A heterozygous 21-bp deletion in \textit{CAPN3} causes dominantly inherited limb girdle muscular dystrophy

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Limb girdle muscular dystrophy type 2A is the most common limb girdle muscular dystrophy form worldwide. Although strict recessive inheritance is assumed, patients carrying a single mutation in the calpain 3 gene (\textit{CAPN3}) are reported. Such findings are commonly attributed to incomplete mutation screening. In this investigation, we report 37 individuals (age range: 21–85 years, 21 females and 16 males) from 10 families in whom only one mutation in \textit{CAPN3} could be identified; a 21-bp, in-frame deletion (c.643\_663del21). This mutation co-segregated with evidence of muscle disease and autosomal dominant transmission in several generations. Evidence of muscle disease was indicated by muscle pain, muscle weakness and wasting, significant fat replacement of muscles on imaging, myopathic changes on muscle biopsy and loss of calpain 3 protein on western blotting. Thirty-one of 34 patients had elevated creatine kinase or myoglobin. Muscle weakness was generally milder than observed in limb girdle muscular dystrophy type 2A, but affected the same muscle groups (proximal leg, lumbar paraspinal and medial gastrocnemius muscles). In some cases, the weakness was severely disabling. The 21-bp deletion did not affect mRNA maturation. Calpain 3 expression in muscle, assessed by western blot, was below 15\% of normal levels in the nine mutation carriers in whom this could be tested. Haplotype analysis in four families from three different countries suggests that the 21-bp deletion is a founder mutation. This study provides strong evidence that heterozygosity for the c.643\_663del21 deletion in \textit{CAPN3} results in a dominantly inherited muscle disease. The normal expression of mutated mRNA and the severe loss of calpain 3 on western blotting, suggest a dominant negative effect with a loss-of-function mechanism affecting the calpain 3 homodimer. This renders patients deficient in calpain 3 as in limb girdle muscular dystrophy type 2A, albeit in a milder form in most cases. Based on findings in 10 families, our study indicates that a dominantly inherited pattern of calpainopathy exists, and should be considered in the diagnostic work-up and genetic counselling of patients with calpainopathy and single-allele aberrations in \textit{CAPN3}.
Limb girdle muscular dystrophy (LGMD) encompasses a group of muscular dystrophies that share common clinical and laboratory findings such as (i) weakness and atrophy of proximal muscles, often affecting lower extremities first; (ii) elevated creatine kinase (CK) levels; and (iii) dystrophic findings on muscle biopsy. More than 35 recessively and dominantly inherited forms of LGMD have been identified so far (http://neuromuscular.wustl.edu/musdist/lg.html).

LGMD type 2A was the first LGMD to be defined at the molecular level (Richard et al., 1995), and remains the most common recessive LGMD subtype worldwide (Zatz and Starling, 2005; Guglieri et al., 2008; Norwood et al., 2009). Regional differences in prevalence exist, and in Denmark, LGMD type 2A is only the third most common LGMD among ethnic Danes, surpassed in frequency by LGMD type 2C and LGMD type 2B (Sveen et al., 2015). The function of calpain 3 is not fully elucidated, but appears to include (i) assembly and remodelling of contractile proteins in the sarcomere (Sorimachi et al., 1995; Kramerova et al., 2004; Cohen et al., 2006); (ii) control of Ca<sup>2+</sup>-efflux from the sarcoplasmic reticulum (Ojima et al., 2011); (iii) membrane repair (Huang et al., 2008); and (iv) muscle regeneration (Hauerslev et al., 2012).

It may be difficult to distinguish patients with LGMD type 2A from other recessive LGMDs, but clinical clues for LGMD type 2A can be (i) absence of cardiac involvement; (ii) asymmetric weakness and atrophy, which is uncommon in other recessive forms of LGMD, except LGMD type 2L and LGMD type 2B (Sveen et al., 2008; Sarkozy et al., 2012; Quick et al., 2015); and (iii) early scapular winging. In fact, upper extremity affection may be the presenting sign in some cases. On muscle MRI, it is characteristic that the posterior thigh muscles and the adductor magnus muscle are preferentially affected with relative sparing of the anterior thigh muscles (Mercuri et al., 2005).

Allelic diseases with both dominant and recessive modes of inheritance have been reported for a number of muscle diseases, such as myotonia congenita and collagen 6-related myopathies. This dual mode of inheritance has also been reported for LGMD, such as desminopathies (Nigro and Savarese, 2014). In this report, we provide evidence that the well-known recessively inherited calpainopathy also exists in a dominantly inherited form. This work began when we re-diagnosed Danish patients with LGMD (Schwartz et al., 2005; Sveen et al., 2006). During this process, we sequenced for aberrations in CAPN3 in all patients that had not been assigned a definite LGMD subgroup, and found three patients from three families, who initially appeared to be sporadic cases, carrying a single, 21-bp deletion of CAPN3 (c.643_663del21) (Duno et al., 2008). Subsequent investigations clearly indicated a dominant trait of the mutation in their families. After reporting this at the 14th World Muscle Society meeting (Vissing et al., 2011), we were contacted by researchers in the UK, Sweden and Norway, who had found families carrying this mutation as well, with apparent dominant mode of inheritance. This report therefore presents the collective experience and findings on this new mode of inheritance for calpainopathy in patients from the UK, Norway, Sweden and Denmark.

**Materials and methods**

**Subjects**

During the course of diagnosing families affected by LGMD at neuromuscular centres in Denmark, Sweden, Norway and the UK, we identified 10 probands from different families, heterozygous for the 21-bp, in-frame deletion in CAPN3 (c.643_663del21). Three families came from Denmark (Copenhagen), three from the UK (Newcastle), two from Norway (Bergen and Tromsø) and two from Sweden (Stockholm and Örebro). Informed consent for diagnostic testing was obtained for all individuals, according to the Declaration of Helsinki.

Participants were tested for muscle strength and atrophy, and questioned about their use of walking aids and clinical
symptoms (Table 1). Serum CK levels, and in some individuals myoglobin levels, were tested in both probands and their family members. Fourteen individuals had a muscle biopsy performed on the vastus lateralis muscle for histological and western blot analyses. Western blot was performed on the muscle tissue and quantified as previously described (Sveen et al., 2006), using mouse anti-human calpain 3, clone 12A2 (Novocastra) recognizing the full size calpain and the 60 kDa fragment, and clone 2C4 recognizing full size calpain and the 30 kDa fragment. The clones 2C4 and 12A2 recognize amino acids 1–9 and 350–370 of the human calpain 3 molecule, and thus their epitopes do not overlap with the mutation found in this study, which affects amino acids 215–225 of calpain 3. Eleven patients had a T1-weighted MRI, and one a CT, performed of paraspinal and lower limb muscles.

**Molecular analyses**

DNA was isolated from EDTA blood samples by standard methods, and the entire coding and exon-flanking sequences of CAPN3 (NM_000070) were PCR amplified and directly sequenced (primers and PCR conditions vary from country to country, but are available on request). Haplotype analysis was performed by assaying three tightly linked microsatellites in Families 2, 3, 9 and 10. The proband of Family 3 is of Polish decent. All affected individuals carried c.643_663del21 on the same haplotype (Supplementary material). cDNA analysis of muscle CAPN3 RNA in the Danish patients and primers used, have been described in Duno et al. (2008).

**Results**

**Segregation of the c.643_663del21 mutation in CAPN3 with muscle disease**

In the 10 families we report here, we identified a total of 37 carriers of the c.643_663del21 mutation (Fig. 1). Evidence of muscle disease segregated with the mutation in 36 of 37 mutation carriers. The verification of muscle disease was based on findings of muscle weakness/atrophy, hyperCKemia, myopathic muscle biopsies and muscle replacement by fat on MRI (Table 1 and Figs 3–5). The seemingly unaffected mutation carrier (Subject 1/II-4, Table 1), however, was not tested by MRI or muscle biopsy, and could therefore still be subclinically affected, similar to other family members. Likewise, all non-mutation carriers, with the exception of one (Subject 5/III-1), showed no evidence of muscle disease. Collectively, this shows a strong association of the c.643_663del21 mutation in CAPN3 to muscle disease and dominant transmission. The non-carrier person (Subject 5/III-1), had been reported with an elevated CK level of unknown magnitude, but was unavailable for further testing. However, a repeat CK measurement in this person at age 15 years showed normal CK, suggesting that the previous elevated CK was due to an episodic phenomenon, such as physical exertion.

**Clinical phenotype**

The clinical phenotype in the dominant families showed similar variability to the recessively inherited form of calpainopathy, ranging from almost asymptomatic to wheelchair dependence. This variation was observed even within families. The pattern of muscle involvement also resembled that seen in LGMD type 2A, with the most affected muscles being the paraspinal, gluteal, hamstring and medial gastrocnemius (Table 1). Unquestionably, however, the phenotype in this dominant form is generally milder than in LGMD type 2A. In line with this, disease onset was on average 34 years, which is 16 years later than British and Danish patients affected by LGMD type 2A (Groen et al., 2007; Hauerslev et al., 2012). Strikingly prevalent symptoms were myalgia and back pain, which were present in about half of the mutation carriers.

**Molecular findings**

The c.643_663del21 deletion in CAPN3 does not compromise RNA maturation, and the transcript appears to be expressed equal to wild-type calpain 3 mRNA (Duno et al., 2008). The amount of calpain 3 protein was severely reduced to less than 15% of normal, as judged by western blot (Fig. 2), in all nine patients in whom this could be investigated. Haplotype analysis through microsatellite analysis in four families from three different countries suggested that the 21-bp deletion is a founder mutation (Supplementary material).

**Muscle imaging**

Eleven patients had MRI and one had CT performed of their muscles. The patients were not selected for imaging according to disease severity; in fact several patients were minimally affected (Table 1). Nevertheless, 11 of 12 patients showed significant muscle replacement by fat in one or several muscle groups, i.e. at least Grade 2a on the Mercuri MRI grading scale (Mercuri et al., 2002). Thus, MRI disclosed severe muscle affection in mutations carriers Subjects 1/II-5, 1/III-2, 1/III-4, and 1/III-6, who otherwise had minimal complaints and findings, besides elevated CK levels. In Subject 1/II-5, an increased muscle fat fraction on MRI and elevated myoglobin levels were the only laboratory anomalies, but as evident from Fig. 3, the paraspinal muscles were almost totally lost in this individual. In line with the pattern of weakness, the most affected muscle groups on imaging were paraspinal, gluteal, hamstring and medial gastrocnemius muscles (Figs 3 and 4).

**Muscle histology**

Muscle histology in the 14 mutation carriers in whom a muscle biopsy was performed, universally showed myopathic changes with increased number of internalized nuclei and variation in fibre size (Fig. 5). Occasionally, cell necrosis, ring fibres and increased fibrosis among
Table 1  Demographic, clinical and laboratory findings in 10 families/37 carriers of the c.643_663del21 mutation in CAPN3

<table>
<thead>
<tr>
<th>Family\b/</th>
<th>person</th>
<th>Age at exam (years)</th>
<th>Age at onset (years)</th>
<th>Sex F/M</th>
<th>Weakness</th>
<th>Atrophy</th>
<th>Ambulatory aids</th>
<th>Other symptoms</th>
<th>Muscle fat infiltration on MRI</th>
<th>Creatine kinase (normal &lt; 150)</th>
<th>Muscular biopsy</th>
<th>WB calpain (% of normal)</th>
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<td>1/II-1</td>
<td>74</td>
<td>50</td>
<td>M</td>
<td>Prox arms</td>
<td>Prox arms</td>
<td>None</td>
<td>None</td>
<td>Back pain</td>
<td>Paraspinal, glut max, adductors</td>
<td>Normal\a</td>
<td>ND</td>
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<tr>
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<td>76</td>
<td>40</td>
<td>M</td>
<td>Prox limbs</td>
<td>Prox limbs</td>
<td>Rollator</td>
<td>None</td>
<td>Back pain, myalgia</td>
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<td>1542</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<td>No</td>
<td>Med gest</td>
<td>None</td>
<td>None</td>
<td>No</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
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<td>7/I-2</td>
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<td>No</td>
<td>Normal\a</td>
<td>ND</td>
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Subject designation in the first column refers to the number shown in the pedigrees (Fig. 1). When listed as fat infiltrated on MRI, the muscle rates 2a as a minimum on the Mercuri scale (Mercuri et al., 2002).

Prox = proximal; ND = not determined; Med gest = the medial gastrocnemius muscle; COLD = chronic obstructive lung disease; Glut max = the gluteal maximus muscle; F = female; M = male; WB = western blot.

*aMyoglobin was elevated in persons with normal creatine kinase levels.

bThis female was studied by CT and not MRI.
intrafascicular myocytes were observed, but generally, dystrophic changes did not reach the level seen in patients with LGMD type 2A (Supplementary Table 1).

Plasma creatine kinase

Serum CK was elevated in most patients, and ranged from normal to 9000. In seven patients, CK levels were normal, but myoglobin was elevated in four of them. In total, CK and or myoglobin were elevated in 31 of 34 mutation carriers in whom it was tested.

Discussion

This report documents evidence of a dominantly inherited form of calpainopathy. In 10 families from four countries, we demonstrate that heterozygosity for the c.643_663del21 mutation in CAPN3 results in limb girdle muscular dystrophy, resembling the recessively inherited form, but with a generally milder phenotype. Fat replacement of muscle, muscle weakness and atrophy, hyperCKemia and/or myopathic histopathological findings were observed in 36 of 37
inheritance was not suggested (Chrobáková et al., 2004; Stehlíková et al., 2007). Interestingly, this patient carried an in-frame deletion (c.598_612del15) close to the location of the deletion found in our patients. A single family has been described where five male members were heterozygous for a single 3-bp deletion in CAPN3, and all showed variable degrees of muscular dystrophy. Remarkably, all five also carried a mutation in the XK gene leading to McLeod syndrome (Starling et al., 2005). We therefore sequenced the XK gene in Patients 1/II-6, 2/II-1 and 3/I-3, but did not detect aberrations in this gene. A single in-frame deletion in CAPN3 (c.759-761delGAA) has also been described in one patient with calpain 3 deficiency and camptocormia (Liewluck and Goodman, 2012), but family members were not investigated.

What could be the mechanism by which CAPN3 aberrations result in a dominantly inherited trait? The active calpain 3 enzyme is a homodimer, and if a mutation does not lead to non-sense mediated mRNA decay of the transcript, the altered calpain 3 protein can potentially polymerize with wild-type calpain 3 and render the complex inactive. Theoretically, this would leave on average 75% of the dimer molecules inactive, and reduce calpain 3 activity to 25% of normal or less dependent on individual allelic expression. In accordance with this, western blotting showed residual calpain 3 expression of <15% in all patients in whom a muscle biopsy was performed. The seemingly unbiased expression of the mutated mRNA in our patients, as inferred from cDNA analyses, and the in-frame nature of the mutation likely led to expression of mutated protein and thus a dominant negative effect on wild-type calpain 3 as described above. Most patients with LGMD type 2A have total loss of calpain 3 expression on western blotting, but far from always (Fanin et al., 2004; Fanin and Angelini, 2013). The consistent finding in our patients of residual calpain 3 on western blotting, albeit seriously reduced to 5–15% of normal, helps to explain the generally milder phenotype of the dominantly transmitted disease versus LGMD type 2A.

A dual mode of inheritance (dominant and recessive), where a protein is either lost by two mutations or lost by protein expression of a mutated allele exerting a dominant negative effect, has been described for several other diseases. These include myotonia congenita (Thomsen and Becker myotonia) (Duno et al., 2004), collagen 6-related muscle diseases (Bethlem myopathy and Ullrich congenital muscular dystrophy) (Foley et al., 2009), carnitine palmitoyltransferase II deficiency (Ørngreen et al., 2005) and glutaryl-CoA dehydrogenase deficiency (Bross et al., 2012). Typically, diseases in which aberrations in the same gene cause recessive and dominant forms differ somewhat phenotypically, with the dominant forms tending to be milder in disease severity. Examples are CLCN1 gene mutations giving rise to recessive (Becker type) and dominant (Thomsen disease) forms of myotonia congenita. Although both forms may exhibit severe myotonia, only the Becker type is associated with the transient and fixed weakness and atrophy later in life.

Figure 2 Multiplex western blots of muscle in five patients from Families 1–3, who all carried the c.643_663del21 mutation. Calpain 3 expression was examined, using the 2C4 antibody. The blot shows that calpain 3 is reduced to 5–15% of normal for both the 94 kDa and 30 kDa fragments. Similar findings were observed for the 60 kDa fragment when examined with the 12A2 (Novocastra) antibody (data not shown). Patients IDs are shown above the blots and correspond to their designation in pedigrees (Fig. 1 and Table 1). C = control muscle and 1/2C = control muscle where the protein loaded on the gel was halved. Protein loading differences are visualized by the myosin heavy chain (MHC) Coomassie blue stained gel at the bottom of the figure. α-SG = α-sarcoglycan; β-DG = β-dystroglycan.
Figure 3  CT and MRI of lumbar muscles in four patients with dominantly inherited calpainopathy. Lumbar paraspinal muscles were visualized by CT in one patient (Patient 8/III-3, age 49), and by MRI in three (Patient 3/II-1, 60 years; Patient 1/II-1, 74 years; Patient 1/II-5, 73 years). All patients carried the c.643_663del21 mutation in CAPN3. Patients IDs are indicated on each image and correspond to their designation in pedigrees (Fig. 1) and Table 1. All four showed severe fatty replacement of the erector spinae muscle (arrows).

Figure 4  CT and MRI of leg muscles in three patients with dominantly inherited calpainopathy. Thigh muscles (left) visualized by CT and MRI in Patients 8/III-1 (age 49 years) and 1/III-6 (age 36 years), and MRI of calf muscles (right) in Patients 1/III-6 (age 36 years) and 6/III-1 (age 42 years). All patients carried the c.643_663del21 mutation in CAPN3. Patients IDs are indicated on each image and correspond to their designation in pedigrees (Fig. 1) and Table 1. Thigh muscle imaging showed selective affection of the right biceps femoris in one patient (arrow, bottom left) and more widespread affection of hamstring muscle in the other (arrow, top left). Calf muscle imaging showed asymmetric involvement of medial gastrocnemius muscles in both patients (arrows, right).
Another example is Ullrich congenital muscular dystrophy, which often is recessively inherited versus Bethlem myopathy, which most often is dominantly transmitted (Foley et al., 2009). Both conditions are explained by COL6A mutations. In line with this difference in phenotypic expression, the dominant calpainopathy we report here also appears clinically milder than the recessive forms. A number of mutation carriers reported no overt symptoms, yet had marked CK elevations, large muscle groups replaced by fat on MRI and myopathic findings on muscle biopsy. Also, disease onset is ~16 years later in dominantly inherited calpainopathy than in patients affected by LGMD type 2A (Groen et al., 2007; Hauerslev et al., 2012). The findings suggest that the clinical affection is not always penetrant in dominant calpainopathy, but laboratory evidence of muscle affection probably is.

A prominent feature of our patients was back pain and myalgia, which was present in more than half of the mutation carriers. It is our collective clinical experience that myalgia and back pain in patients with LGMD type 2A are also common, but no systematic evaluations have been carried out to document this. The back pain could be hypothesized to relate to the severe affection of the paraspinal muscles in our cohort, but such a link was not present in patients with facioscapulohumeral muscular dystrophy, who like our patients have weakness and prominent fat replacement of the paraspinal muscles and a prevalence of back pain, which is 2-fold that in healthy persons (Dahlqvist et al., 2014). The use of MRI in the present study was helpful in demonstrating clearly abnormal muscle structure in otherwise seemingly asymptomatic patients. Particularly the paraspinal muscles were affected, and considering the dominant trait, suggests that axial myopathies due to aberrations in the ryanodine 1 receptor gene, should be considered as a differential diagnosis (Laseth et al., 2013).

In conclusion, this study provides strong evidence that the single, in-frame c.643_663del21 deletion of CAPN3 leads to muscle disease, indicating that a dominantly inherited form of calpainopathy exists. This should be taken into consideration in the diagnostic work-up and genetic counselling of patients with calpainopathy and single-allele aberrations of CAPN3. Our findings suggest that the many patients with LGMD or other myopathies, who have been reported with single mutations in CAPN3 and calpain 3 deficiency, should be revisited to discern potential dominant negative effects of single CAPN3 aberrations on calpain 3 expression.
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Supplementary material

Supplementary material is available at Brain online.

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