mutations in the currently 13 genes directly or putatively involved in the glycosylation pathway (POMT1, POMT2, POMGnT1, FKR, Fukutin, LARGE, ISPD, GTDC2, B3GALNT2, B3GNT1, TMEM5, GMPPB, SGK196) [26,27]. A single mutation in dystroglycan (DAG1) that specifically interferes with its glycosylation can lead to an  $\alpha$ DG-RD [28]. Mutations in the dolichyl-phosphate mannosyltransferase subunit genes DPM1, DPM2 and DPM3 cause overlap syndromes of muscular dystrophy with under-glycosylated  $\alpha$ DG in the muscle [29–31], while mutations in the dolichol kinase DOLK are a cause of dilated cardiomyopathy [32].  $\alpha$ DG-RD classifications have been proposed to accommodate the very broad clinical spectrum, ranging from syndromic CMD forms with very severe brain involvement (including WWS, FCMD and Muscle-Eye-Brain disease) to the LGMD spectrum [5,33–42]. It may be difficult to unequivocally classify patients with transitional milder CNS abnormalities including microcephaly, cerebellar hypoplasia with or without cysts or patients with learning

disability with normal appearing MRI presenting with either CMD or LGMD like age of symptom onset with or without achieved ambulation as it may be difficult to assign a delay in acquisition of motor milestones to the global developmental delay as opposed to muscle weakness. Normal cognitive abilities have been encountered in patients with FKR, FKTN, and ISPD mutations while cognitive impairment which ranges from profound mental retardation to mild learning disability has been observed in patients with mutations in all 17 genes (DAG1, POMT1, POMT2, POMGnT1, LARGE, FKR, FKTN, ISPD, GTDC2, B3GNT1, B3GALNT2, GMPPB, TMEM5, SGK196, DPM1, DPM2, DPM3).

In the  $\alpha$ DG-RDs, the spectrum of the muscle involvement is broad for all subtypes, ranging from prenatal onset weakness precluding ambulation, to Duchenne and Becker-like severities (Fig. 2H, I). The distribution of muscle weakness is proximal with a tendency for muscle hypertrophy and pseudohypertrophy in both upper and lower extremities. Scapular winging, lumbar lordosis and a Trendelenburg gait can be present. Some patients have experienced myositis-like rapid decline in function that was partially responsive to steroid treatment [43–45]. Dilated cardiomyopathy is most commonly found in  $\alpha$ DG patients due to FKR and FKTN mutations, especially in those patients at the LGMD end of the clinical spectrum, and less commonly in POMT1 mutations. However, echocardiographic surveillance has to be considered in any dystroglycanopathy patient [46,47]. The hallmark of central nervous system involvement in the  $\alpha$ DG-RD on brain MRI (Fig. 3C–F) is represented by the cobblestone complex, ranging from complete lissencephaly (type II) to more focal pachygyria or polymicrogyria showing a frontal predominance. Similar to LAMA2-RD there may also be an occipital cortical dysplasia with a smooth appearing cortex and an underlying heterotopic band of neurons. Characteristic infratentorial findings may include midbrain hypoplasia, a relatively thick tectum, fused colliculi, a pontomesencephalic kink, ventral pontine cleft, pontocerebellar hypoplasia, abnormalities of cerebellar foliation and cerebellar cysts, which are frequently observed in POMGnT1, and FKR mutations and have recently been described in POMT2 and LARGE patients [48,49]. Some patients may only have frontal polymicrogyria without infratentorial involvement (seen in POMT1, POMT2 and LARGE), while some may only have infratentorial involvement (ISPD) [50]. MRI findings may also include hydrocephalus, and occipital encephalocele. There may be high signal in the white matter on FLAIR or T<sub>2</sub>-weighted images showing patchy or more confluent involvement. In contrast to LAMA2-RD, these white matter abnormalities can regress over time [48,51,52], and are not typically observed in dystroglycanopathy patients with preserved intelligence.

*Selected genotype–phenotype correlations:* The number of  $\alpha$ DG-RD diagnosis without a mutation in one of the



currently known genes is not entirely clear, but it is still significant and additional genes will likely be found. While point mutations are the most common mutation type in all genes, genomic deletions or deletion-insertions have been reported in particular *POMT2* and *LARGE* [49,53,54]. Mutations in *POMGnT1* showed the highest correlation with the typical MEB phenotype [38,55]. Patients homozygous for the ancestral Japanese mutation (insertion of a retrotransposon) in *FKTN* have a comparatively milder phenotype (FCMD), while the disease severity increases towards the MEB and WWS range in patients who are compound heterozygous for this ancestral mutation and a more severe loss-of-function mutation on the other allele [56]. Homozygous null mutations in the human *FKTN* gene have resulted in a WWS-like phenotype [57]. *FKRP*, *FKTN* and *ISPD* mutations are associated with the broadest clinical spectrum to date ranging from WWS to a Becker-like limb girdle muscular dystrophy [51,58,50,59]. The c.826C>A (p.Leu276Ile) mutation in the *FKRP* gene is particularly common in *LGMD2I* patients, but can be associated with a more severe phenotype in the compound heterozygous state depending on second mutation [37,58]. In contrast, most of the CMD associated *FKRP* mutations are unique to individual patients. In the *POMT1* and *POMT2* genes, mutations leading to severe functional defects appear to be associated with severe WWS or MEB phenotypes [53], whereas less disruptive missense changes result in milder phenotypes such as CMD or even *LGMD* with mental retardation and normal MRI [41,60–62].

New genes associated with alphaDG-RD are continuously added reducing the percentage of patients in the alphaDG spectrum without genetic basis: Recessive mutations in the *ISDP* (isoprenoid synthase domain containing protein) have recently been identified as a novel cause for WWS [63,64], but ranging to the milder spectrum with isolated cerebellar involvement and *LGMD* like presentations without cognitive involvement [50]. Mutations in *CTDC2* were found in consanguineous WWS families [65], while *TMEM5* mutations have been identified in aborted fetuses with severe cobblestone lissencephaly typical of aDGpathy [66] and in WWS and MEB [67].  $\beta$ -1,3-*N*-acetylgalactosaminyltransferase 2 (*B3GALNT2*) mutations were shown to cause CMD with brain and eye abnormalities consistent with the alphaDG-RD spectrum [68], while mutations in GDP-mannose pyrophosphorylase B (*GMPPB*) were associated with a spectrum from severe CMD with brain involvement to milder *LGMD* [69], and *SGK196* mutations in one family with WWS [67].

The congenital disorders of glycosylation associated with mutations in the *DPM1*, *DPM2* and *DPM3* [29–31] while showing reduced alpha-dystroglycan and elevated CK, also present with cognitive impairment, microcephaly, cerebellar hypoplasia, feeding difficulties and notably severe myoclonic epilepsy. Recessive mutations in *DOLK* so far present mostly as a dilated cardiomyopathy [32].

### 3.3. Collagen VI related dystrophies (*COL6-RD*)

**Diagnostic considerations:** Collagen VI is an important component of the muscle extracellular matrix where it interacts with the basement membrane of all muscle fibers. Mutations in one of the three collagen 6 alpha genes (*COL6A1*, *COL6A2*, *COL6A3*) can have recessive as well as dominant modes of action and inheritance patterns, leading to the *COL6-RD* spectrum, ranging from early onset, severe Ullrich CMD (UCMD) to an intermediate severity phenotype to milder Bethlem myopathy (BM). (For reviews see [70–73].)

UCMD (Fig. 2A–C) typically presents in the newborn period with striking distal joint hypermobility of the hands and often feet with prominent calcanei, while talipes equinovarus can also occur [1]. Congenital hip dislocation is frequently present. Proximal elbow and knee contractures, kyphoscoliosis, and torticollis may be also present at birth and may improve initially with physical therapy and orthopaedic treatment. Later in life, joint contractures return and progress, in particular in the long finger flexors, shoulders, elbows, knees and hips, and spine becomes stiff with risk of kyphoscoliosis. While some UCMD patients may not achieve the ability to walk, more commonly walking is achieved for some years, and then is lost again in the late first or early second decade of life due to combined progressive hip contractures and increasing weakness. A steady decline in percent predicted forced vital capacity is observed in virtually all Ullrich patients, leading to predominantly night-time respiratory insufficiency [74–76] in which the diaphragm is disproportionately affected [77,78].

In the allelic Bethlem phenotype onset may either be as early as the congenital period but with few conspicuous findings in early childhood such as mild weakness and some degree of joint hypermobility, or clinical recognition may be later. Contractures of the Achilles tendons, pectoralis muscle, elbows and long finger flexors develop progressively. The weakness itself is slowly progressive. Respiratory compromise is also less conspicuous in the Bethlem phenotype, although weakness may progress in adulthood [74,79]. Clinical phenotypes that are intermediate between these two classic presentations include patients with early presentation who are ambulating to late teenage years or young adulthood but still presenting progressive respiratory failure, even while still ambulating [76,78,80]. Characteristic skin findings are diagnostically helpful and include a tendency for keloid or atrophic scar formation, striae, soft velvety skin on palms and soles and hyperkeratosis pilaris [81]. Cognition is normal and often advanced for age. CK is normal or mildly elevated.

Differential diagnostic considerations for milder *COL6-RD* phenotypes with prominent joint contractures include *LAMA2-RD* with partial deficiency, *LMNA-RD* as well as other Emery–Dreifuss muscular dystrophies. In contrast to *LMNA-RD* and Emery–Dreifuss muscular

dystrophy and FHL1-related disorders, the COL6-RD do not develop cardiac involvement. For patients with very prominent joint hypermobility, the relevant differential diagnostic considerations are kyphoscoliotic Ehlers–Danlos syndromes (type VI) and the hypermobile type caused by mutations in tenascin X [82]. Recently recognized EDS/myopathy overlap syndromes to consider in the differential diagnosis include a form with severe kyphoscoliosis and myopathy due to FKBP14 mutations [83] and forms due to mutations in collagen XII ranging from severe and precluding ambulation to milder presentations [84,85]. Analysis of collagen VI in dermal fibroblast cultures may add sensitivity to the biochemical testing [73,86–88]. The availability of fibroblast cultures also allows for genetic testing and confirmation of splice mutations on fibroblast derived cDNA. This type of analysis is currently only available in research laboratories and is often needed to confirm a molecular diagnosis given the important role of splice mutations.

*Selective genotype–phenotype correlations:* Even though two new collagen VI related genes have been identified in humans (COL6A5 and COL6A6 [89,90]), all cases of COL6-RD identified to date are related to mutations in the original COL6A1–3 genes with genotype–phenotype correlations established in larger published cohorts of similar mutations [11,21,73,91,92]. Mutations underlying the severe end of the spectrum are typically recessive loss of function mutations that prevent any chains from assembling [21], occasional recessive missense mutations [93], and importantly de novo dominant negative mutations [21,92]. Dominant negatively acting mutations are usually in-frame exon skipping mutations or glycine missense mutations of the collagenous Gly-X-Y motif at the N-terminal end of the triple helical domain, allowing them to be carried forward in the assembly [21,91,92,94,95]. Bethlem myopathy is typically caused dominantly acting mutations with less severe functional impact, [91,94], while recessive mutations are less common [96,97]. In particular dominantly acting glycine missense mutations are associated with a phenotypic range that extends from typical Ullrich CMD to Bethlem, and are also responsible for large number of patients in the intermediate severity group discussed earlier [73,91]. Large exonic or even the whole gene deletions that will not be recognized by exon sequencing based testing can occur in COL6A1 and COL6A2 in particular [98,99].

### 3.4. *SEPN1* related myopathy (*SEPN1*-RM)

*Diagnostic considerations:* *SEPN1*-RM is a congenital muscle disorder caused by autosomal recessive mutations of the *SEPN1* gene, which encodes selenoprotein N (SelN) and plays a key role in protecting human cells against oxidative stress [100,101]. Poor or delayed head control in the first months of life is the most common presenting sign, although almost all patients continue to

acquire motor milestones and achieve independent ambulation often at a normal age. Neonatal respiratory failure, severe feeding difficulties, congenital contractures or major joint hyperlaxity would be highly unusual presenting features. During later infancy and childhood, muscle weakness and slenderness remain more marked in axial groups, particularly in neck flexors and sometimes extensors (dropped head) [102–104]. In contrast, limb strength and therefore walking ability are usually preserved, although difficulties climbing stairs, walking long distances and easy fatigability are common. Marked progression has been observed in several cases after the fourth decade. Other characteristic features are a relative atrophy of the inner thigh muscles, mild hyperlaxity of hands and wrists and mild facial weakness with a typical nasal voice. Mild ophthalmoparesis is uncommon but can be seen particularly in severe cases. In a series of patients with hirsutism, signs of insulin resistance were detected [105]. Joint contractures are absent or mild but they are severe in the spine leading to a spinal stiffness which may appear around 5–6 years of life or even earlier. Later on, thoracic lordoscoliosis with lateral translation is a frequent complication (Fig. 2E). Progressive restrictive respiratory failure frequently manifests by the end of the first decade of life as nocturnal hypoventilation even in children with fairly preserved vital capacity. As in Ullrich patients, diaphragmatic failure may be observed and most patients require non-invasive ventilation while still ambulant, at an average age of 13.9 years with a range of 1 to 33 years, suggesting that respiratory surveillance should be initiated at diagnosis [104]. CK is normal or mildly elevated (less than 4-fold).

*SEPN1*-RM needs to be differentiated from other conditions with prominent spinal rigidity, particularly Emery–Dreifuss muscular dystrophy, FHL1 related myopathies, Pompe disease, COL6-RD (UCMD, Bethlem myopathy), and some cases of RYR1 related core disease. Joint contractures are not typical of *SEPN1* and this is a differential feature with most of these entities, but this complication may not be present at young ages. Drop head and spinal rigidity are also observed in LMNA-RD, but CK levels are usually higher in LMNA-RD and muscle weakness distribution in the limbs is different (proximal in upper extremities and distal in lower limbs in LMNA-RD).

*Selective genotype–phenotype correlations:* Mutations are distributed along the whole gene, except exon 3 and the majority are nonsense mutations, microdeletions or insertions leading to frameshifts, as well as splice-site mutations leading to aberrant pre-mRNA splicing (reviewed in Lescure et al., [106]) [100,104,99]. Interestingly, several mutations affect the cis sequences (3' UTR SECIS element, Sec codon redefinition element (SRE)) required for selenocysteine insertion which needs to be evaluated if Sanger sequencing of coding exons does not reveal a mutation [107–109].

### 3.5. Recessive *RYR1*-related myopathy (*RYR1*-RM) presenting as CMD (*RYR1*-CMD)

Patients with recessive mutations in the *RYR1* gene coding for the sarcoplasmic reticulum calcium release channel may present with a distinct CMD like presentation (*RYR1*-CMD) which falls into the larger context of recessive *RYR1*-RM that now includes centronuclear (CNM), central core, multi-minicore, and fiber type disproportion histological presentations [110,111]. *RYR1*-CMD lacks evidence for typical core formation on muscle biopsy staining with NADH and other oxidative stains, but presents with a histological and clinical picture most suggestive of CMD. Like *SEPN1* mutations, *RYR1* mutations can present as disorders sharing features of both a congenital myopathy and a CMD. There is evidence to suggest common aspects to the pathogenesis in both of these disorders and that they may physically interact [101]. Clinically, patients with *RYR1*-CMD may present with significant congenital onset hypotonia, including facial weakness and early onset severely progressive scoliosis. Nocturnal ventilatory support due to pulmonary insufficiency and gastrostomy due to feeding and swallowing difficulties may be required. Although not frequent, CK can be mildly elevated. Ophthalmoplegia/paresis as seen in the the centronuclear and multi-minicore presentations of recessive *RYR1* mutations may be absent in the CMD like presentation of *RYR1*-CMD.

### 3.6. *LMNA* related CMD (*LMNA*-CMD)

Mutations in the lamin A/C (*LMNA*) gene, cause a wide range of genetic disorders in humans, including muscular dystrophies (*LMNA*-RD) [112,113]. The typical neuromuscular disorder associated with lamin A/C mutations is Emery-Dreifuss muscular dystrophy (EDMD), characterized by scapulo-peroneal muscle weakness, contractures of elbows, heel cords and spine, scoliosis, cardiomyopathy and cardiac arrhythmias. More recently mutations in *LMNA* have also been identified in patients with an early onset CMD form (*LMNA*-CMD) [114,115].

In *LMNA*-CMD, weakness becomes evident in infancy, sometimes including a brief phase of more rapid progression during the first 24 months of age with loss of early motor milestones. Characteristic weakness of axial and neck muscles (flexors and extensors) causes the clinical phenomenon of head-drop or “dropped head syndrome” (Fig. 2D) [115–117], due to very weak neck extensors. In addition there is pronounced lumbar hyperlordosis at a very early age, arm and hand weakness as well as peroneal predominant weakness while hip flexors are better preserved demonstrating good antigravity strength. Thus, weakness resembles an early axial–scapulo–peroneal pattern in addition to the early and severe axial weakness. Contractures manifest in the

Achilles tendon, knees, hips and spine with considerable spinal rigidity, with less contractures in the elbows and finger flexors or extensors when compared to classic Emery–Dreifuss phenotype and *COL6*-RD. In the most severe cases, sitting and head support may never be achieved. More commonly, walking ability is acquired but it is lost later in life, often after a short period of time. Night-time respiratory insufficiency with hypoventilation and hypercapnea may manifest early [115]. Similar to Emery–Dreifuss phenotype, cardiac involvement in *LMNA*-CMD may take the form of an initially atrial arrhythmogenic cardiomyopathy with conduction block, and also ventricular tachyarrhythmias, necessitating the use of an AICD. Cognition is unaffected. CK levels can be mildly to moderately elevated. The most important differential diagnostic consideration is *SEPN1*-RM (see section 3.4.).

*Selective genotype–phenotype correlations:* All identified mutations so far have been heterozygous de novo mutations that act in a dominant negative way [114]. Some mutations seem unique to *LMNA*-CMD, while other mutations also occur in patients at the severe end of the spectrum of the Emery–Dreifuss phenotype [115,118].

### 3.7. Mutations in metabolic pathway genes presenting as CMD

Several genetic causes for CMD like presentations have been described recently and involve mutations in genes that are involved in metabolic pathways (see Table 2).

*CHKB-related CMD:* Mutations in choline kinase B, which is involved in phosphatidylcholine biosynthesis, cause a congenital onset muscular dystrophy with large appearing mitochondria (megacolonial or giant mitochondria) on oxidative stains and ultrastructure [119]. Affected patients in addition show cognitive impairment but normal brain MRI findings and also skin findings including acanthosis nigricans like lesions with intense pruritus. This clinical constellation together with the biopsy findings is diagnostic [2].

### 3.8. Paraclinical Diagnosis of CMD

#### 3.8.1. Muscle pathology

The careful evaluation of the muscle biopsy often is essential to suggest or support a genetic diagnosis. Proper performance, handling, and processing of the biopsy specimen need to be assured [120]. The muscle biopsy should be obtained from a skeletal muscle that is clinically affected but not to a degree that makes it unsuitable for diagnosis due to near complete replacement of muscle by connective and fatty tissue. Although the degree of involvement of the muscle can be suspected on clinical grounds, it may be very helpful to utilize muscle imaging (MRI, ultrasound, or CT) to estimate the degree of involvement. It is important to

Table 2

Summary of currently recognized Congenital Muscular Dystrophies.

| Disease entity  | Protein product<br>Gene symbol   | Salient clinical features   | CNS imaging findings   | Immuno-histochemical<br>diagnosis  |
|---|--|---|--|--|
| <i>Laminin alpha 2 related CMD (primary merosin/laminin2 deficiency)</i>                          |  |   |  |  |
| CMD with primary laminin-211 (merosin) deficiency (MDC1A)   | Laminin- $\alpha$ 2<br>LAMA2   | Complete deficiency: Maximal motor ability is sitting and standing with support. Milder (contractural) presentations with partial deficiency. Generally normal mental development, epilepsy in about 30%,       | Abnormal white matter signal (T2 weighted MRI), 5% occipital pachy- or agyria, pontocerebellar atrophy (rare)                                | Complete or partial deficiency for laminin $\alpha$ 2                                  |
| <i>Alpha-dystroglycan related Dystrophies</i>   |  |   |  |  |
| CMD with partial merosin deficiency (MDC1B)   | Not known<br>(locus: 1q42)   | Variable severity, delayed onset possible, proximal limb girdle weakness, muscle hypertrophy, early respiratory failure possible.   | Abnormal white matter and structural grey matter changes possible. Expanding spectrum.   | Variable deficiency of the glycosylated aDG epitope, secondary reduction of laminin211 |
| LARGE related CMD (MDC1D)   | LARGE  | Variable. CMD with significant mental retardation, may eventually blend with the MEB/WWS spectrum.  | White matter changes, mild pachygyria, hypoplastic brainstem, cerebellar abnormalities incl cysts.   | Same   |
| Fukuyama CMD (FCMD)   | Fukutin<br>FCMD  | Frequent in the Japanese population, walking not achieved, mental retardation, epilepsy common, more limited eye findings but clinical overlap with MEB.  | Lissencephaly type II/pachygyria, hypoplastic brainstem cerebellar abnormalities, including cysts.   | Same   |
| Muscle-eye-brain disease (MEB)  | POMGnT1<br>FKRP, Fukutin, ISPD, TMEM5  | Significant congenital weakness, walking is rarely achieved, motor deterioration because of spasticity. Mental retardation, significant ocular involvement (e.g. severe myopia, retinal hypoplasia).            | Lissencephaly type II/pachygyria, brain stem and cerebellar abnormalities, including cysts   | Same   |
| Walker–Warburg syndrome (WWS)   | POMT1<br>POMT2, FKRP, Fukutin, ISPD, CTDC2, TMEM5, POMGNT1, B3GALNT2, GMPPB, B3GNT1, SGK 196 | Often lethal within first years of life because of severe structural CNS involvement. Congenital weakness may be less apparent in the setting of the brain involvement. Significant ocular involvement possible | Lissencephaly type II, pachygyria, hydro-cephalus, occipital encephalocele, hypoplastic brainstem, cerebellar atrophy.                       | Same   |
| CMD/LGMD with MR  | FKRP, POMT1, POMT2, ISPD, GMPPB  | Early onset weakness but ambulation is often achieved, or early onset LGMD phenotype, with mental retardation, some patients with microcephaly.   | May be normal, or show cerebellar cysts, or mild cortical abnormalities. Microcephaly without any other obvious structural changes possible. | Same   |
| CMD/LGMD without MR (including MDC1C)   | FKRP, Fukutin, ISPD, GMPPB   | Early onset weakness but often ambulation, or early onset LGMD phenotype, without mental retardation, may have steroid responsive progression of weakness, cardiomyopathy.                                      | No   | Same   |
| <i>Congenital Disorders of Glycosylation (CDG) with abnormal alpha-dystroglycan glycosylation</i> |  |   |  |  |
| CDG I (DPM3)  | Dolichol-Phosphate-Mannose Synthase-3<br>DPM3  | 1 patient: CMD/LGMD with elevated CK, cardiomyopathy and stroke like episode, mild developmental disability (IQ 85)   | Unexplained stroke-like episode without clear neuroimaging correlate   | Mild reduction in $\alpha$ DG glycoepitope, variable laminin 211 reduction             |
| CDG I (DPM2)  | Dolichol-Phosphate-Mannose Synthase-2<br>DPM2  | CMD with MR and severe myoclonic epilepsy, elevated CK  | Cerebellar vermis hypoplasia, microcephaly.  | Same   |
| CDG Ie (DPM1)   | Dolichol-Phosphate-Mannose Synthase-1<br>DPM1  | Initially described as CDG Ie, now emerging evidence of the presence of a dystrophic myopathy with abnormal alpha DG  |  | Same   |

(continued on next page)



Table 2 (continued)

| Disease entity   | Protein product<br>Gene symbol                                 | Salient clinical features  | CNS imaging findings   | Immuno-histochemical<br>diagnosis  |
|--|--|--|--|--|
| DOLK-CDG   | DOLK   | Non-syndromic AR dilated cardiomyopathy  |  | Mild reduction in aDG glycoepitope in cardiac muscle   |
| <i>Collagen VI and Integrin-related CMD forms</i>                              |  |  |  |  |
| Collagen VI Related Myopathies Ullrich/ Intermediated/ Bethlem spectrum (UCMD) | $\alpha 1/2$ and $\alpha 3$ collagen VI COL6A1, COL6A2, COL6A3 | Distal joint hyperextensibility, proximal contractures, motor abilities variable, precludes independent ambulation in severe cases, soft palmar skin.  | No   | Deficiency of collagen VI immunoreactivity, in dominant cases only apparently deficient from the basement membrane |
| Integrin $\alpha 7$  | Integrin $\alpha 7$ ITGA7                                      | Very rare, delayed motor milestones, walking with 2–3 years  | No   | Reduced (difficult stain)  |
| CMD with hyperlaxity (CMDH)  | 3p23–21  | French Canadian, presenting with weakness, proximal contractures, distal laxity, milder compared to UCMD with ambulation preserved into adulthood  | No   | Not clear yet  |
| <i>Intracellular and nuclear CMD forms</i>                                     |  |  |  |  |
| SEPN1 Related Myopathy   | Selenoprotein N SEPN1  | Delayed walking, predominantly axial weakness with early development of rigidity of the spine, restrictive respiratory syndrome  | No   | No diagnostic immunohistochemical deficiency   |
| Lamin A/C related Dystrophy  | LMNA   | Absent motor development in severe cases, more typical: “dropped head” and axial weakness/rigidity, proximal upper and more distal lower extremity weakness, may show early phase of progression | No   | Same   |
| RYR1 related CMD   | RYR1 (recessive)   | Congenital weakness and early scoliosis, facial weakness +/- ophthalmoplegia   | No   | Same   |
| CHKB related CMD   | CHKB (recessive)   | Congenital weakness, cognitive impairment, pruritus, giant mitochondria in biopsy.   | No   | Same   |
| PTRF related PCGLP4 with CMD   | PTRF (recessive)   | Congenital onset generalized progressive lipodystrophy, later rippling muscle  | No   | Same   |
| CMD merosin-positive   | 4p16.3   | Severe muscle weakness of trunk and shoulder girdle muscles, and mild to moderate involvement of facial, neck and proximal limb muscles. Normal intelligence.                                    | No   | Same   |
| CMD with adducted thumbs   | Nesprin  | Rare, adducted thumbs, toe contractures, generalized weakness, delayed walking, ptosis, external ophthalmoplegia, mild mental retardation.   | Mild cerebellar hypoplasia   | Not clear yet  |
| CMD with cerebellar atrophy  | Not known  | Delayed motor milestones, mild intellectual impairment.  | Moderate to severe cerebellar hypoplasia, no white matter abnormalities. | No diagnostic immuno-histochemical deficiency  |

anticipate the need for future analysis of biological materials and assure proper storage of muscle fixated for ultrastructural analysis, frozen muscle, genomic DNA and if possible fibroblast culture. When available,

establishment of a myoblast culture may be useful for future studies in unclear CMD presentations.

Correlation with the clinical picture is often required to arrive at a correct biopsy interpretation given the

variability of possible compatible morphologic findings [2,121,122], the possible variability inherent in performance and interpretation of the commercial antibodies used to evaluate specific protein deficiencies by immunohistochemistry [123], and the lack of the specific stains in certain CMD subtypes. One of the most immediate uses of a muscle biopsy is to recognize or exclude other disorders that are important in the differential diagnosis of CMD. Lack of any morphological changes could indicate the presence of a central nervous system disorder causing significant hypotonia, and does not exclude a metabolic problem.

On the *Hematoxylin and Eosin (H&E) stain*, CMD is usually characterized by abnormal variation in fiber size for age without obvious grouping. The fiber shape may be rounded, and there is an increase in internalized nuclei (but not usually central nuclei as seen in the centronuclear myopathies). RYR1-RM in particular may have high numbers of centralized nuclei as can congenital DM1 as an important differential diagnosis. There is a variable increase in endomysial connective and adipose tissue, while the width of perimysium is increased (but note that it is wider in general in neonates) [2,124]. There may be necrosis, which however may not be readily apparent on H&E so that its absence does not exclude a CMD diagnosis. The presence of basophilic fibers suggests regenerative activity, but not all basophilic fibers are regenerating fibers. The analysis of neonatal and foetal myosins might be very helpful in these cases [120]. In addition, foci of inflammatory cells may be present. Other fiber abnormalities that may be seen occasionally and which are still consistent with a CMD diagnosis include various types of vacuoles (however, these are never a prominent finding in the biopsy), whorled and/or split fibers and hyper-contracted fibers (though fewer compared to the dystrophinopathies). In neonates, the observation of some large Wohlfart B fibers is considered normal.

In addition, the *modified Gömöri Trichrome (mGT)* stain may be helpful in recognizing other conditions such as rods in nemaline myopathy and ragged red fibers in mitochondrial myopathy. The mGT stain also reveals the degree and distribution of fibrosis present in the biopsy.

The *oxidative stains* as well as *ATPase* stains reveal fiber typing. Fiber type 1 predominance is common in the CMDs but is not specific for any particular diagnosis. Fiber typing can be indistinct, particularly in neonates. In this case myosin heavy chain immunofluorescence can be helpful. Absent clear typing is referred to as fiber type uniformity and could suggest a RYR1-RM. Fibertype grouping (of both types) is not a feature in the CMDs and suggests the presence of a neurogenic disorder.

Cores can be diagnosed if observed on all oxidative stains: *COX*, *SDH* and *NADH-TR* stains. The presence of large and longitudinally extended cores would usually suggest the presence of a RYR1-RM or RYR1-CMD.

However, unevenness in staining is more common and can even be seen in COL6-RM. The presence of multiple minicores (in particular in longitudinal section) suggests the presence of either a SEPNI-RM or RYR1-RM, but if infrequent is not a very specific finding. Peripheral aggregation of mitochondria sometimes resembling lobulated fibers may occur in UCMD, although true lobulated fibers are not usually a feature in children with any neuromuscular disorder. The presence of COX negative and/or COX negative SDH positive fibers suggest a mitochondrial cytopathy. Very large mitochondria, in particular towards the periphery of fibers, is indicative of a phosphatidylcholine defect (CHKB) [125]. The size of mitochondria, degree of glycogen and lipid accumulation can vary for a variety of reasons, including diet and type of feed in nasogastric fed neonates. Electron microscopy is sometimes helpful to interpret the significance of subtle loss of oxidative stains as it can differentiate the myofibrillar abnormalities found in myofibrillar myopathies from the disruption associated with typical core lesions.

### 3.8.2. Subtype specific findings

The *immunohistochemical examination* is of particular importance in the pathological workup of a patient with suspected CMD [126]. There is a basic panel of antibodies that need to be available for comprehensive evaluation of the biopsy (see Table 3). In the following section we briefly summarize the general and immunohistochemical findings for the CMD forms in which this analysis can be diagnostic:

*Laminin  $\alpha 2$  related dystrophy (LAMA2-RD)*: General histology may show a particularly pronounced buildup of fibrosis and fatty replacement, as well as sometimes prominent presence of inflammatory cells, along with evidence for degeneration and regeneration. These findings are present early. On immunostaining typically there will be a complete absence or near absence of laminin  $\alpha 2$  immunostaining from all muscle fibers and nerves. In cases of a partial deficiency, there will be a reduction on some muscle fibers while the staining on nerves may appear normal. Partial laminin  $\alpha 2$  deficiency can be seen in both primary LAMA2-RD and  $\alpha$ DG-RD. If the deficiency is subtle, confirmation with a second laminin  $\alpha 2$  antibody to the 300 kDa fragment (or one that behaves similar to it) and review of the clinical presentation to determine consistency with a diagnosis of LAMA2-RD versus  $\alpha$ DG-RD are required. Fibers that are deficient in laminin  $\alpha 2$  immunostaining will show a compensatory upregulation of laminin  $\alpha 5$  immunofluorescence [17,126]. Upregulated laminin  $\alpha 5$  can also be seen on regenerating fibers, thus, those will have to be excluded from this assessment. Laminin  $\alpha 2$  immunostaining in skin from a patient with LAMA2-RD will show absence of laminin  $\alpha 2$  from the epidermal/dermal junction, the sensory nerves and all other components seen in skin (e.g. sebaceous glands). Intramuscular nerves typically will also be

Table 3  
Antibodies used routinely with consideration of CMD specific findings.

| Antibody                         | Findings   |
|----------------------------------|--|
| Laminin $\alpha 2$               | Absence (in muscle fibers and nerves, skin biopsy can also be used) = MDC1A<br>Primary reduction = MDC1A, check laminin $\alpha 5$ (should be elevated)<br>Secondary reduction = suggestive of dystroglycanopathy-check 2H6 (glycosylated $\alpha$ DG) labeling (in LGMD2I this reduction is seen on blots only)<br>Partial reduction may need antibody against 300 kDa fragment to appreciate the reduction   |
| Laminin $\beta 1$ and $\gamma 1$ | Should be normal in all CMDs – serves as basement membrane control in laminin $\alpha 2$ deficiency  |
| Laminin $\alpha 5$               | There is secondary over-expression in MDC1A (note that regenerating fibers have higher expression, while moderate levels may be present in neonatal muscle biopsies)   |
| $\alpha$ -Dystroglycan           | Immunolabeling with antibody against glycosylated $\alpha$ DG (such as 2H6). Absence or virtual absence of immunolabeling = seen in severe CMD forms, including MEB, WWS, FCMD. Also seen in LG forms with Fukutin mutation.<br>Incomplete or mild reduction = seen with abnormal $\alpha$ DG glycosylation of various severity, including LGMD presentations (unclear genotype/phenotype correlations, some will have FKRP mutations)   |
| $\beta$ -Dystroglycan            | Should be normal in most CMDs – serves as a control for $\alpha$ -dystroglycan (some mild reduction may sometimes be seen)<br>Marked reduction is exclusion criterion for CMD (seen in dystrophinopathies)   |
| Collagen VI                      | Complete absence = suggestive of recessive UCMD<br>Reduction from sarcolemma only, with good preservation of sarcolemma (Labeled with perlecan or collagen IV) = suggestive of dominant UCMD, some recessive missense mutations show similar behavior.<br>Normal appearance does not exclude Bethlem in particular<br>Cultured skin fibroblasts may be more sensitive for subtle changes.  |
| Dystrophin                       | Absence-exclusion criterion for CMD = seen in DMD<br>Some mild reduction – can be secondary in some dystroglycanopathies<br>(no genotype correlation), if more prominent, suggestive of BMD<br>Note that labeling with dys 2 may be non-specifically weak in some neonates (age related)   |
| Sarcoglycans                     | Should be normal in all of the CMDs<br>Reduction – exclusion criterion   |
| Utrophin                         | Mild to moderate elevation of immunostaining may be non-specific, seen in regenerating fibers<br>Consistent high levels of expression: exclusion criterion, suggestive of dystrophinopathy   |
| Myosins                          | Co-expression of fast and slow isoforms in several fibers = suggests abnormal muscle, but is non-specific<br>Predominance of slow fibers may occur in CMD and is nonspecific<br>Presence of several fibers with developmental/neonatal myosin- may indicate regeneration, and/or delayed development, and/or non specific abnormality<br>Note: in neonates a direct correlation of myosin immunolabeling with ATPase staining is difficult as the presence of neonatal myosin (in relation to immaturity) makes many fibers stain as type 2 but they may in fact react positively for slow myosin. The decline in the number of fibers with neonatal myosin in normal muscle is not known but by 3–6 months there are generally very few. The presence of many fibres with neonatal myosin at 1 yr is indicative of an abnormality |

negative for laminin- $\alpha 2$  in LAMA2-RD, while the staining is preserved in  $\alpha$ DG-RD.

*Alpha-dystroglycanopathy related dystrophy ( $\alpha$ DG-RD):* General histology shows dystrophic features with degeneration, necrosis and regeneration and fibrofatty replacement that are most similar to those seen in the dystrophinopathies and the sarcoglycanopathies. In contrast to LAMA2-CMD in neonatal or very early biopsies dystrophic features may be subtle. Immunohistochemical findings on muscle biopsy in the  $\alpha$ DG-RD are similar irrespective of the primary gene involved. The degree of deficiency can be variable and does not necessarily correlate with the severity of the clinical phenotype. It is important to utilize an antibody raised against the glycosylated form of  $\alpha$ -dystroglycan and not against the core protein. This glycoepitope-sensitive antibody will show absence, near absence, or reduced labeling on most or some of the fibers. In less pronounced cases, there may just be uneven labeling on some fibers, in which case it may be difficult to clearly recognize the

findings as a primary deficiency. Western-blot analysis for glycosylated  $\alpha$ -dystroglycan may be helpful by showing a reduction as well as a downward shift of the broad band of glycosylated  $\alpha$ -dystroglycan. Normal labeling of  $\beta$ -dystroglycan on all fibers will help recognize a secondary  $\alpha$ -dystroglycan deficiency seen in the dystrophinopathies (DMD, BMD). Commercially available antibodies to glycosylated  $\alpha$ -dystroglycan have to be validated carefully by comparing established disease controls with normal samples as they may produce variable results. Laminin  $\alpha 2$  reduction (with preservation of laminin  $\beta 1$ ,  $\gamma 1$ ) will be seen as a secondary change in primary  $\alpha$ -dystroglycan deficiency ( $\alpha$ DG-RD). As degeneration and regeneration is seen early on in this group of conditions, several fibers will be positive for developmental and/or neonatal myosin labeled regenerating fibers.

*Collagen VI Myopathy (COL6-RD):* Muscle biopsy findings in the COL6-RD are quite variable and depend on the disease severity and stage. In very young children and very mildly affected patients there may only be

minimal myopathic changes or features of fiber type disproportion [124]. Later in the disease, myopathic findings become more pronounced and dystrophic features more apparent. Core-like lesions may also be present [127]. In cases with recessive null mutations, an overall deficiency or absence of collagen VI immunofluorescence in the muscle will be apparent (including sarcolemma, endomysium, epimysium and perimysium), although some may still be seen around blood vessels. In patients with dominantly acting mutations, collagen VI immunoreactivity will be absent from the sarcolemma/basement membrane specifically, while there may be no discernible deficiency in the interstitial connective tissue of the endomysium, epimysium and perimysium [128,129]. For proper recognition of this phenomenon in particular in partial deficiencies it is necessary to co-label the sarcolemma/basement membrane with a second basement membrane antibody (i.e. perlecan, collagen type IV) using two color double immunofluorescence technique. While many Bethlem cases also show a recognizable sarcolemmal specific deficiency, in mild cases in particular in the mild Bethlem myopathy range, the collagen VI immunoreactivity in the biopsy may appear normal in amount and localization. Degenerating and regenerating fibers are not a prominent feature early on in biopsies from patients with COL6-RD, however, there may be fibers present that stain for developmental and/or neonatal myosin. A sarcolemmal reduction of laminin  $\beta 1$  may be seen in some adult or adolescent cases of Bethlem myopathy but is not specific to collagen VI disorders. Immunohistochemical examination of skin sections (as opposed to fibroblasts in culture) is only helpful if there is a complete absence of collagen VI immunoreactivity, although more recently the application of techniques such as FACS may allow to appreciate more subtle reduction in collagen VI expression [88].

**SEPN1-RM and RYR1-RM:** The muscle pathology spectrum of SEPN1-RM is broad and includes most but not all cases of Rigid Spine Muscular Dystrophy (RSMD1) [130], classic multi-minicore disease [102], desmin-related myopathy with Mallory body-like inclusions [131] and in a small percentage of congenital fiber type disproportion (CFTD) cases [105]. Most SEPN1-RM muscle biopsies show small focal areas of mitochondria depletion and sarcomere disorganization (minicores) on oxidative stains in muscle fibers, together with type 1 fiber predominance and variable atrophy, protein aggregates and/or endomysial fibrosis. Necrosis and/or regeneration are less frequent but may be present. There currently is no immunohistochemical diagnostic stain for SEPN1-RM yet. In RYR1-RM cases presenting as CMD histological findings are extremely variable [111]. Extreme fiber atrophy, frequent central nucleation, fiber type uniformity, irregular oxidative enzyme stains including core-like areas are all features. Overt

degeneration and regeneration is not conspicuous while fatty-fibrous replacement can be prominent.

**LMNA-CMD:** The histological appearance of the muscle biopsy is variable, ranging from a myopathic appearing biopsy with mostly type 1 atrophic fibers to more overtly dystrophic findings, mainly reflected as increased fibrosis and less so by overtly necrotic fibers. Findings may be different between sections in a given biopsy and between different muscles. Conspicuous cellular infiltration suggesting inflammation is a feature in some biopsies and may provide rationale for anti-inflammatory steroid therapy [132]. Immunohistochemical examination for LMNA in the biopsy will be normal as there is no appreciable deficiency or mislocalization of lamin A/C even in the presence of a mutation causing severe disease. LMNA-CMD shows no characteristic protein deficiencies by immunohistochemical analysis.

### 3.8.3. Muscle imaging (ultrasound and magnetic resonance imaging)

Imaging techniques, such as computed tomography or resonance magnetic imaging, and ultrasound [133,134] have assumed increasing importance in the diagnostic approach for patients with muscle disease and show specificity for several genetic entities [62,135–137]. Within the diagnostic work up of CMDs they have proved to be particularly useful when suspecting a COL6-RD, SEPN1-RM, LMNA-CMD and RYR1-CMD [62,138–140]. MRI should be regarded as a gold standard technique. Standardized T1 weighted spin echo sequences of the lower limb, particularly of the thigh muscles are probably the most informative and should be favored when time and resources are limited. Whole body MRI has also been successfully used for the purpose of pattern recognition, in particular when lower limbs are not specific enough or if the myopathy has selective involvement in other parts of the body [62,141]. The acquisition of images is generally easy to accomplish in conventional imaging units. However, the identification of a specific pattern of muscle involvement requires a high level of expertise and one should consider sending the images for advice to international centers of CMD expertise.

In COL6-CMD (Fig. 3G), muscle MRI shows a characteristic pattern with diffuse involvement of fatty infiltration within thigh muscles with relative sparing of sartorius, gracilis, adductor longus. Localization of fatty infiltration typically takes the form of a rim of hypodensity at the periphery of muscles particularly in vasti muscles, with a relative sparing of the central part indicative of endomysial fibrosis tracking along the muscle fascia. In the rectus femoris muscle fatty infiltration occurs along the central fascia specifically with a centrally located abnormal signal denoted as a “central shadow sign” on ultrasound [138–140].

In SEPN1-RM (Fig. 3H), selective involvement of sartorius, semimembranosus and great adductor muscles



with sparing of the gracilis is very suggestive of the diagnosis [142,143]. This pattern may overlap with RYR1 at the thigh level but using WB MRI, selective axial involvement with striking hypotrophy of neck flexors will allow differential diagnosis [62]. A useful imaging based differential diagnosis of rigid spine myopathies is provided by Mercuri et al. [135]. LMNA-RD in ambulatory patients shows vastus lateralis and gastrocnemius medialis selective initial involvement. In the congenital forms with severe weakness, muscle imaging is informative by regarding the pattern of relatively spared muscles, (cranial, psoas and forearm muscles) [141].

Muscle imaging is less used for diagnostic purposes in the CMD types with central nervous system involvement or increased CK levels (LAMA2-RD and  $\alpha$ DG-RD), since diagnosis is usually oriented by other complementary tests (brain MRI, immunohistochemistry).

#### 4. Diagnostic algorithm schematics

The subtype specific schematics (Supplemental Figs. A–E) aim to guide the diagnostic workup starting from a clinical suspicion of CMD with a prioritization of possible subtype involvement to genetic confirmation of a CMD diagnosis. Although it is advantageous (less invasive) and sometimes possible to go directly to genetic testing for a suspected CMD diagnosis, the algorithms proposed here favor inclusion of a muscle biopsy (provided that it can be expertly done, interpreted and stored). However, given a strong clinical suspicion, difficult access to quality biopsy services and easier access to genetic services, a muscle biopsy can sometimes be skipped. The algorithm can also be used in reverse order – i.e. when a genetic change is found on panel genetic testing the algorithm can be followed backwards to assess whether the gene the mutation was found in is plausible as a cause of the phenotype in the patient.

#### 5. Final practical considerations and pitfalls

##### 5.1. Interpretation of molecular genetic results

The interpretation of the results of mutation analysis can be quite straightforward if the pathogenicity of the mutations is obvious and the mutations are consistent with the known pattern(s) of inheritance in a given condition. In the following we address three of the more likely scenarios and pitfalls that occur in the genetic confirmation of CMD.

“Missing” second allele: Lack of detection of a second allele using current methods may occur in particular in LAMA2-RD, with upwards of 25% of LAMA2-RD patients having a gene rearrangement (deletion/duplication of one or more exons) not identified on standard Sanger sequencing [20]. Access to quantitative allele assessment (including MLPA and comparative

genomic array technology) will be important to appropriately detect this type of mutation in order to complete the genetic workup. Similar types of genomic mutations may be found in COL6-RD, however given the multiple inheritance patterns of COL6-RD one must carefully evaluate a single variant found on one allele for its potential to act dominantly before suspecting a missing allele. Also, in both LAMA2-RD and COL6-RD cases with only a single allele identified, there should be clear diagnostic evidence for the presence of the disease (such as unequivocal deficiency of immunostaining for laminin  $\alpha$ 2 or collagen VI in the muscle biopsy) before suspecting a missing allele.

It is also important to confirm apparent homozygous mutations by parental analysis. If only one of the parents carries the mutation it is possible that the patient is in fact hemizygous for the initially detected mutation because of the presence of a larger deletion on the other allele. Other important possibilities for a “missing” second allele are mutations in regulatory regions of the gene as well as deep intronic mutations that could influence splicing but elude mutation analysis based on exonic sequencing alone. In such situations research laboratory based analysis of cDNA from muscle or from dermal fibroblast cultures (for COL6-RD,  $\alpha$ DG-RD, SEPN1-RM and LMNA-CMD analysis) can be helpful to detect additional deep intronic mutations that may affect splicing of exons. In SEPN1 it is important to not forget to include the SECIS sequence located in the 3'UTR [107].

*Recognizing dominantly acting mutations:* Autosomal dominant mutations are required to be co-inherited with the phenotype in families with a positive family history, or confirmed as dominant “sporadic” and de novo confirmed by parental testing. The possibility of somatic and germline mosaicism in dominant de novo mutations should always be considered and has been reported in LMNA-CMD and COL6-RM, with obvious implications on genetic counseling. All hitherto recognized LMNA-CMD mutations are dominantly acting. In COL6-RD, mutations acting in a dominant fashion are common in Ullrich, intermediate and Bethlem phenotypes, but recessive mutations can also cause all three phenotypes. For accurate genetic counseling and disease recurrence risk for family planning it is thus essential to decide whether a single detected mutation would be expected to act in a dominant manner, or whether only one of two recessive mutations has been detected (missing allele). Clearly dominantly acting mutations have been identified as such with solid supporting evidence and are usually annotated in genetic reports. Genomic deletions and deep intronic mutations may also lead to dominantly acting exon skipping (see earlier discussion for COL6-RD). Missense mutations elsewhere in the collagen VI genes that have not been previously convincingly reported as pathogenic are much harder to interpret and one cannot automatically assume the mechanism of its action and counsel for recurrence risk.

*Unclear pathogenicity of identified sequence changes (Variant of Unknown Significance, VOUS):* To reduce the chance of having to deal with sequence changes of unclear significance it is best to focus the sequence analysis on the gene(s) that is most likely to be responsible for the disease phenotype in the patient, using clinical and paraclinical analysis outlined above. Undirected shot-gun approaches to genetic testing, as in parallel sequencing array platforms and whole exome or genome sequencing will result in a number of VOUS potentially confusing genetic confirmation for the individual patient. Testing parents for the variant is important to establish whether the change follows the pattern of inheritance predicted for a mutation in the suspected CMD subtype, including de novo occurrence in the patient or co-inheritance with the disease from an affected parent for forms with a possible dominant mechanism (COL6-RD, LMNA-CMD). Finding a single variant initially detected in both a sporadic patient and in unaffected family members suggests a benign sequence variant under consideration of a dominant mechanism, or, under consideration of a recessive mechanism, that the required other allele has not been detected. Literature review to identify additional publications describing the variant in question and in silico analysis should be performed by the testing laboratory to determine the presumed variant's effect based upon the secondary protein structure and degree of evolutionary conservation of the affected amino acid. An innocent appearing missense mutation or even a synonymous change (a mutation that does not change an amino acid) could still be pathogenic by interfering with an exonic splice enhancer, thereby leading to exon skipping. In silico analysis can also be performed for this type of change but is imperfect at such predictions. However, cDNA analysis in muscle or in fibroblast culture may provide a direct answer by confirming the presence or absence of an abnormal splicing event. Helpful ancillary investigations may include additional stainings on the muscle biopsy, and dermal fibroblast analysis to assess collagen VI matrix formation in the case of COL6-RD. For the  $\alpha$ DG-RDs, fibroblasts or lymphoblasts [144] can be used for direct assays of enzymatic activity for POMT1, POMT2 and POMTGnT1. It is also possible to assess fibroblast  $\alpha$ DG glycosylation and perform complementation assays directed at pinpointing the defective gene in selected situations [64,68].

It is equally important to keep track of patients with convincing clinical and paraclinical phenotypes but without genetic confirmation (i.e. mutation analysis was performed but was negative or inconclusive) as new genes can be expected to be discovered in the future, eventually allowing for a diagnosis in such patients.

*Massive parallel sequencing of groups of disease genes and whole exome sequencing as the primary diagnostic tool:* Next generation based sequencing will be increasingly available in the diagnosis of the CMDs [145].

This can take the form of targeted massively parallel re-sequencing of groups of disease implicated genes, or be based of whole exome sequencing. These technologies can be very beneficial in that they can lead efficiently and directly to the genetic diagnosis, provided the mutation found are clear and unequivocal. Even in those situations it is mandatory to compare the sequencing results with the ascertained clinical and morphological phenotype as outlined here to make sure the genetic and clinical results are congruent. A more challenging situation arises if potentially pathogenic variants are detected in more than one relevant disease gene, a relatively common occurrence. In these scenarios it will be very helpful to carefully follow the algorithms outlined here to arrive at the most likely genetic diagnosis from a clinical point of view. With clinical direction it will be considerably easier to weigh the changes found on next generation sequencing. Finally, if the clinical analysis strongly suggests a specific diagnosis that is not reflected in the results obtained from next generation sequencing it is important to interrogate the genetic platform used for types of mutation that could have been missed because of poor coverage of certain exons, insensitivity to larger deletions and genomic rearrangements and lack of detection of deep intronic changes. A careful clinically informed approach to the diagnosis of the CMDs thus will not become obsolete but only gain in importance in conjunction with the application of next generation genetic technology.

### Conflict of interest

Authors declared that there is no conflict of interest.

### Acknowledgements

Members of the International Committee for Standard of Care for Congenital Muscular Dystrophies: Annie Aloysius, MRCSLT, HPC, London, United Kingdom; Gyula Ascadi, MD, Detroit, MI; Robert O. Bash, MD, Dallas, TX; Vanessa Battista, CPNP, Boston, Massachusetts; Kate Bushby, MD, Newcastle, United Kingdom; Ronald D. Cohn, MD, Baltimore, MD; Anne M. Connolly, St. Louis, MO; Trak Davis, RD, London, England; Isabelle Desguerre, MD, Paris, France; Denis Duboc, MD, Paris, France; Michelle Eagle, PhD, Newcastle, United Kingdom; Brigitte Estournet-Mathiaud, MD, Garches, France; Richard Finkel, MD, Orlando, FL; Josef Finsterer, MD, Vienna, Austria; Dominic Fitzgerald, MD, Sydney, Australia; Julaine M. Florence, DPT, St. Louis, MO; Raimund Forst, MD, PhD, Erlangen, Germany; Albert Fujak, MD, Erlangen, Germany; Danielle Ginisty, MD, Paris, France; Allan Glanzman, DPT, Philadelphia, PA; Nathalie Goemans, MD, Leuven, Germany; Madhuri Hegde, PhD, Atlanta, GA; Robert Anthony Heinle, MD, Wilmington, DE; Brittany Hofmeister, RD, Stanford, CA; Susan T.

Iannaccone, MD, Dallas, TX; Patricia Jouinot, PhD, Garches, France; Yuh-Jyh Jong, MD, Kaohsiung, Taiwan; Annie Kennedy, Washington, DC; Janbernd Kirschner, MD, Freiburg, Germany; David Little, PhD, Sydney, Australia; Ian MacLusky, MD, Toronto, Canada; Marion Main, MA, MCSP, London, United Kingdom; Agneta Markstrom, MD, Stockholm, Sweden; Asa Martensson, MD, Vanersborg, Sweden; Hank Mayer, MD, Philadelphia, PA; Renee McCulloch, PhD, London, United Kingdom; Paola Melacini, MD, Padua, Italy; Eugenio Mercuri, MD, Rome, Italy; Soledad Monges, MD, Buenos Aires, Argentina; Wolfgang Mueller-Felber, MD, PhD, Munich, Germany; Craig Munns, MD, Sydney, Australia; Leslie L. Nelson, MPT, Dallas, Texas; Makiko Osawa, MD, Tokyo, Japan; Jes Rahbek, MD, PhD, Aarhus, Denmark; Claudio Ramaciotti, MD, Dallas, TX; Umbertina Reed, MD, PhD, Sao Paulo, Brazil; Kristy Rose, PT, Sydney, Australia; David Rosenthal, MD, Stanford, CA; Ulrike Schara, MD, Essen, Germany; Pamela M. Schuler, MD, Gainesville, FL; Thomas Sejersen, MD, PhD, Stockholm, Sweden; Anita Simonds, MD, London, United Kingdom; Susan Sparks, MD, PhD, Charlotte, NC; David Spiegel, MD, Philadelphia, PA; Kari Storhaug DDS, PhD, Oslo, Norway; Beril Talim, MD, Ankara, Turkey; Brian Tseng, MD, PhD, Boston, MA; Haluk Topaloglu, MD, Ankara,

|           |   |
|-----------|---|
| GMPPB     | GDP-mannose pyrophosphorylase B   |
| GTDC2     | glycosyltransferase-like domain containing 2                                      |
| ISPD      | isoprenoid synthase domain containing   |
| LMNA-CMD  | LMNA related CMD, LAMA2, laminin $\alpha 2$ gene                                  |
| LAMA2-CMD | Laminin $\alpha 2$ related CMD, Merosin Deficient CMD, MDC1A                      |
| LARGE     | Glycosyltransferase-Like Protein LARGE1   |
| Liss      | lissencephaly   |
| Liss-CH   | lissencephaly cerebellar hypoplasia   |
| LGMD      | limb-girdle muscular dystrophy  |
| LMNA      | lamin A   |
| MDC1A     | congenital muscular dystrophy type 1A synonymous with LAMA2-RD                    |
| MG        | myasthenia gravis   |
| MYH 7     | myosin heavy chain 7  |
| MRI       | magnetic resonance imaging  |
| MSS       | Marinesco Sjogren syndrome  |
| PCH       | ponto cerebellar hypoplasia   |
| POMGnT1   | protein O-mannose beta-1,2-N-acetylglucosaminyltransferase 1, an $\alpha$ DG gene |
| POMT1     | protein-O-mannosyl transferase 1, an $\alpha$ DG gene                             |
| POMT2     | protein-O-mannosyl transferase 2, an $\alpha$ DG gene                             |
| RYR1      | ryanodine receptor 1  |
| RYR1-RM   | ryanodine receptor 1 related myopathy   |
| SEPN1     | selenoprotein 1   |
| SEPN1-RM  | SEPN1 related myopathy  |
| SGK 196   | sugen kinase 196  |
| TMEM5     | transmembrane protein 5   |
| TSEN      | tRNA-splicing endonuclease  |
| VRK1      | vaccinia-related kinase 1   |

## Abbreviations

|                |   |
|----------------|---|
| $\alpha$ DG-RD | alpha dystroglycanopathy related dystrophy, whereas alpha dystroglycan (protein is spelled out) |
| B3GNT1         | $\beta$ -1,3-N-acetylglucosaminyltransferase 1  |
| B3GALNT2       | $\beta$ -1,3-N-acetylgalactosaminyltransferase 2  |
| CK             | creatine kinase   |
| CM             | congenital myopathy   |
| CMS            | congenital myasthenic syndrome  |
| CNM            | centronuclear myopathy  |
| CNS            | central nervous system  |
| COFS           | cerebro oculofacial syndrome  |
| COL6           | collagen 6  |
| COL6-RD        | collagen 6 related dystrophy  |
| CAPN3          | calpain 3   |
| DAG1           | dystroglycan gene   |
| DM1 and DM2    | myotonic dystrophy 1 and 2  |
| DOLK           | dolichol kinase   |
| DOK7           | docking protein-7   |
| DPM2           | dolichyl-phosphate mannosyltransferase 2, regulatory subunit                                    |
| DPM3           | dolichyl-phosphate mannosyltransferase 3, regulatory subunit                                    |
| EDS            | ehler danlos syndrome   |
| FHL1           | four-and-a-half LIM domains 1   |
| FKRP           | fukutin related protein, a $\alpha$ DG gene   |
| FKTN           | fukutin, a $\alpha$ DG gene   |
| FSHD           | facio scapulo humeral dystrophy   |

Turkey; Andrea Vianello, MD, Padua, Italy; Karim Wahbi, MD, Paris, France; Tom Winder, PhD, Marshfield, WI; Nanci Yuan, MD, Stanford, CA; Edmar Zanoteli, MD, Sao Paulo, Brazil; Reinhard Zeller, MD, Toronto, Canada.

This project is supported by grants from the following groups: CureCMD (www.curecmd.org), TREAT-NMD (www.treat-nmd.edu), AFM – Association Française contre les Myopathies (www.afm-france.org/), and Telethon, Italy (www.telethon.it), and U54-NS053672 Wellstone Muscular Dystrophy Cooperative Research Center Grant, 1R13AR056530-01, and MDA Special Grant for the preceding Congenital Muscular Dystrophy Workshop held in July 2008 at the University of Iowa. The support of the National Specialist Commissioning team to F. Muntoni and of NINDS intramural funds to C. Bönnemann is also gratefully acknowledged.

## 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.2013.12.011>.

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