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Review

# Congenital muscular dystrophy: molecular and cellular aspects

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**Abstract.** The congenital muscular dystrophies are a clinically and genetically heterogeneous group of neuromuscular disorders. Each form has a characteristic phenotype, but there is overlap between some entities and their classification is based on a combination of clinical features and the primary or secondary protein defect. Recent studies have identified the genetic basis of a number of congenital muscular dystrophies (11 genes in total) and have recognised a novel pathological mechanism that highlights the importance of the correct posttranslational processing of proteins, in particular $\alpha$-dystroglycan. Diagnosis of these conditions has been aided by the availability of specific antibodies for each protein and a better understanding of the protein changes that accompany each condition. In this review we present the major molecular, clinical and diagnostic aspects of each group of congenital muscular dystrophy with an emphasis in the more recent developments.

**Key words.** Muscular dystrophy; congenital; dystroglycan; glycosylation; laminin; collagen VI; integrin; selenoprotein.

## Introduction

The congenital muscular dystrophies (CMDs) are a clinically and molecularly heterogeneous group of inherited neuromuscular disorders (table 1). Recent clinical studies have categorised the various forms of CMD, and major advances have been made in identifying causative gene mutations and alterations in the immunolocalisation of proteins in relation to these phenotypes. Several of the proteins affected (primarly and secondarly) in CMDs are associated with the sarcolemma and are involved in the interaction between the muscle cell and the extracellular matrix (fig. 1). These include cell surface receptors, such as integrins, basal lamina proteins, such as laminin-$\alpha$2, and extracellular matrix proteins, such as collagen VI. Disruption of the mechanical and probably also the signalling links provided by these proteins is thought to be at the centre of the pathological mechanism in each condition. The other major group of proteins includes those with known or putative enzyme activity that reside, and probably act, intracellularly, such as POMT1 (protein O-mannosyl transferase 1) and POMGnT1 (protein O-mannose $\beta$1,2-N-acetylglucosaminyltransferase 1) (fig. 2). In addition, the function of some of the proteins involved has yet to be proven. These include fukutin, fukutin-related protein (FKRP), LARGE and selenoptotein-1 (SEPN1). There is a degree of association between all these proteins either by means of a direct interaction (such as laminin-$\alpha$2 and integrin $\alpha$7), a common binding partner (such as dystrolgycan, collagen VI and biglycan) or by acting upon each other (the glycolsyltransferases that act on dystroglycan). This article aims to review current knowledge of the cellular and molecular aspects

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Table 1. Congenital muscular dystrophies.

CMD forms where causative gene is known

| Name | Brain involvement | Gene symbol | Location | Protein |
|---|---|---|---|---|
| Merosin deficient | white matter hypodensity | *LAMA2* | 6q | laminin-$\alpha$2 |
| Fukuyama | mental retardation + structural changes | *FKTN* | 9q | fukutin |
| Muscle-eye-brain disease | mental retardation + structural changes | *POMGnT1 (FKRP)* | 1p | POMGnT1 |
| Walker-Warburg syndrome | structural changes | *POMT1 (FKRP, POMGnT1)* | 9q | POMT1 |
| MDC1C | variable | *FKRP* | 19 | FKRP |
| MDC1D | mental retardation + structural changes | *LARGE* | 22q | LARGE |
| RSMD1 | none | *SEPN1* | 1p | selenoprotein-1 |
| Integrin a7 deficiency | none | *ITGA7* | 12q | integrin $\alpha$7 |
| Ullrich's disease | none | *COL6A1, A2, A3* | 2 and 21q | collagen VI |

CMD forms where causative gene/s is unknown

| Name | Brain involvement | Gene symbol | Location | Protein |
|---|---|---|---|---|
| WWS not linked to POMT1 (80% of WWS) | structural changes | ? | ? | ? |
| MDC1B | none | ? | 1q | ? |
| Italian MEB | mental retardation + structural changes | ? | ? | ? |
| CMD with PNS and CNS involvement | structural changes + white matter hypodensity | ? | ? | ? |
| CMD with adducted thumbs | mental retardation + cerebellar hypoplasia | ? | ? | ? |

of those primary and secondary defects that are of diagnostic value and which are contributing to our understanding of the pathogenesis of this group of disorders.

## Main clinical features of CMD

Classification of the CMDs has become increasingly complex, and a wide spectrum of clinical features are now apparent. Patients present at birth, or within the first few months of life, with hypotonia, muscle weakness and often with joint contractures. Serum creatine kinase (CK) levels are markedly elevated in some variants while normal in others.

A major distinction between the various forms is the involvement of the central nervous system (CNS) which may include white matter abnormalities, structural changes, mental retardation and involvement of the eyes. Early and severe rigidity of the spine, distal joint laxity, muscle hypertrophy and respiratory insufficiency are also features of note in distinct entities.

## Pathological features of CMD

The key features of CMDs are muscle wasting, muscle fibre necrosis and fibrosis (fig. 2). These characterisitics, however, are variable in the different forms and may also depend on the muscle biopsied since selective muscle involvement occurs in some CMDs. Similarly, other features, such as internal nuclei and adipose tissue, are variable.

Oxidative enzyme histochemistry may also show staining abnormalities, such as areas of mitochondrial depletion, aggregation (lobulated fibres) and myofibrillar disruption (e.g. in rigid spine muscular dystrophy (RSMD1) and Ullrich's congenital muscular dystrophy (UCMD) (fig. 2). Fibre regeneration is a consequence of necrosis, and such fibres can be identified by antibodies to developmentaly regulated proteins, such as neonatal/foetal myosin. Immature fibres as a consequence of age rather than disease may also be present and will be recognised by antibodies to neonatal myosin. The number of regenerating fibres with neonatal myosin varies in the different forms of CMD. For example, a large number of positive fibres of varying size are observed in the severe forms with marked ongoing damage and very elevated CK levels, such as MDC1A and MDC1C, in contrast to only very few positive fibres in cases with a normal or slightly elevated CK levels, for example in RSMD1.

## Laminin-$\alpha$2 and CMD

Laminins are essential components of basement membranes which provide tissue compartmentalisation by acting as barriers to cell penetration and filtration. There are at least 15 different heterotrimers formed from 5$\alpha$, 3$\beta$ and 3$\gamma$ chains encoded by different genes. The three chains bind together via their central coiled-coiled domains. Laminins are able to self-assemble via their short arms and through multiple interactions with other proteins play a crucial role in basement membrane integrity both during development and in adult life. In

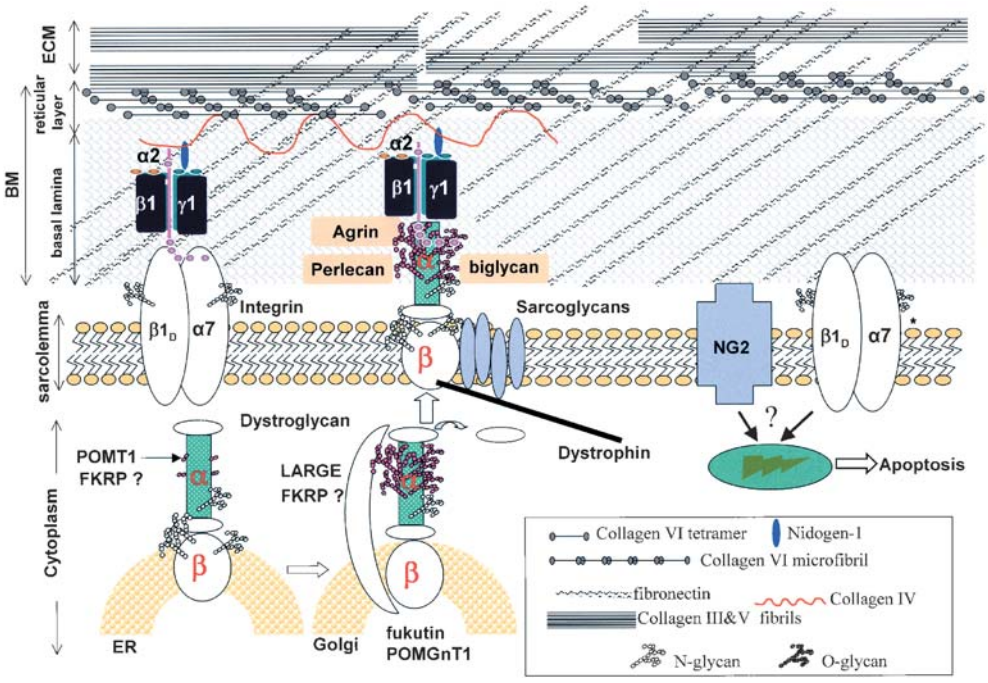


Figure 1. Diagram showing the location of the main proteins involved in CMD and the protein interactions relevant to this review. BM, basement membrane; ECM, extracellular matrix; ER, endoplasmic reticulum.

muscle this role is faciliated by interactions between the C-terminal globular LG domains and α-dystroglycan (LG 4–5) and integrin α7B1D (LG 1–3) on the muscle cell surface [1, 2] (fig. 2).

The predominant laminin form in the skeletal muscle basal lamina is laminin-2 (merosin), which is composed of α2, β1 and γ1 chains. The expression of different laminin chains is developmentally regulated and cell type specific. In skeletal muscle, laminin-α2 is found around the muscle fibre, in the Schwann cell basal lamina and at the neuromuscular (NMJ) and myotendinous junctions (MTJ) [1]. The laminin β1 and γ1 chains are also found in capillaries and blood vessels. Laminin-α2 is not expressed in capillaries and blood vessels in muscle, but it is expressed in the brain vasculature [3]. There are also appreciable amounts of laminin-4 (α2, β2, γ1) . The β2 chain is detected around the muscle fibre basal lamina (where it is developmentally regulated) but is more abundant in blood vessels, at the NMJ and in peripheral nerves in the perineurium [4].

Many laminin chains undergo spontaneous posttranslational proteolytic cleavage within the G-domain or short arms (hereby the 80-kDa and 300-kDa fragments of laminin-α2). The exact purpose of this processing is unknown, but it may regulate binding to cell surface receptors; for example, mutation of the cleavage site in laminin-α2 reduces its binding affinity to α-dystroglycan [5].

Approximately one-third of all CMD cases are due to mutations in the *LAMA2* gene in 6q22, which encodes for the laminin-α2 chain (MDC1A [MIM156225]); but regional variations in the frequency of this form are known to occurr [6, 7]. Since laminin-2 and -4 both contain the α2 chain, both heterotrimers are affected in these patients. Most mutations in the *LAMA2* gene result in complete absence of laminin-α2 protein; however, rare allelic mutations can result in partial protein reduction [8].

Laminin-2 performs important functions in muscle [9, 10], and mouse models have been instrumental in elucidating these functions. Briefly, these models include the naturally occuring *dy* and $dy^{2J}$ mice with very little and reduced expression of laminin-α2, respectively, and the genetically engineered $dy^{W}$ and $dy^{3K}$ mice, which are partly deficient and complete knockout respectively [11–14]. In accordance with its proposed role in the correct assembly and maintenance of the muscle fibre basal lamina, ultrastructural studies have shown an abnormal basal lamina surrounding the fibres of MDC1A patients and the *dy* mouse models [15].

Whilst polymerisation of laminin requires binding to dystroglycan and α7B1D integrin and reorganisation of the actin cytoskeleton, laminin is in turn necessary to organise dystroglycan, integrin, dystrophin and spectrin upon polymerisation [1]. It has been shown on teased mouse muscle fibres that laminin is distributed around the fibre in an ordered spatial pattern that resembles the costameric distribution of α-dystroglycan. However, in fibres from $dy^{2J}$ mice this pattern was not maintained, and

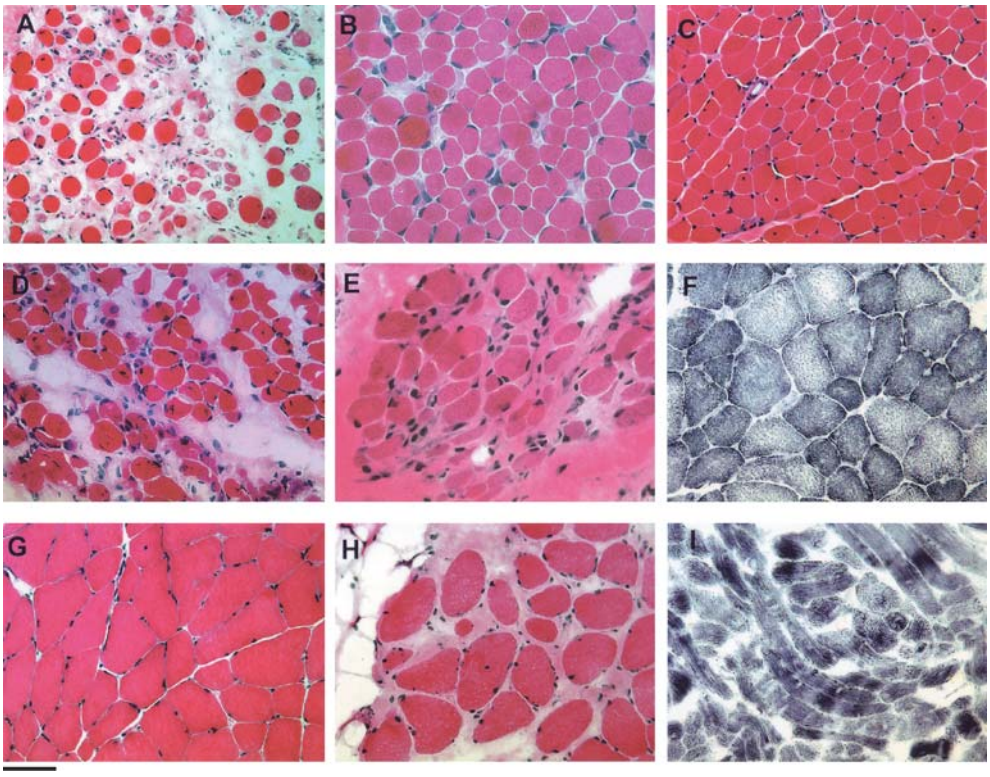


Figure 2. Muscle pathology of CMD. (*A*, *B*, *C*, *D*, *E*, *G* and *H*): Haematoxilin and eosin showing the variable degree of fibrosis, variation in muscle fibre diameter and degeneration. (*F* and *I*) Oxidative enzyme stain (NADH-TR). Type I fibres are more intensely stained because of their higher mitochondrial content. Pale areas reflect focal mitochondrial depletion and myofibrillar disruption in *F* and *I* (cut longitudinally). (*A*) MDC1C, (*B*) MEB, (*C*) MDC1D, (*D*) WWS, (*E*) MDC1A, (*F*, *G*) RSMD1, (*H* and *I*) UCMD. Scale bar represents 118 μm in *A*, *C*, *D*, *F*, *G*, *H*, *I* and 38 μm in *B* and *E*.

laminin staining was diffuse. The costameric distribution of dystrophin and vinculin was similarly affected. This could be physiologically important since the costameres are thought to be the sites of force transmission across individual basement membranes [16].

Animal experiments show that injection of Evans blue dye (which accumulates in fibres with membrane damage) does not accumulate inside the muscle fibres of *dy* and $dy^{2J}$ mice as it does inside *mdx* muscle, suggesting that muscle membrane leakage is not central to the pathogenis of MDC1A [17].

## Clinical phenotype of MDC1A

MDC1A is a severe form characterised by hypotonia, markedly elevated CK (10–150 fold), delayed motor milestones, respiratory insufficiency and feeding difficulties. Children sit unsupported but almost invariably do not achieve independent ambulation. Progressive joint contractures, rigidity and scoliosis of the spine are common, making ambulation more difficult. Respiratory failure followed by death (if untreated) in the first decade of life has been observed in 30% of patients with complete laminin-$\alpha$2 deficiency. Most patients have normal intelligence, but some have been reported to show moderate mental retardation and epilepsy [18].

In brain, laminin- $\alpha$2 is found in the basement membrane of brain blood vessels, epithelial cells lining the choroid plexus, oligodendrocytes tracts and glia-limitans [3, 19]. The most characteristic finding in MDC1A patients brain is a specific and invariable pattern of white matter changes on magnetic resonance imaging (MRI) after 6 months of life which are thought to represent dysmyelination [18]. Interestingly, these changes are not observed in the *dy* mice [20, 21]. A breach in the blood-brain barrier has been suggested as a possible explanation, but this has yet to be proven and has not been supported by studies in MDC1A patients and *dy* mice [authors' personal observations]. Therefore, the causal relationship between these white matter changes and laminin-$\alpha$2 is still unclear. Additional structural brain abnormalities include hypoplasia of the cerebellum (up to one-third of cases) and more rarely neuronal migration defects, mostly localised in the occipital lobes [18].

Laminin-$\alpha$2 is also expressed in the Schwann cell basal lamina, and both MDC1A patients and laminin-$\alpha$2-deficient mice have reduced nerve conduction velocity (mainly motor nerves) [18]. In *dy* mice this could be due

to disrupted Schwann cell basal lamina and defective myelination and/or the observed widening of the nodes of Ranvier [21]. In humans, dysmyelination is a feature, but in contrast to the *dy* mouse, the Schwann cell basement membrane seems well preserved, and other ultrastructural abnormalities have not been found [22]. It is possible that upregulation of other laminin chains ($\alpha$4) [4] at the Schwann cell basement membrane has an undesired negative effect.

**Genotype-phenotype correlation**

While complete absence of laminin-$\alpha$2 always leads to a severe phenotype, partial deficiency can result in a mild or severe phenotype depending on the effect of the specific mutation on laminin-$\alpha$2 function [23]. For example, the mutation in the *LAMA2* gene responsible for the $dy^{2J}$ mouse phenotype is a splice site mutation leading to an in-frame deletion within the polymerising N-terminal domain and partial laminin-$\alpha$2 deficiency and a mild phenotype compared to the severe phenotype of the *dy* and $dy^{3k}$ mice, which have very little or no laminin-$\alpha$2, respectively. In contrast, misensense mutations in the conserved cysteine residues involved in trimer assembly result in partial deficiency but a severe phenotype [8]. Mutations affecting the LG domains often result in a severe MDC1A phenotype because they potentially interfere with the interactions with $\alpha$-dystroglycan and/or integrin $\alpha$7/$\beta$1D [5].

**Diagnosis of MDC1A**

The muscle biopsies of MDC1A patients show a variable degree of fibre necrosis and regeneration, increased endomysial connective tissue and occasional inflammatory infiltration (fig. 2). It is important to use a range of antibodies that recognise both 80-kDa C-terminal and 300-kDa N-terminal fragments since labelling with one antibody may be well preserved but may be very reduced or absent with another antibody [24, 25]. Western blotting can also be used to detect laminin-$\alpha$2 defects.

$\alpha$-Dystroglycan may often be reduced, making it difficult sometimes to distinguish between a partial laminin-$\alpha$2 deficiency and a dystroglycanopathy (see below). In addition, in *dy* mice, levels of laminin-$\alpha$2 in peripheral nerve are lower than those observed in the sarcolemma, suggesting tissue-specifc regulation of expression [3].

Laminin $\beta$2 can be a useful indirect diagnostic marker since it is reduced at the sarcolemma and in the nerve in primary laminin-$\alpha$2 deficiency cases [26]. However, it is important to look at age-matched controls when assessing $\beta$2 levels because it is developmentally regulated. Other secondary markers that can assist diagnosis are laminin $\alpha$5 and $\alpha$4 chains that are upregulated at the sarcolemma [27] and $\alpha$7 integrin, which can be reduced [28].

Laminin chains are expressed at the epidermal dermal junction and in the basal lamina of blood vessels, sebaceous and sweat glands and hair follicles. When laminin-$\alpha$2 is absent from muscle basal lamina, it is also absent from the epidermal-dermal junction and other structures in the skin where it would normally be expressed [29]. However, quantitatively, laminin-$\alpha$2 expression may be different in skin and muscle taken from the same patient, suggesting that it is worth labelling both tissues when possible. Although assessment of laminin-$\alpha$2 status in the skin is useful, secondary alterations must also be considered since laminin-$\alpha$2 can also be reduced in patients with proven mutations in FKRP and with other dystroglycanopathies [authors' personal observations].

**Prenatal diagnosis**

Laminin chains are expressed in chorionic villi (CV) in a developmentally regulated fashion [30]. They are found in the basal lamina underneath the trophoblast, in the mesoderm and in the basal lamina of intramesodermal blood vessels.

Absence of laminin-$\alpha$2 from trophoblast is highly suggestive of a foetus affected by MDC1A, but a combined molecular genetic approach is recommended [31]. Studies of CV samples from foetuses with a potential reduction of laminin-$\alpha$2 have not been reported; and it is not known if a secondary reduction might occur in association with other gene defects, although we have seen normal laminin-$\alpha$2 in an MDC1C CV sample from a family in which the proband had reduced laminin-$\alpha$2 in muscle [author's personal observation].

Immunolabelling for laminin-$\beta$2 is reduced in MDC1A foetuses (with total absence of laminin-$\alpha$2), and preliminary data suggest it is normal in foetuses affected by MDC1C, implying that laminin-$\beta$2 may be a useful distinguishing marker for primary defects in the LAMA2 gene as opposed to FKRP gene [32].

**Future perspectives**

Further understanding of the functions of laminin-$\alpha$2 in brain and peripheral nerve will help clarify the reason underlying the reduced nerve conduction velocity and brain white matter changes.

Potential therapies currently revolve around observations that the transgenic expression of human laminin-$\alpha$2 in $dy^{w}$ and $dy^{2J}$ mice results in partial correction of the muscle phenotype (but do not alleviate the neurological symptoms) [14], and myoblast transplantation in *dy* mice has also been used to restore laminin-$\alpha$2 [33]. More recently, agrin and laminin-$\alpha$1 transgenes have been used to succesfully compensate for the missing laminin-$\alpha$2 chain in the linkage between dystroglycan and the basement membrane [34, 35].

## Dystroglycan and its role in CMD

The dystroglycan gene, *DAG1,* encodes for a polypeptide that is posttranslationally modified to yield two glycoproteins known as $\alpha$- and $\beta$-dystroglycan (fig. 3) which remain non-covalently associated in the cell surface where $\alpha$-dystroglycan binds to a variety of ligands within the extracellular matrix that include laminin-$\alpha$2, perlecan, biglycan, neurexin and agrin. $\beta$-Dystroglycan spans the cell membrane and binds to dystrophin, utrophin, actin and Grb2. Thus, the dystroglycan complex provides a link between the extracellular matrix and the cytoskeleton [36–40].

Dystroglycan has been implicated in a variety of cell processes including development, cell adhesion, and signalling in both muscle and various non-muscle tissues [38, 40, 41]. However, it is its role in basement membrane formation that has received the most attention. This work has been carried out predominantly in ES (embryonic stem) cells and attributes dystroglycan and integrin $\alpha 7\beta 1_D$ with a role in the organisation of laminin near the cell surface [42–44].

Because of the early lethality of DG-null embryos [42], dystroglycan chimaeric mice and Cre-loxP muscle-specific conditional knockouts have been generated [45]. These mice develop a muscular dystrophy and display neuromuscular junction defects. In addition, removing dystroglycan from zebrafish leads to a disruption of the dystrophin-associated complex and loss of muscle integrity and necrosis [46].

The molecular weight of $\alpha$-dystroglycan in skeletal muscle is 156 kDa due to extensive and tissue-specific posttranslational modifications of the original 72-kDa polypeptide. Differential patterns of glycosylation are thought to confer functional variability to $\alpha$-dystroglycan, and this is supported by the presence of at least three different $\alpha$-dystroglycan glycoforms in skeletal muscle [47].

Electrophoretically, $\alpha$-dystroglycan runs as a broad smear which is not diminished after PNGaseF treatment, suggesting that the main modification is O-rather than N-linked carbohydrate addition [37]. Protein O-glycosylation [48] involves the addition of glycans to the hydroxyl groups on either serine or threonine residues. One particular glycosylation, O-mannosylation, is very rare inmammals but is crucial for confering specific ligand binding properties to dystroglycan.

The domain structure of dystroglycan is well characterised (fig. 3) [38], and studies using deletion constructs have shown that the N-terminal and the first half of the mucin-like domain of dystroglycan are necessary for laminin and perlecan clustering. The N-terminal domain is recognised by LARGE (fig. 2 and see below), an enzyme that induces the glycosylation of the central mucin-like region of $\alpha$-dystroglycan [49].

The O-linked carbohydrate moieties are crucial for $\alpha$-dystroglycan function since they mediate its binding to laminin-$\alpha$2 and agrin, and indeed dystroglycan from patients with defects in $\alpha$-dystroglycan glycosylation have reduced laminin-binding activity [50–52]. The structure of the major O-linked mannose glycan on $\alpha$-dystroglycan has been elucidated [50] (fig. 4), and its formation involves the action of several enzymes (collectively refered as glycosyltransferases) that add different monosaccharides in a stepwise manner.

A number of forms of CMD are now thought to be associated with the inteference of this glycosylation process and the subsequent hypoglycosylation of $\alpha$-dystroglycan. These are often refered to as dystroglycanopathies or as defects of $\alpha$-dystroglycan glycosylation [53]. The defect, however, is secondary, although suggesting a pathogenic pathway, unlike other disorders named after the primary protein defect, such as dystrophinopathy. These conditions include Fukuyama congenital muscular dystrophy (FCMD, MIM 253800, [54]), muscle-eye-brain disease, (MEB, MIM 253280, [55]), Walker-Warburg syndrome (WWS, MIM 236670, [56]), , MDC1C (MIM 606612, [57]) and MDC1D [58]. The primary protein defects for these disorders lie in Fukutin, POMGnT1, POMT1, FKRP and LARGE, respectively.

Fukutin, FKRP and POMGnT1 are type II transmembrane proteins (N-terminus outside and C-terminus inside) and contain a motif (Asp-Xaa-Asp) in their C-terminus conserved in several glycosyltransferases [59]. The Asp-Xaa-Asp motif is not present in POMT1. The enzymatic activity of fukutin and FKRP and their substrates have yet to be directly demonstrated, although the observation that $\alpha$-dystroglycan appears hypoglycosylated in FCMD patients, fukutin chimaeric mice and in MDC1C patients provides indirect evidence that both proteins exert an effect on the glycosylation of $\alpha$-dystroglycan [51].

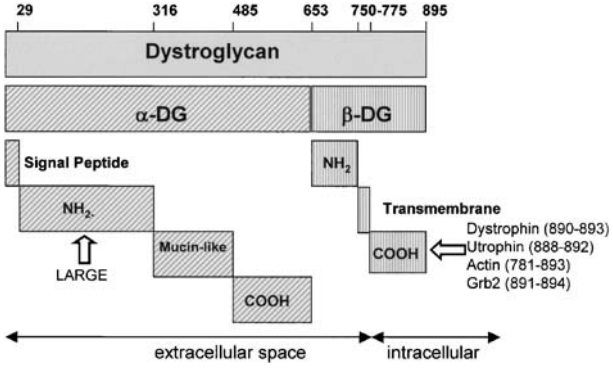


Figure 3. Schematic representation of the structure of the dystroglycan precursor showing the extent (numbers indicate aa) of the main domains of $\alpha$- and $\beta$-dystroglycan (SwissProt Q14118). O-linked glycans are situated in the mucin-like domain. Interactions with other proteins are indicated by arrows.

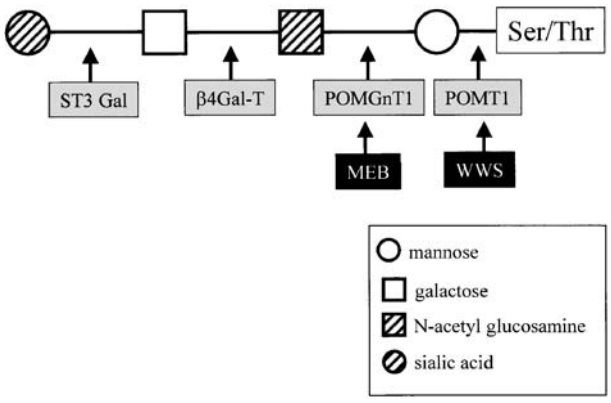


Figure 4. Structure of the main O-mannosyl glycan (Sia$\alpha$2-3Gal$\beta$1-4GlcNac $\beta$1-2 mannose) modification on $\alpha$-dystroglycan.

POMT1 in yeast catalyses the transfer of a mannosyl residue (the first step of O-linked glycosylation) and has been shown to act on recombinant $\alpha$-dystroglycan in vitro together with its closely related isoform POMT2 [52, 60, 61]. POMGnT1 catalyses the subsequent addition of GlcNAc (N-acetyl glucosamine) to a peptide-linked mannose residue (fig. 4).

Fukutin and POMGnT1 are thought to reside in the Golgi system [62, 63]. However, the localisation of FKRP may vary from one cell type to another and also between different stages of development For example, in C2C12 myoblasts, neuronal, oligodendroglial and cardiac cells we have observed FKRP in the Golgi apparatus, but in well-differentiated C2C12 myotubes and in transverse sections of normal striated muscle endogenous FKRP appears to co-localise with components of the nuclear membrane [62, 63, authors' personal observation].

*Fukutin, FKRP, POMT1 and POMGnT1* genes have a wide tissue distribution and are expressed in a similar set of tissues that include skeletal and cardiac muscle, brain and testes but with some individual differences. During foetal life, Fukutin and POMT1 messenger RNAs (mRNAs) are expressed mainly in developing CNS, muscle and eye [57, 62, 64–66].

Targeted inactivation of fukutin leads to embryonic death at 7 days, suggesting that fukutin is crucial for normal development. Heterozygous mice have no phenotype, and as a consequence chimaeric mice were generated. Mice with more than a 50% contribution of fukutin-deficient cells had difficulties walking and suffered significant muscle weakness. Histologically, chimaeric mice muscle showed signs of necrosis and subsequent regeneration and reduced $\alpha$-dystroglycan immunolabelling and laminin binding. The brain was also affected, with abnormal cerebral cortex and cerebellum histogenesis and abnormalities in the meningeal basal lamina. In the brain, dystroglycan was also reduced on Western blots, and laminin binding activity was similarly reduced. These mice also showed abormal retina and lens development [67]. POMT1-null mice die at very early stages of development, and similarly to DG-null mice, lethality is likely to be due to the disruption of Reichert's membrane [65].

LARGE is characterised by two separate catalytic domains with Asp-Xaa-Asp motifs [68]. One of them is similar to a bacterial $\alpha$-glycosyltransferase involved in the synthesis of membrane lipopolysaccarides, and the second one is similar to a glucosaminyltransferase. The precise function of LARGE is unknown, although its interaction with dystroglycan seems to facilitate the glycosylation of $\alpha$-dystroglycan [49].

Indeed, $\alpha$-dystroglycan is severely hypoglycosylated in the muscle and brain of the myodystrophic *myd* mouse, the only known naturally occuring mammalian model of dystroglycanopathy, which carries a mutation in the murine *large* gene. These mice show the muscle, brain and eye pathology seen in MEB, FCMD, WWS and MDC1C, and also the cardiac and tongue muscle abnormalities seen in MDC1C and MDC1D. Neuronal migration is affected throughout the brain areas affected in humans with the CMD muscle-brain forms [69]. Another related animal model is the chicken with muscular dystrophy and abnormal glycosylation of $\alpha$-dystroglycan [70]. The primary genetic defect in this animal model is not known.

**Clinical phenotype of dystroglycanopathies**

FCMD, MEB, WWS and MDC1D are characterised by the combination of muscular dystrophy and brain malformations. In addition, brain malformations have now been described in patients at the severe end of the MDC1C spectrum [71]. Severe ocular abnormalities involving the retina and the anterior chamber (myopia, cataracts and retinal detachment) are also present in WWS and MEB and more rarely in FCMD and MDC1C.

Although initially described as defined individual clinical entities, recent molecular data rather suggest that the dystroglycanopathies represent a continuous spectrum, the severity of the affected individuals being determined by the specific mutations in each of the genes affected [72, 73]. Broadly speaking, WWS is the most severe variant and MDC1C the mildest, with FCMD, MEB and MDC1D in an intermediate position. However, severe non-sense mutations in the fukutin gene can result in a WWS phenotype, and the clinical severity of patients with mutations in FKRP ranges from WWS-like to mild limb-girdle muscular dystrophy (LGMD), depending on the severty of the individual mutations [74].

Most of these CMD forms share common structural brain abnormalities. The most characteristic abnormality is type II (cobblestone) lyssencephaly. This is caused by neurons overmigrating and passing through the glia limitans during the development of normal cortex layering.

Other changes include flattened brainstem, ventricular dilation and a delay in myelination. Seizures are a feature of WWS, FCMD and MEB.
Cardiac involvement invariably presents after the first decade in FCMD and in MDC1C and the milder allelic LGMD2I [18, 75].

**Diagnosis**
The muscle biopsies of patients with these forms of CMD show features of muscular dystrophy (fig. 2) with a significant proportion of fibres expressing neonatal myosin.
Immunocytochemistry and immunoblotting of muscle biopsy reveals an apparent reduction in $\alpha$-dystroglycan but normal $\beta$-dystroglycan (in contrast to a reduction of both $\alpha$- and $\beta$-dystroglycan as seen in dystrophinopathies). The majority of studies have used a combination of polyclonal antibodies raised to the primary amino acid sequence of an $\alpha$-dystroglycan peptide, [51, 76, 77] and monoclonal antibodies to the glycosylated epitopes of $\alpha$-dystroglycan (clones IIH6 and VIA4-1, Upstate Biotechnologies). IIH6 recognises the laminin binding site on $\alpha$-dystroglycan. The epitope recognised by VIA4-1, is still unknown. Although commercially available, there is significant performance variability between different batches of the IIH6 and VIA4-1, and this variability can affect the Western blot and immunohistochemistry results [authors' personal observation]. There is currently no commercial antibody to the core protein.
The extent of the reduction of $\alpha$-dystroglycan labelling is variable amongst these diseases, broadly correlating with disease severity. For example, in MEB [78] $\alpha$-dystroglycan immunolabelling is significantly reduced with IIH6 and VIA4-1 antibodies but only slightly reduced with the sheep antibody to core dystroglycan [51, 78], whereas in WWS $\alpha$-dystroglycan labelling can be completely absent [79]. A similar correlation between disease severity and the extent of the reduction of $\alpha$-dystroglycan has been demonstrated in MDC1C and LGMD2I (with confirmed mutations in FKRP) [74].
Another common feature of the dystroglycanopathies is a reduction in labelling for the laminin-$\alpha$2 chain. The extent of the immunocytochemical reduction is variable but is never completely absent as in primary laminin-$\alpha$2 deficiency. However, on immunoblots of LGMD2I and WWS muscle extracts, the 80-kDa fragment of laminin-$\alpha$2 may be very reduced or almost absent. This discrepancy may relate to the solubility of laminin-$\alpha$2 [57, 79].
The other major laminin receptor in skeletal muscle is integrin $\alpha7\beta1$D. In MDC1C, some authors have reported that both $\alpha$- and $\beta$-chains were reduced [80], while others have noted an apparent upregulation of this integrin [74] in a proportion of fibres. Other proteins may be reduced, i.e. laminin-$\beta$2 [81], perlecan [80] and P180 [75, 82]. Ultrastructural analysis has revealed some changes, but it is not clear if these are specific or due to muscle regeneration/degeneration and generalised basal lamina damage [80, 83].
$\alpha$- and $\beta$-dystroglycan are expressed in the skin (in the epidermal-dermal junction and around keratinocytes) [84]. However, a reduction of dystroglycan immunolabelling in the skin in patients with muscular dystrophy or indeed any other disorder has not yet been described. Immunolabelling of the skin with laminin antibodies is also helpful since a secondary reduction of laminin-$\alpha$2 at the epidermal-dermal junction can also be seen in MDC1C patients [authors' observation]. At present, it is possible to analyse POMGnT1 enzymatic activity in extracts obtained from a muscle biopsy and from cultured fibroblasts [85, 86].

**Prenatal diagnosis**
Genetic analysis allows prenatal diagnosis in families where the primary genetic defect has been identified and prenatal scans are useful [78] in this context.

**Future perspectives**
There are several important questions outstanding, the first being what the precise enzymatic function of fukutin and FKRP is and whether dystroglycan is in fact the primary target/substrate. It will also be important to identify any additional targets that may contribute to the clinical phenotype.
Other proteins that contribute to the glycosylation of $\alpha$-dystroglycan are obviously primary candidates for the glycosylation syndromes of unknown genetic aetiology, such as a form of CMD linked to chromosome 1q42 [87] and Italian MEB [88], in which $\alpha$-dystroglycan immunolabelling is reduced [authors' personal observation].
Animal models will be instrumental in understanding the pathogenesis and will also be valuable as tools with which to try future therapeutic strategies.
Very recently gene transfer of a LARGE construct into the legs of *myd* mice resulted in expression of a highly glycosylated form of $\alpha$-dystroglycan, the restoration of its binding capacity to laminin, agrin and neurexin and an improvement of the muscle pathology [89]. These studies raise the possibility that modulating the expression of LARGE may be a possible therapy for all these defects of dystroglycan glycosylation.

**Integrin $\alpha$7 and CMD**

The laminin-binding integrin $\alpha7\beta1$ is a major laminin receptor of skeletal, cardiac and smooth muscle cells [90]. During muscle development, the laminin-specific

$\alpha$7 integrin is alternatively spliced in both the cytoplasmic ($\alpha_{7A}$, $\alpha_{7B}$) and the putative ligand binding domains ($\alpha 7_{X1}$ and the $\alpha 7_{X2}$). Similarly, the partner $\beta$1 integrin cytoplasmic domain is converted from the $\beta$1A to the $\beta$1D splice variant. In skeletal muscle, integrin $\alpha 7\beta 1$ is located predominantly at the myotendinous and neuromuscular junctions and in the sarcolemma [91, 92]. The critical role of $\alpha_7$ integrins in muscle function became evident after inactivation of both alleles of the *itga7* gene in mice. Deficient mice developed a myopathy, accompanied by disruption of the myotendinous junctions [93]. Subsequently, human patients were identified with similar defects associated with mutations in the *ITGA7* gene (MIM 600536, [94]).

**Clinical phenotype**

Only three Japanese cases have been reported so far. These patients presented with delayed motor milestones, proximal weakness and mildly elevated CK. One patient had mental retardation and two patients had congential torticollis.

**Diagnosis**

Muscle pathological changes are rather myopathic than overtly dystrophic, although regenerating fibres were seen in one patient. On immunohistochemistry the $\alpha$7 subunit was completely absent and the $\beta$1D was slightly reduced. Laminin-$\alpha$2 was normal [94].

**Future perspectives**

Because this form of CMD is very rare, patients are not well characterised at the clinical and pathological level. Together with dystroglycan, integrin $\alpha 7\beta 1$ is the main muscle surface receptor, and its crucial role is underscored by the fact that trangenic overexpression of $\alpha$7 chain in dystrophin/utrophin-deficient mice attenuates the muscle pathology and extends the life span of these mice [95].

## Collagen 6 and Ullrich's CMD

Collagen VI is a major extracellular matrix protein which consists of three $\alpha$ chains, namely $\alpha$1(VI) $\alpha$2(VI) and $\alpha$3(VI), encoded by the *COL6A1* and *COL6A2* genes on chromosome 21q22.3 and *COL6A3* gene on chromosome 2q37. Each chain is made up of two large globular domains connected by a short triple-helical stalk consisting of Gly-Xaa-Yaa amino acid repeat sequences. All three chains contain potential N-glycosylation sites, and the $\alpha$3 chain contains a potential O-linked glycosylation site. The three polypeptide chains form a trimer which further assembles into disulfide-bonded antiparallel dimers and then tetramers (via cysteines in the triple-helical domain) which are secreted from the cell and associate in an end-to-end fashion to give rise to the final microfilament network (5-nm diameter) with a characteristic beaded appearance and 100-nm periodicity [96].

Several mutations in the *COL6A2* and *COL6A3* genes and one mutation in the *COL6A1* gene have been reported in Ullrich's (UCMD) families [97–99]. Mutations in the three collagen VI genes also cause the milder Bethlem myopathy (MIM 158810). In addition, mice null for coll6a1 have been generated by targeted inactivation of the *COL6A1* gene [100].

Collagen VI is present in most connective tissues and in skeletal and heart muscle localises in the reticular layer of the basement membrane around each fiber, perimysium and endomysium. It is also expressed in Schwann, endoneurial and perineurial cells [101] (fig. 2). Collagen VI microfibrils interact with other components of the basal lamina, including collagen IV, fibronectin, biglycan, decorin and perlecan [102–105]. Collagen VI also interacts with cell transmembrane receptors such as integrins and NG2 proteoglycan [106], and in this way serves to transmit signals from the pericellular to the intracellular space. For example, collagen VI induces DNA synthesis and proliferation [107], spreading of fibroblasts [106], promotes survival and inhibits apoptosis [108]. In the extracellular matrix collagen VI interacts with other collagens (collagens I, II and XIV) and with fibronectin [109].

Electron microscopy supports a role of collagen VI in anchoring the muscle fibre basement membrane to the extracellular matrix. Consistent with this there are data showing absence of collagen VI microfibrils from the area immediately adjacent to the basal lamina in the muscle of UCMD patients [110, 111]. Collagen VI may also be important for organisation of the components of the extracellular matrix, since in fibroblast cultures from mice deficient for collagen VI the three-dimensional organisation of fibronectin fibrils is altered [112].

A study of muscle fibers from the *COL6A1* knockout mouse [113] has revealed mitochondrial and sarcoplasmic reticulum ultrastructural abnormalities and increased opening of the permeability transition pore in the mitochondrial membrane. These defects as well as the resulting increased spontaneous apoptosis were rescued in vitro by growing the cells on collagen VI (therefore proving that a defect in collagen VI was causative) and in vivo by cyclosporin treatment which was followed by amelioration of the contractile strength of the mice, suggesting that pharmacological intervention in UCMD may be possible.

**Clinical features of UCMD**

Typically a UCMD patient will present in the neonatal period with muscle weakness, kyphosis of the spine, joint contractures, torticollis, hip dislocation and hyperextensi-

bility of the distal joints. The contractures may improve with physiotherapy but typically recur and may eventually also affect those initially laxed joints [18]. Some patients may never achieve ambulation, while others will be able to walk independently, although ambulation usually decreases with time due to the progressive contractures. CK levels are usually normal or mildly elevated. Rough skin (follicular hyperkeratosis) is a constant feature, and impaired wound healing resulting in the formation of cheloids is common. Many UCMD patients have a characteristic rounded face and prominent ears. Respiratory insufficiency invariably appears in the first or second decade of life, and patients may require ventilation. UCMD appears to be the second most common form of CMD after MDC1A in the West and after FCMD in Japan [114, 115]. Collagen VI is expressed in cardiac muscle; however, heart involvement is not recognised as part of the UCMD phenotype.

Until recently, it was believed that dominant mutations resulting in haploinsuficiency or in normal levels of a mutated collagen VI caused the mild Bethlem phenotype, whereas recessive mutations resulting in greatly reduced or no collagen VI caused the Ullrich phenotype. However, dominant mutations have also been reported even in severe patients [99]. Therefore, it seems that it is the effect of each specific mutation in the production and function of collagen VI that determines the severity of the outcome.

**Diagnosis**

Muscle pathology in the severe cases shows marked variation in fibre size, necrosis and regeneration, increased peri- and endo-connective tissue and a dramatic increase in intramuscular fat (fig. 1). Histochemical reactions for oxydative enzymes may show core-like areas of mitochondrial depletion within muscle fibres or areas of peripheral clusters of mitochondria resembling lobulated fibres. There is a spectrum of collagen VI immunolabelling anomalies. Collagen may be completely absent both from the basal lamina and endomysial and perimysial connective tissue, or the changes may be subtle, with only absence at the basal lamina and apparently normal labelling of the peri- and endomysium [99, 114, 116]. The subtle reduction makes it important to make comparisons with the localisation of another protein such as perlecan or collagen IV or V to asses the integrity of the basal lamina and amount of interstitial connective tissue. The extent of immunolabelling reduction may be more marked with some antibodies than others [115]. Normal or nearly normal collagen VI immunolabelling does not exclude a diagnosis of UCMD [117]. In addition, a reduction in collagen VI immunolabelling has also been found in patients with no detectable mutations in the COL6A genes [116].

In the dermis, collagen VI is found in the connective tissue and in the basal lamina of glands, hair follicles, blood vessels, peripheral nerves and erector pili muscles. In some UCMD cases collagen VI is completely absent from skin, while in others it may be only reduced or even look normal [117, 118]. There is one report of reduced fibronectin receptor expression in the dermis of a UCMD skin biopsy [119].

Primary fibroblasts can be induced to secrete collagen VI in vitro using ascorbic acid in the medium. The secreted collagen VI can be visualised by immunohistochemistry of confluent cultures. Using this approach several reports [99, 112] have described absence or a reduction in the amount of collagen VI in fibroblast cultures from UCMD patients. Collagen VI synthesis can be monitored in vitro using radiolabelled methionine and detetcted by autoradiography [97].

**Prenatal diagnosis**

Collagen VI is abundant in placenta in the mesoderm within the villi and in the blood vessels, and therefore CV samples can be used for prenatal diagnosis by immunohistochemistry, coupled with haplotype analysis [120].

**Future perspectives**

It is becoming increasingly apparent that de novo mutations are a frequent event in UCMD [99; author's personal observation]. This may partly explain the genetic basis of the UCMD cases unlinked to any of the collagen VI loci [115]. In addition, mutations in two different collagen VI genes can also occur, making molecular diagnosis and genetic counselling very complex.

The report of Irwin and colleagues last year (see above) suggests that there is scope for pharmacological intervention, but more work is needed to elucidate the intracellular events mediated by collagen VI [113].

Despite the complete absence of collagen VI from the extracellular matrix, coll6a1-null mice have a mild myopathy compared to the severe phenotype of collagen VI-null humans. For this reason, it will be useful to develop additional animal models, including knock-in mice carrying some of the dominant mutations described in humans.

It is likely that genes other than collagen VI genes are involved in UCMD [115]. Obvious candidates are other collagens and extracellular matrix proteins that interact with collagen VI.

**Seleno-binding protein and RSMD1**

Selenoproteins are a family of enzymes that contain a selenium atom in the form of a seleno-cysteine in the catalytic site and are involved in oxidation-reduction reactions.

The gene responsible for RSMD1 encodes for selenoprotein N (SEPN1), which is a membrane-bound glycoprotein localising to the rough endoplasmic reticulum and expressed in several tissues, including skeletal muscle, heart, brain, lung and placenta [121]. The protein is found at high levels in diaphragm, which could explain the respiratory impairment in RSMD1 patients. In all cases, it is more abundant during foetal than adult life [122].

The specific function of SEPN1 is not known, but given its location it may be involved in protein trafficking and processing or perhaps in calcium homeostasis. Mutations in SEPN1 also cause multi-minicore-disease (MMD) [123] and a form of desmin related myopathy with Mallory-body-like inclusions [124].

### Clinical features of RSMD1

RSMD1 (or rigid spine syndrome, RSS, MIM 602771) is characterised by rigidity of the spine, scoliosis and respiratory insufficiency requiring ventilation. In addition to severe axial weakness there is also mild proximal weakness. The severity is variable, but most patients achieve and maintain ambulation. Patients are usually thin due to muscle wasting and poor weight gain; muscle hypertrophy is not observed. Mental retardation and brain structural abnormalities are not a feature of RSMD1.

Levels of serum CK are normal or mildly elevated. RSMD1 patients have a characteristic nasal speech due to palatal weakness. Muscle MRI reveals a selective pattern of involvement with wasting of the medial aspect of the thighs. The absence of contractures at birth, distal joint laxity and skin hyperkeratosis helps to clinically distinguish RSMD1 from UCMD.

### Diagnosis

Muscle biopsy shows myopathic changes (fig. 1) with increased variation in fibre diameter, internal nuclei, mild increase in endomysial connective tissue and predominance of type 1 fibres. There are no reported secondary protein abnormalities.

In some cases core-like lesions can be seen in the muscle biopsy, but similar features can also be seen in cases with a mutation in the *RYR1* gene [123, 125]. Antibodies to SEPN1 have been used to show the absence of the 70-kDa SEPN1 band in fibroblasts from a RSMD1 patient with nonsense mutations. At present, changes in protein levels cannot be ascertained on sections by immunohistochemistry.

### Future perspectives

This is the first example of a selenoprotein involved in muscular dystrophy, and therefore very little is known about the possible pathomechanism. Further research in skeletal muscle and other tissues needs to be carried out to elucidate its function and identify interacting proteins.

### Conclusions

Despite the advances made in the last few years in the identification of new CMD loci, there are still a number of CMD forms in which the molecular basis remains unknown (e.g. CMD with adducted thumbs) [126], and it is likely that in the next few years the molecular and primary protein defects in some of those will be identified. In some cases, they may lie within proteins related to the main protein groups described in this review, but new pathological pathways may also emerge.

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