# Limb girdle muscular dystrophy type 2I: No correlation between clinical severity, histopathology and glycosylated α-dystroglycan levels in patients homozygous for common FKRP mutation.

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## Abstract

Limb Girdle Musclular Dystrophy type 2I (LGMD2I) is a progressive disorder caused by mutations in the FuKutin-Related Protein gene (*FKRP*). LGMD2I displays clinical heterogeneity with onset of severe symptoms in early childhood to mild calf and thigh hypertrophy in the second or third decade. Patients homozygous for the common *FKRP* mutation c.826C>A (p.Leu276Ile) show phenotypes within the milder end of the clinical spectrum. However, this group also manifests substantial clinical variability.

FKRP deficiency causes hypoglycosylation of α-dystroglycan; a component of the dystrophin associated glycoprotein complex. α-Dystroglycan hypoglycosylation is in turn associated with loss of interaction with -, and hence, depletion of laminin α2. Here, we have attempted to clarify if the clinical variability seen in patients homozygous for c.826C>A, is related to alterations in muscle fiber pathology, α-DG glycosylation levels, levels of laminin α2 as well as the capacity of α-DG to bind to laminin. We have assessed *vastus lateralis* muscle biopsies from 25 LGMD2I patients harboring the c.826C>A/c.826C>A genotype by histological examination, immunohistochemistry and immunoblotting. No clear correlation was found between clinical severity, as determined by self-reported walking function, and the above features, suggesting that more complex molecular processes are contributing to the progression of disease.


Keywords: Limb Girdle Muscular Dystrophy, FKRP, dystroglycan, laminin α2

## 1. Introduction

Limb Girdle Muscular Dystrophy type 2I (LGMD2I) is a progressive, autosomal recessive, disorder that results from mutations in the FuKutin Related Protein gene (*FKRP*) [1, 2]. The spectrum of LGMD2I phenotypes ranges from mild calf and thigh hypertrophy with disease onset in the second or third decade to early onset Duchenne-like disease with loss of ambulation in the teens [3]. Cardiac and respiratory involvement is frequent [4-6].

The phenotypic spectrum caused by *FKRP* mutations is heterogeneous and may also result in severe disease with inability to achieve walking function (Congenital Muscular Dystrophy; MDC1C) [7], with structural brain abnormalities (Walker-Warburg syndrome, WWS), or with muscle-eye brain involvement (Muscle-Eye Brain disease; MEB) [8].

FKRP is thought to be involved in the *O*-glycosylation of α-dystroglycan (α-DG), a component of the dystrophin associated glycoprotein complex (DGC). This was based on findings of reduction in molecular weight and band intensity seen on western blots of muscular extracts from MDC1C patients, using glycan dependent anti α-DG antibodies [7]. Likewise, immuno-histochemistry demonstrates reduced levels or absence of glycosylated α-DG in muscle sections from MDC1C and LGMD2I patients [1, 7].

The *O*-glycan moiety of α-DG anchors the laminin α2 chain, and plays an important role in stabilizing the muscle surface membrane and creating a link to the extracellular matrix. Phenotypic severity has been correlated to depletion of glycosylated α-DG [1, 7, 9-11] and secondary reduction in laminin α2 [6, 12]. However, exceptions have been reported since severe depletion of glycosylated α-DG was found in patients with relatively mild limb-girdle phenotypes [4, 13].

Approximately 100 different FKRP mutations have been identified, of which 60 cause LGMD2I. The c.826C>A mutation (p.Leu276Ile), is the most frequent among LGMD2I patients from Northern Europe and, in general, patients homozygous for this mutation have a

milder phenotype than those who are compound heterozygous or homozygous for other mutations [5, 14]. Correspondingly, in compound heterozygous patients the presence of the c.826A allele has been suggested to reduce the clinical severity caused by other mutations *in trans* [9]. Although they are in the mild end of the clinical spectrum, we have reported substantial clinical variability among LGMD2I patients harbouring the c.826C>A/c.826C>A genotype [14]. Hence, in this work we have sought to clarify if the clinical variability, determined by walking function and age of onset, is related to one or more of the following features: alterations in muscle histopathology, α-DG glycosylation levels, levels of laminin α2 as well as the capacity of α-DG to bind to laminin in an overlay assay. To achieve these gaols, we have enrolled 25 patients homozygous for the *FKRP* c.826C>A allele.

## 2. Patients, Materials and Methods

### *2.1 Patients*

The 25 LGMD2I patients (15 females and 10 males) enrolled here have previously been subjected to investigation by Stensland *et al* [14], and of these six patients have also been studied by Rasmussen *et al* [15]. All patients have consented on the use of diagnostic muscle biopsies for research.

The 25 patients included were all homozygous for the c.826C>A *FKRP* mutation. Patients 2 and 19 (males), and patients 21 and 23 (females) are siblings. A severely affected, compound heterozygous patient (patient 27) (c.826C>A/c.962C>A, p.Leu276Ile/p.Ala321Glu), was included to serve as a α-DG negative control in immunohistochemistry and immunoblot experiments. Information concerning clinical history, age at onset of disease, presenting symptom(s) and current ambulatory status was collected via a questionnaire as explained in Stensland *et al* [14]. The clinical onset of disease was defined as first time the patient noticed

one or more of the following symptoms: reduced walking distance, difficulty climbing stairs or rising from a seated or crouched position, muscle pain after physical exertion, or muscle weakness in the arms. Ambulatory status was scored as following: Walking without aid (0), walking with an aid (1) (e.g. stick, crutch or frame), the need for a wheelchair at walking distances <200 m (2), and wheelchair dependence (3) [14].

This study (#159/2007, #12/2008) was approved by The Regional Committee for Medical Research Ethics (REK Nord). With authorization in the ACT 2008-06-20 no.44: the Health Research Act, § 20, REK Nord waived the need for consent for the use of an anonymous human *rectus femoris* normal control sample.

*2.2 Genetic testing*

*FKRP* gene analysis was carried out as explained in Stensland *et al* [14].

*2.3 Biopsies*

Muscle biopsies were obtained from the *vastus lateralis*. The specimens were flash frozen in isopentane, cooled in liquid nitrogen, and then stored at -80$^0$C.

*2.4 Histological assessment*

Standard techniques were applied for histochemical staining [16]. Frozen sections, 10µm thick, were processed for H&E, modified Gomori trichrome, NADH dehydrogenase, ATPase (pre incubation at pH 4.3, 4.5, and 9.4), acid phosphatase, Periodic-acid Schiff (PAS), Oil red O, and non-specific esterase staining.

A semi-quantitative evaluation of histological changes within each biopsy was carried out by visual inspection as explained in [17]. The histological features were graded as follows:

***Fibrosis***: no fibrosis (0), endomyseal hyaline (1), endomyseal connective tissue fibrosis (2), endomyseal & perimyseal connective tissue fibrosis (3). ***Regeneration***: no regenerated fibers (0), single fiber (1), small groups (2), large groups (3). ***Atrophy***: normal (0), single fiber (1), small groups; <5 fibers (2), large groups; >5 fibers (3). ***Fibers with central nuclei***: no fibers (0), <2% of the fibers (1), 2% - 10% of fibers (2), >10% fibers (3). ***Necrosis***: no necrotic fibers (0), single fiber (1), <5 fibers (2), >5 fibers (3). ***Inflammation***: no inflammation cells (0), 1-3 cells (1), 3-10 cells (2), >10 cells (3).

*2.5 Antibodies*

For immunohistochemistry (IHC) analysis, the following primary antibodies were used: Mouse anti α-Dystroglycan monoclonal antibody, clone VIA4-1 (05-298, Millipore, USA), mouse monoclonals anti laminin α2, clone 2G9/31 (anti-merosin 80 kDa, AdD Serotec, UK) and clone 3/22B2 (anti-merosin 300 kDa, Novocastra, UK). Primary antibodies were detected with either *i*View (Cat no. 760-091, Ventana/Roche, USA) or *ultra*View DAB ICH Detection Kit (Cat no. 760-500, Ventana/Roche, USA). For Western blot analysis the following antibody combinations were used: i) Sheep anti human dystroglycan polyclonal antibody (AF6868, R&D Systems, USA) and Donkey anti sheep IgG-HRP conjugated antibody (HAF016, R&D Systems), ii) Mouse anti rabbit α-Dystroglycan, IgM monoclonal antibody, clone IIH6C4 (05-593, Millipore, USA) and Rabbit anti mouse IgG-HRP (sc-358914, Santa Cruz Biotechnology, USA), iii) Mouse anti laminin α2 (anti-merosisn 80 kDa), clone 5H2 (MAB1922, Millipore, USA) and Rabbit anti mouse IgG-HRP, (sc-358914, Santa Cruz Biotechnology, USA). For laminin overlay assays the following antibodies were used: Rabbit anti laminin (L9393, Sigma, Norway) and Chicken anti rabbit IgG-HRP (sc-2963, Santa Cruz Biotechnology, USA).

*2.6 Immunohistochemistry (IHC)*

Muscle sections from 25 patients were subjected to incubation with primary antibodies against α-DG or laminin α2 and subsequently subjected to detection with either *i*View or *ultra*View DAB ICH Detection Kits, followed by counter staining with haematoxylin, according to manufacturer's specifications. The incubation steps were carried out using an automated slide stainer (Ventana, Roche, USA). Sections were evaluated using a Leica MD 6000 B microscope interfaced to a Leica QWin Semi-quantitative morphometric system. For each biopsy 100 individual fibers were examined. The proportion of glycosylated α-DG and laminin α2 stained fibers were estimated by labelling intensity and percentages of labelled fibers.

*2.7 Western blot and laminin overlay*

Skeletal muscle biopsies from 23 LGMD2I patients, were available for Western blot analysis. Biopsies were homogenized by adding tissue protein extraction reagent (T-PER) (PIERCE, USA) and by the use of a MagNA Lyser Instrument (Roche, USA). Muscle homogenates were centrifuged and 20 μg total protein from the supernatants were electrophoresed on NuPAGE 4-12% Bis-Tris gel (Invitrogen, USA). Proteins were transferred to PVDF membranes in 20% methanol Tris-Glycine buffer at 40-50 constant voltage for 6 hours in cold room for DG detection and laminin overlay immunoblots and for 90 minutes at room temperature for laminin α2 immunoblots. Immunoblot membranes for DG and laminin detection were blocked in 2% non-fat milk low salt Tris-buffered saline (pH 7.4) and subsequently incubated with primary antibodies and their appropriate secondary antibodies. For laminin overlay assays membranes were blocked in laminin Binding Buffer (LBB: 10 mM trietanolamine, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2 pH 7.4) with 5% non-fat milk for one hour, followed by incubation

overnight in LBB/BSA with 1.25 mg/ml laminin (Engelbrect-Holm-Swarm murine sarcoma) (Sigma, Norway). After washing in LBB with 5% non-fat milk 5x5 min, the laminin binding to α-DG was detected by standard immunoblotting method with the appropriate anti laminin antibody and the corresponding HRP conjugated secondary antibody. For both immunoblot and laminin overlay assays membranes were treated with SuperSignal west Dura Extended Duration Substrate (Thermo Scientific, USA). Immunoreactive bands were visualized using a FUJIFILM Luminescent Image Analyzer LAS-3000 (Fuji photo film, Co., LTD, Japan). Band sizes were determined by comparison with MagicMark XP western protein standard (Life Technologies, USA) and the relative band intensities were approximated by visual inspection.

## 3. Results

### *3.1 Clinical findings*

Among the 25 patients the age at onset ranged from 0 to 45 years (one patients with missing information) (Tables 1 and 2). Thirty six percent showed onset of disease before 10 years of age. Disease duration at biopsy ranged from 1 to 40 years. Sixty three percent had a disease duration at the time of biopsy of < 10 years, 21 % had disease duration at biopsy of 10-20 years, and 16 % had >20 years of disease duration at the time of biopsy (Tables 1 and 2). Difficulties in walking/running and/or climbing stairs and/or rising from a seated position were the presenting symptoms in 22 of the patients (88%). One patient (patient 10) reported weakness in the arms as an additional symptom. Exertional pain was the first presenting symptom in one patient (patient 2). Exertional pain was also reported as an additional onset symptom in patients 1, 13, 14, 16, 17, 20 and 22. Two patients reported exertional pain with episodes with dark urine (patients 2 and 16). The majority of the patients were walking without aid (60 %, n=15) while only one patient had already lost ambulation/was wheelchair dependent at the time of study

(patient 21). Patient 27, the patient control included, was one year old at onset and never showed the ability to walk (Table 2).

*3.2 Histopathological alterations*

There was a great variability in the degree of structural changes among the patients. The majority of the 25 muscle biopsies showed non-specific myopathic changes, consistent with mild to severe degrees of a slowly progressive muscular dystrophy. Semi quantitative grading of fibrosis, regeneration, atrophy, centralized nuclei, necrosis and inflammation is presented in Table 2. Biopsies could be categorised into three groups according to the main histological alterations as follows:

**Mild structural changes (patients 1, 2, and 7):** These biopsies revealed unaltered skeletal muscle with focal scattered necrotic fibers and small groups regenerating muscle fibers, no or minimal endomyseal fibrosis was detected. Less than 3% of the muscle fibers showed internal or centralized nuclei.

**Moderate structural changes (patients 3, 10, 11, 12, 16, 20, 21 and 22):** In these biopsies clusters of necrotic fibers invaded by macrophages and hypercontracted fibers were observed. Variable degrees of endomyseal fibrosis and increased number of hypertrophic/atrophic fibers were detected. ≤ 20% of the muscle fibers showed internal nuclei.

**Severe structural changes (patients 5, 6, 8, 9, 13, 14, 15, 17, 18, 19, 23, 24, 25, and 26):** In biopsies with the most severe myopathic changes, necrotic and regenerated fibers were increased in numbers. Fibers splitting and both endomyseal and perimyseal fibrosis were seen. These biopsies also showed increased numbers of hypertrophic fibers and small groups of atrophic fibers. More than 20% of the muscle fibers showed internal nuclei. Most of these biopsies showed increased inflammatory changes with scattered infiltrates of lymphocytes.

*3.3 α-DG glycosylation and laminin α2 status in LGMD2I muscle sections*

Muscle sections from 25 patients, immunolabelled for glycosylated α-DG demonstrated variable levels of α-DG glycosylation, ranging from normal to complete absence (Fig. 1). The proportion of glycosylated α-DG was ≥ 80% in 12 patients (disease duration at biopsy: mean 17.5, median 11, range 3-40), 40-70% in three, and ≤ 30% in 10 patients (disease duration at biopsy: mean 6.8, median 7.5, range 1-16). The compound heterozygous, patient control, showed complete absence of glycosylated α-DG.

Although the three patients with mild structural alterations (patients 1, 2 and 7) displayed normal levels of glycosylated α-DG, no linear relationship was noticed between the extents of pathological changes, and the level of α-DG glycosylation seen by IHC. By comparing α-DG glycosylation status with walking function, no clear correlation was observed. Among the 12 patients with ≥ 80% glycosylated α-DG three needed walking aid whereas nine patients were walking without aid at the time of the study. At the other end of the spectrum, four out of 10 patients with ≤ 30% glycosylated α-DG demonstrated normal walking function whereas six were wheelchair dependent, either partially or completely.

Laminin α2 labelling, using anti 80 kDa and anti 300 kDa laminin α2 antibodies, appeared normal in all (Fig. 1), except in biopsies from two patients where laminin α2 labelling was somewhat patchy and slightly reduced.

α-DG glycosylation levels were judged by visual inspection after immunoblot analyses, using anti core DG antibody and the glycan specific anti α-DG antibody; IIH6C4, on muscle homogenates from 23 patients. When compared with normal control muscle a reduction in α-DG molecular weight (MW) (~ 80 kDa) was noticed in all patients when using the anti-core DG antibody. Some patients displayed a faint high MW smearing (Fig 2, upper panel). This feature was associated with relatively strong α-DG glycan specific signals by IIH6C4 (patients

2, 3, 9, and 17). The remaining 19 patients showed either reduced levels, or absence of glycosylated α-DG (patients 8, 13, 18, and 24) (Fig 2, panel 2). β-DG was normal in all patients and served as a loading control (Fig. 2, upper panel).

There was no obvious correlation between α-DG glycosylation levels, seen by immunoblotting, and walking function. At the time of the study three (patients 2, 9, and 17) of the four patients displaying strong of α-DG glycosylation had normal ambulation. However, normal walking function was also the case for three patients (patients 8, 13, and 18) with severe reduction of glycosylated α-DG.

Overall, as observed by laminin overlay assays, the lack of α-DG glycosylation corresponded to its reduced capacity to bind laminin (Fig. 2, panel 3). However, no depletion of the endogenous laminin α2 80 kDa, C-terminal fragment was noticed (Fig. 2, panel 4).

*3.4 Intrafamilial variability*

Among the patients there were two pairs of siblings; two brothers (patients 2 and 19) and two sisters (patients 21 and 23) (Table 2). The two sisters (patients 21 and 23) showed similar age at onset, 10 and 8 years of age, respectively. They both had impaired walking function and displayed similar levels of α-DG glycosylation and histopathological alterations, except that necrosis was pronounced in the younger one. The two brothers (2 and 19) had preserved walking function, however, they differed with regard to age of onset; 12 vs 27 years of age and levels of α-DG glycosylation; 100 vs 30% of normal, respectively. The younger brother (19) with the shortest disease duration displayed the most severe histopathological alterations, in particular with regard to necrosis, inflammation and regeneration (Table 2).

## 4. Discussion

Inter- and intrafamilial clinical heterogeneity, among LGMD2I patients, have been reported in several studies [4-6, 12, 14, 15, 18-20]. The cause of this variability may in part be explained, by correlation of phenotype with *FKRP* genotype [1, 7, 9, 11]. In this work all LGMD2I patients included were c.826C>A homozygotes. This allowed us to assess walking function, histopathological alterations, α-DG glycosylation status, and potential endogenous laminin α2 depletion, on a homogeneous *FKRP* genotypic background.

The histopathological alterations were typically dystrophic but variable (Table 2). The extent of histopathological alterations did not correlate with disease duration at biopsy, age of onset, or walking function. Only a few studies have investigated the relationship between histopathological alterations and clinical severity in c.826C>A homozygotes. The studies include few patients and have employed different methods for clinical and histopathological assessment. However, five homozygous patients studied by Boito *et al* [21] showed an apparent correlation between the clinical grading and pathology severity score, with an inverse correlation to age of onset. In contrast, four homozygous patients studied by Yamamoto *et al* [12] all showed a mild clinical course with no obvious correlation with age of onset and histopathological alterations. Likewise, in a study of 16 young homozygous patients (of whom six are included in this work) there appeared to be lack of correlation between ages at first evaluation, clinical severity and biopsy morphological sum-score [15]. Although comparison of clinical and histological findings across studies are hampered by the different the methods employed, it seems fair to conclude that there exist extensive clinical and histopathological variability, and that these two features appear not to be mutually related in LGMD2I patients homozygous for *FKRP* c.826C>A.

Proper *O*-glycosylation of α-DG in muscle cells is crucial for sarcolemma stability and the linking of the Extra Cellular Matrix (ECM) components to the actin cytoskeletal network [22].

Among the patients reported here variable α-DG glycosylation was observed by IHC, ranging from 5% to 100% of normal muscle. In some patients a patchy pattern of α-DG labelling was observed. A patchy labelling pattern has previously been described in LGMD2I [12, 20, 23] and might be due to transient expression of glycosylated α-DG in regenerating muscle fibers [24]. By immunoblot analysis with anti-core DG antibody, α-DG hypoglycosylation was observed as a down-shift in α-DG molecular weight (MW) in all patients, and variable levels of α-DG hypoglycosylation was observed using *O*-glycan specific antibodies. α-DG glycosylation levels detected by immunoblot correlated broadly with those detected by IHC.

The relationship between α-DG glycosylation and clinical severity have previously been studied in LGMD2I patients harbouring various *FKRP* mutant genotypes [1, 7, 10, 13, 21]. By employing IHC Brown *et al* [10] demonstrated a correlation between α-DG glycosylation levels and clinical phenotype. Whereas c.826C>A homozygotes (four patients) showed a mild form of LGMD2I, with a subtle alteration in α-DG glycosylation, severely affected patients harbouring other mutations showed profound reduction of glycosylated α-DG. However, Jimenez-Mallebrera *et al* [13] found some inconstancies between levels of α-DG glycosylation determined by IHC and the clinical course in patients harbouring mutations in *fukutin* and *FKRP*. Furthermore, Boito *et al* [21] found variable reduction of glycosylated α-DG in patients investigated by IHC and, interestingly, by immunoblot analysis they detected only trace amounts of glycosylated α-DG in three c.826C>A homozygotes of whom one was asymptomatic [4]. The result presented in this work demonstrates a huge variability in the levels of glycosylated α-DG among the homozygous patients, however, we observed no systematic correlation between the level of α-DG glycosylation and walking function or histopathological alterations.

Since *O*-glycosylation of α-DG is crucial for laminin α2 binding [22, 25], the capacity of α-DG to bind laminin α2 is expected to be reduced in LGMD2I patients. Various degrees of laminin

α2 depletion, ranging from normal to severe, have been reported in LGMD2I patients homozygous for c.826C>A [1, 6, 12]. In the patients reported here there was a clear correlation between the level of glycosylated α-DG and its ability to bind laminin in laminin overlay assays. However, no significant depletion of endogenous laminin α2 was observed, neither by IHC nor by immunoblot analysis.

The availability of only two pairs of siblings restricts observations and, hence, a discussion on intrafamilial variability. However notably, whereas the two sisters (patients 21 and 23) were similar regarding disease onset, histopathology and α-DG glycosylation levels, the two brothers (2 and 19) demonstrated great variation in age at onset, 12 vs 27 years of age. Moreover, their disease durations at biopsies were 24 and 7 years, respectively, but still patient 19 displayed more pronounced histopathological alterations and lack of α-DG glycosylation. The phenotypic variability within sib-ships seen here, is in agreement with earlier reports [4, 14, 19].

Although the 25 of the patients investigated here harbour the same genotype we found no relationship between walking function, histopathological alterations and α-DG glycosylation levels in affected muscle. Neither was there any consistent relationship between histopathological alterations and gender, age at onset or disease duration at biopsy. The lack of correlation among these features could potentially be caused by modifying factors, such as environmental factors and molecular factors. The function of muscle groups other than the proximal, are also essential to the complex performance of walking. Thus, factors such as general physical condition, nutrition and availability to physiotherapy might contribute as compensating mechanisms in this regard.

Furthermore, secondary molecular processes have been suggested to affect the phenotype of LGMD2I. By employing quantitative reverse transcription PCR on RNA derived from LGMD2I muscle, Boito et al (2007) [21] demonstrated upregulation of *GRP78* and *CHOP*

transcription. GRP78 and CHOP are proteins involved in the endoplasmic reticulum stress response that is induced by the unfolded protein response signalling system (UPR). A similar conclusion was made by Lin et al (2011) [26], who studied *FKRP* and *fukutin* knock-down Zebrafish embryos. They suggested further that knock-down of FKRP or fukutin function may affect protein secretion beyond α-DG glycosylation resulting in acute ER stress and consequent UPR activation. Moreover, in a study by Hauerslev *et al* (2013) the muscle biopsy of a severely affected patient with LGMD2I histopathology did not reveal the complex molecular processes affecting the progression of disease. Instead, muscle mass impairment and atrophy were suggested to be caused by increase in protein turnover and myostatin levels [27]. Altogether, these studies indicate that mechanisms other than the impairment of α-DG glycosylation and the disruption of its interaction with laminin, might play important roles LGMD2I muscle pathogenesis. Furthermore, activation of the ubiquitin-proteasome-system (UPS) and the autophagic lysosomal system was observed in muscles from LGMD2A and LGMD2B patients [28, 29]. Hence, detailed investigations of the potential involvement of the UPR, UPS and the autophagic lysosomal system in LGMD2I pathology needs to be carried out.

In summary, we have demonstrated that the clinical heterogeneity in LGMD2I patients, homozygous for *FKRP* c.826C>A, cannot be explained by histopathological alterations, levels of α-DG hypoglycosylation or laminin α2 depletion. Hence, in our opinion none of these features appear to be suitable as prognostic markers for the gradual impairment of walking function in LGMD2I.

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**Table 1**.

Characteristics of the 25 patients homozygous for *FKRP* c.826C>A.

| | |
|---|---|
| Patients, n | 25 |
| Sex male/female, n | 10/15 |
| Mean average age, years | 32.0 (range 12-56) |
| Mean age at onset, years | 14.7 (range 0-45) |
| Mean disease duration at biopsy, years | 11.4 (range 1-40) |
| Ambulation | |
| Without walking aid (n) | 60 % (15) |
| With walking aid (n) | 4 % (1) |
| Wheelchair at distance>200 m (n) | 28 % (7) |
| Lost ambulation (n) | 4 % (1) |
| Histological sum-score (range 0-18) | 8,2 (range 1-15) |
| α-DG, IHC, % (range 0-100) | 58 (20-100) |

**Table 2** Clinical and histopathological characteristics of patients included in this study.

| Pat. No | Gender | Age | Age at onset | Onset symptoms | Disease duration | Disease duration at biopsy | Walking function | Fibrosis | Regen | Atrophy | Centr nuclei | Necrosis | Inflam | Alpha Dys IHC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | M | 56 | 11 | Low extrem | 45 | 40 | 0 | 2 | 1 | 2 | 1 | 1 | 1 | 100 |
| 11 | M | 53 | 9 | Low extrem | 44 | 37 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | 80 |
| 17 | F | 54 | 17 | Low extrem[3] | 37 | 37 | 0 | 1 | 2 | 1 | 3 | 1 | 1 | 100 |
| 2[1] | M | 38 | 12 | Exert pain | 26 | 24 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 100 |
| 16 | M | 44 | 14 | Low extrem[3] | 30 | 16 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 30 |
| 6 | F | 20 | 1 | Low extrem | 19 | 15 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 100 |
| 24 | F | 35 | 16 | Low extrem | 19 | 13 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 20 |
| 10 | F | 18 | 0 | Low extrem[3] | 18 | 11 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 80 |
| 13 | F | 38 | 16 | Low extrem[3] | 22 | 10 | 0 | 2 | 2 | 3 | 2 | 2 | 2 | 80 |
| 26 | F | 37 | 18 | Low extrem | 19 | 8 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 5 |
| 8 | F | 14 | 1 | Low extrem | 13 | 8 | 0 | 1 | 2 | 3 | 1 | 0 | 1 | 20 |
| 20 | M | 15 | 4 | Low extrem[3] | 11 | 8 | 2 | 1 | 3 | 0 | 1 | 0 | 0 | 20 |
| 12 | M | 31 | 18 | Low extrem | 13 | 7 | 1 | 2 | 1 | 1 | 1 | 2 | 0 | 80 |
| 19[1] | M | 34 | 27 | Low extrem | 7 | 7 | 0 | 1 | 2 | 2 | 3 | 2 | 2 | 30 |
| 7 | F | 15 | 7 | - | 8 | 6 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 90 |
| 15 | F | 38 | 27 | Low extrem | 11 | 5 | 0 | 3 | 2 | 2 | 2 | 2 | 2 | 50 |
| 25 | F | 13 | 6 | Low extrem[3] | 7 | 4 | 2 | 2 | 3 | 2 | 2 | 2 | 1 | 50 |
| 1 | M | 56 | 45 | Low extrem[3] | 11 | 3 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 90 |
| 9 | M | 39 | 34 | Low extrem | 5 | 3 | 0 | 2 | 2 | 3 | 3 | 3 | 2 | 90 |
| 21[2] | F | 24 | 10 | Fatigue | 14 | 3 | 3 | 2 | 1 | 1 | 1 | 0 | 0 | 20 |
| 23[2] | F | 21 | 8 | Low extrem | 13 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 20 |
| 18 | F | 28 | 24 | Low extrem | 4 | 2 | 0 | 2 | 2 | 2 | 1 | 2 | 1 | 30 |
| 22 | M | 12 | 6 | Low extrem[3] | 6 | 1 | 0 | 1 | 2 | 1 | 0 | 2 | 1 | 20 |
| 14 | F | 35 | 26 | Low extrem | 9 | 1 | 0 | 2 | 2 | 2 | 2 | 2 | 1 | 60 |
| *27 | M | 3 | 1 | - | 2 | 1 | - | 1 | 1 | 2 | 1 | 0 | 2 | 0 |
| 3 | F | 37 | - | Low extrem | - | - | 2 | 1 | 0 | 2 | 2 | 0 | 0 | 100 |

**Table 2.**

Patients with LGMD2I are sorted according to disease duration at biopsy. All patients, except 27 are homozygous for the c.826C>A FKRP mutation (p.Leu276Ile). *Patient 27 is compound heterozygous for mutations and c.826C>A/c.962C>A (p.Leu276Ile/p.Ala321Glu). Patients 2[1] and 19[1], and 21[2] and 23[2] are siblings. Onset symptoms: Lower extremities (Low extrem) refers to problems walking/running and/or climbing stairs and/or problems rising from a seated position. [3] Refers to exertional pain as an additional onset symptom.

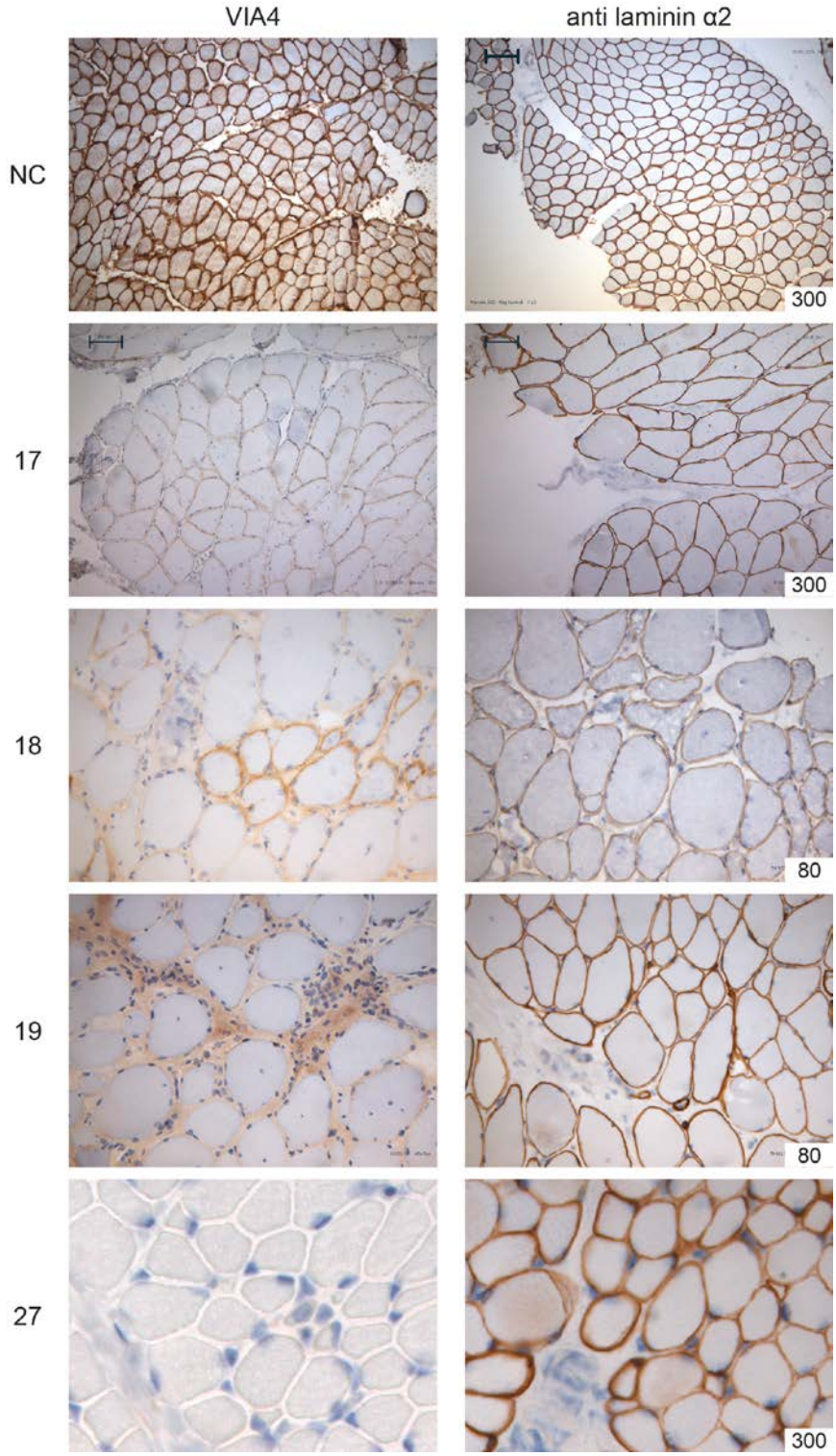


**Figure 1.**

Immunohistochemical stainings of a *rectus femoris* normal control (x5) and the vastus lateralis muscles of patients 17, 18, 19 and 27 (x20), with antibodies directed towards the glycan epitope of α-DG (left) and either the laminin α2, 300 kDa fragment, or the 80 kDa fragment as indicated (right).

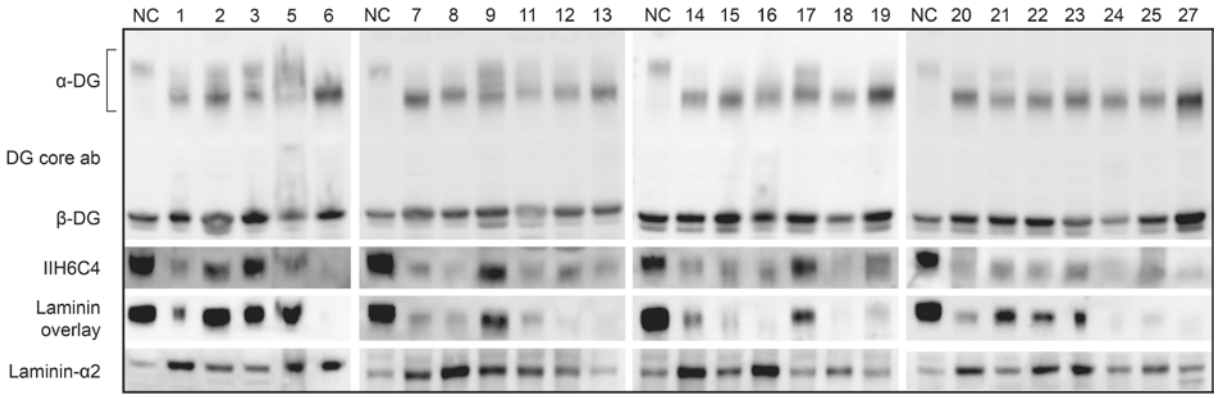


**Figure 2.**

Assessment of glycosylated α-DG and its capacity to bind laminin in LGMD2I muscle homogenates. Upper panel: α/β-DG was detected by sheep anti human core DG. Second panel: The level of α-DG glycosylation was detected with glycan dependent mouse anti human α-DG antibody (IIH6C4). Third panel: Laminin overlay assay where the membrane was coated with laminin followed by detection with rabbit anti laminin antibodies. Fourth panel: Laminin α2 was detected with mouse anti human laminin α2 antibodies (80 kDa fragment). NC; is the normal muscle control. The molecular weights were determined by use of the Magic Mark™ western protein standard.

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