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BRIEF REPORT

A single c.1715G>C calpain 3 gene variant causes dominant calpainopathy with loss of calpain 3 expression and activity

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Abstract

Recessively inherited limb girdle muscular dystrophy (LGMD) type 2A is the most common LGMD worldwide. Here, we report the first single missense variant in *CAPN3* causing dominantly inherited calpainopathy. A 43-year-old proband, his father and two sons were heterozygous for a c.1715G>C p.(Arg572Pro) variant in *CAPN3*. Affected family members had at least three of the following: muscle pain, a LGMD2A pattern of muscle weakness and wasting, muscle fat replacement on magnetic resonance imaging, myopathic muscle biopsy, and elevated creatine kinase. Total calpain 3 protein expression was 4 ± 3% of normal. In vitro analysis of c.1715G>C and the previously described c.643_663del variant indicated that the mutant proteins lack autolytic and proteolytic activity and decrease the quantity of wild-type *CAPN3* protein. Our findings suggest that dominantly inherited calpainopathy is not unique to the previously reported c.643_663del mutation of *CAPN3*, and that dominantly inherited calpainopathy should be considered for other single variations in *CAPN3*.

KEYWORDS

calpain 3, calpainopathy, dominant inheritance, limb girdle muscular dystrophy

1 | INTRODUCTION

Limb girdle muscular dystrophy (LGMD) type 2A is the first reported and most common type of LGMD in the world (Richard et al., 1995). LGMD type 2A is transmitted by recessive inheritance, but recently we demonstrated that a single, 21-bp deletion of *CAPN3* (c.643_663del), in 37 individuals from 10 families, segregated with a dominantly inherited form of calpainopathy (Vissing et al., 2016). This was confirmed in three other families carrying the same mutation (Martinez-Thompson et al., 2018), and reported in 17 patients with unclassified LGMD (Nallamilli et al., 2018). In the study by Nallamilli et al. (2018), a second 15 bp deletion (c.598_612del) was also described in 16 unrelated patients, but the significance of this finding for dominant calpainopathy is still unclear. A dominant form of

calpainopathy could, besides lack of in-depth genetic analyses, explain the unusually high frequency (about 10%) of unresolved LGMD cases carrying just one pathogenic variant in *CAPN3* (Liewluck & Goodman, 2012; Nallamilli et al., 2018). This fits our own experience as 3 of 40 unclassified LGMD patients in our clinic have single missense *CAPN3* mutations and low calpain expression, but uninformative family history. In this report, we present evidence for the first missense mutation in *CAPN3* that leads to a dominantly inherited myopathy resembling LGMD type 2A. This observation prompted us to investigate the mechanism of the dominance in vitro. We observed that this variant, as well as the previously reported dominant c.643_663del, allele both induce loss of the autolytic and proteolytic properties of the protein. Cotransfection with a normal calpain demonstrated that both pathogenic alleles induce

degradation of the normal protein, explaining the dominant nature of the variants.

2 | SUBJECTS

Patients from three generations in a family were included in this study as well as tissue samples from previously diagnosed patients with dominant calpainopathy due to the c.643_663del deletion in *CAPN3*. Informed consent for diagnostic workup on muscle and blood samples was obtained for all individuals, including patients serving as controls, and all investigations were carried out in accordance with the Declaration of Helsinki.

3 | MAGNETIC RESONANCE IMAGING

All family members had a T1-weighted magnetic resonance imaging (MRI) performed of paraspinal and lower limb muscles. In the proband and his father, the scan was repeated with a 6-year follow-up to assess progression.

4 | MOLECULAR ANALYSES

DNA was isolated from ethylenediaminetetraacetic acid blood samples by standard methods. The entire coding and exon-flanking sequences of *CAPN3* (NM_000070.2) were polymerase chain reaction (PCR) amplified in the proband, and directly sequenced (*CAPN3_13F*: AACTGTGACATGGGTGACCA and *CAPN3_13R*: CCTCATGCCTCAACCTCTG were used for segregation analysis). Complementary DNA (cDNA) analysis of muscle calpain 3 messenger RNA (mRNA) was performed in the proband, using methods described elsewhere (Duno, Sveen, Schwartz, & Vissing, 2008). After initially analyzing a targeted panel of 256 genes involved in muscle disease, we performed whole exome sequencing of DNA from the proband to rule out potential other muscle diseases in the family. The analysis was performed by standard techniques, using the Human Core Exome Kit (Twist Bioscience, San Francisco, CA), and sequenced on a NovaSeq S1 flow cell (Illumina, San Diego, CA), to mean coverage of $<100\times$ ($98\% > 20\times$), using GATK best practice guidelines for standard bioinformatic processing. VarSeq (Golden Helix, Bozeman, MT) was used for variant annotation and filtering. Related family members were assessed for the identified mutation by PCR followed by direct Sanger sequencing and results were reported to LOVD (<https://databases.lovd.nl/shared/individuals/00301694>) and ClinVar databases (<https://www.ncbi.nlm.nih.gov/clinvar/variation/287131/>).

5 | MUSCLE BIOPSY ANALYSES

Family members were tested for serum levels of creatine kinase (CK) and biopsies, obtained from vastus lateralis muscle in three of the four

family members, were used for histological, western blot and enzymatic analyses. Western blot was performed and quantified as previously described (Krag et al., 2016), using antibodies against calpain 3 (Clone 2C4; Leica Biosystems, Nussloch, Germany) and GAPDH (Abcam, Cambridge, UK) for loading control and normalization.

6 | COTRANSFECTION EXPERIMENTS

The *CAPN3* cDNA was generated from RNA extracted from patient muscle biopsies harboring the variants p.(Arg572Pro) and c.643_663del, respectively. These two cDNA strains, as well as the human wild-type calpain 3, were then cloned into an expressing plasmid under the control of the CMV promoter. HEK-293 cells were cotransfected at different ratios with these plasmids alone or with a reporter plasmid carrying an inactive calpain 3 (C129A). In this construct, the inactive human calpain 3 coding sequence fuses with the fluorescent proteins YFP at the N-terminus and green fluorescent protein (GFP) at the C-terminus. Twenty-four hours after transfection, cell extracts were prepared in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) with Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). A BCA protein quantification (Thermo Fisher Scientific) was performed to normalize the quantity of protein loaded on the gel. After transfer on a nitrocellulose membrane, a western blot was performed using antibodies against GFP (rabbit antibody Ab6556; dilution 1:500; Abcam) and Calpain 3 (Mouse antibody NCL-CALP-12A2; dilution 1:200; Leica Biosystems, and Goat antibody COP-080049; dilution 1:1,000; Operon Biotechnologies). Detection was performed using the Odyssey system (Li-Cor, Lincoln, NE).

7 | CALPAIN ACTIVITY ASSAY

A protein extract was prepared from muscle biopsies of the patients as well as additional controls that were obtained through Eurobiobank (<http://www.eurobiobank.org/>) and for which an adequate informed consent was signed. Extracts from untransfected HEK293-cells and extracts from HEK293 cells transfected with empty plasmids were added as controls. The extracts were mixed with an activity-deficient calpain C129A fused to GFP in presence of 4 mM calcium for 2 hr at 37°C. We selected a calpain 3 fragment since this, from our experience, is the only fragment that is specific to calpain 3 and not cleaved by other calpains. The reaction was stopped by placing the samples at 4°C. After denaturation at 95°C for 10 min, 30 μ l were loaded on a 4–12% Bis-Tris gel and separated by electrophoresis. After dry-transfer on nitrocellulose membrane, a western blot was performed using antibodies against GFP (Rabbit antibody Ab6556; dilution 1:500; Abcam) and calpain 3 (Mouse antibody NCL-CALP-12A2; dilution 1:200; Novocastra and Goat antibody COP-080049; dilution 1:1,000; Operon Biotechnologies). Detection was performed using the Odyssey system (Li-Cor, Lincoln, NE).

8 | CLINICAL FINDINGS AND IDENTIFICATION OF A SINGLE CALPAIN 3 VARIANT SEGREGATING OVER THREE GENERATIONS IN A FAMILY WITH LGMD

A now 44-year-old man (II-1) was referred to our neuromuscular clinic at age 28 after examinations for unrelated symptoms and findings of a plasma CK level of 2,200 U/L. Clinical examination at that time was unremarkable, but in the following 16 years, his CK levels remained elevated and he developed back pain and progressive limb weakness (Table 1). His 77-year-old father (I-1) had worked as a butcher, a physically demanding job, until he retired at the age of 60. Shortly thereafter, he developed walking difficulties and started using a cane from age 62 and a walker from age 74. The oldest son of the proband (III-1), currently 20 years old, was examined at age 16 because of back pain and unspecific myalgias related to exercise. Clinical examination for muscle force and atrophy was unremarkable, but CK was intermittently elevated. His 14-year-old brother (III-2) was examined at age 12, because the parents had noted scapular winging and complaints about shoulder weakness, which was confirmed on clinical exam.

Sequencing of leukocyte DNA revealed that the proband, his father and both of his sons had a single c.1715G>C p.(Arg572Pro) variant in exon 13 of *CAPN3* (Figure 1a). cDNA analysis of DNA from the proband supported that only one allele was affected and showed that the variant did not compromise RNA maturation (Duno et al., 2008). Targeted sequencing of 256 muscle disease genes in the proband did not reveal any other candidate genes that could explain a dominantly inherited muscle disease. This was followed by whole-exome sequencing, which did not reveal any other obvious candidate gene than *CAPN3*. Western blot analysis showed that calpain 3 protein expression in muscle was reduced to 2% of normal ($N = 8$ controls) in the proband, 7% in the father and 4% in the oldest son (Figure 1b). The c.1715G>C variant and the previously reported dominantly acting variant (c.643_663del) were positioned in the crystal structure of the calcium-free (1KFU) and calcium-bound (3BOW) calpain-2 structures (Figure 1c). Calpain-2 structures were used in the absence of a three-dimensional structure for calpain 3, which is not available due to the rapid autolysis of this protease in vitro. However, it has been postulated that calpain 3 adopts the same overall fold as the large subunit of the ubiquitous calpains due to the conservation of the domain organization and amino acid composition with the exception of the two insertion regions, IS1 and IS2 (Garnham et al., 2009). It appears that the two variants, although quite far in the linear sequence of calpain 3, are both located near the bottom of the proteolytic domain in the 3D-structure (Figure 1c).

9 | THE FAMILY MEMBERS HETEROZYGOUS FOR THE c.1715G>C p.(Arg572Pro) VARIANT PRESENT A MILD MUSCULAR DYSTROPHY

Like recessively inherited cases of calpainopathy, the most clinically affected muscles in the two oldest family members (proband

and his father) were the paraspinal, gluteal, hamstring and medial gastrocnemius muscles (Table 1 and Figure 1h), while the youngest son of the proband had bilateral scapular winging and force 4 on the Medical Research Council scale for abduction and inward rotation of shoulders, but no weakness of lower limb muscles. The oldest son had no muscle weakness but had myalgia on exercise. Myalgia and back pain were present in all mutation carriers (Table 1). Serum CK was elevated in the proband and the two sons (Table 1).

MRI of lower extremity muscles and lumbar paraspinal muscles in the proband and father showed remarkable fatty replacement of muscle in paraspinal, gluteal, hamstring and medial gastrocnemius muscles with clear progression seen in MRIs performed 6 years apart (Figure 1h). Muscle imaging in the two sons did not reveal abnormal findings (Figure 1i).

Muscle histology in vastus lateralis from the proband showed myopathic changes with increased number of internalized nuclei, fiber size variation, focal necrosis, ring fibers, and minor increase in intrafascicular fibrosis. In the father and oldest son, histopathology was myopathic but milder than in the proband.

Collectively, evidence of muscle disease in the affected family was indicated by four or more of the following symptoms and signs in all individuals: muscle pain, muscle weakness and wasting, significant fat replacement of muscles on MRI, myopathic changes on muscle biopsy and elevated creatine kinase activity.

Analyses of proteolytic activities of the mutant: Following the identification of this dominantly acting variant, we investigated the properties of the protein arising from the c.1715G>C missense variant, and the c.643_663del deletion previously reported by us to cause dominant calpainopathy. To define the consequences of the mutation on calpain 3 activity, protein extract from patient biopsies were mixed with inactive calpain 3 as substrate. For comparison, biopsies from two patients with recessively inherited LGMD2A were used. Calpain 3 level and activity were determined by western blot (Figure 1d). The absence of cleavage with all variants indicates that the mutant alleles have lost their ability to produce protein with proteolytic activity.

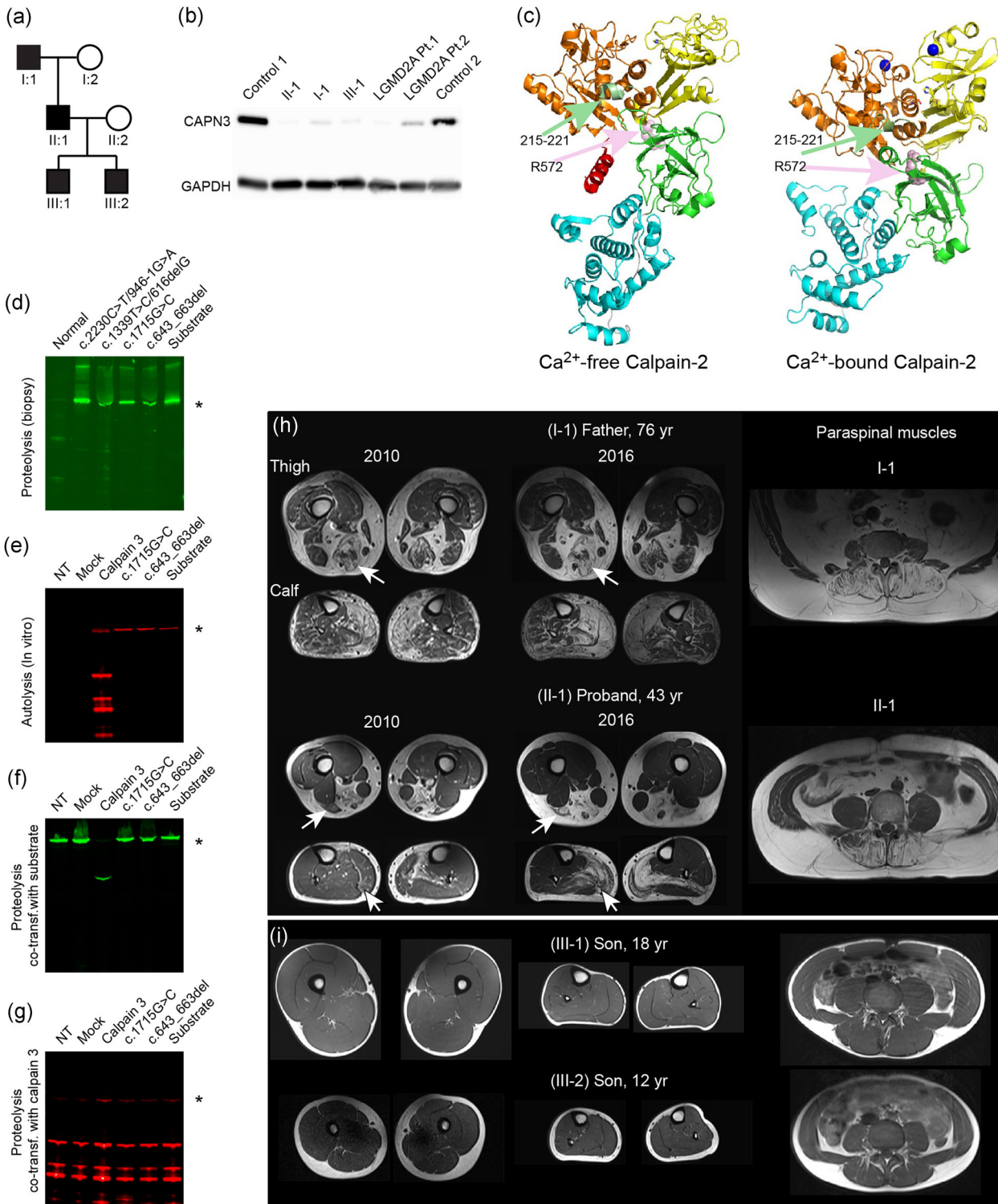
In a second step, cell transfections with plasmids containing cDNA corresponding to the c.1715G>C and c.634_663del variants were performed. The results analyzed on western blot demonstrated that both mutated calpains had lost their autolytic properties compared to the wild-type (Figure 1e). The c.634_663del deletion appears weakly expressed.

This was followed by a cotransfection with the previously mentioned inactive calpain acting as a substrate. The western blot results demonstrated that both variants were inactive towards the substrate, thus had no proteolytic activity (Figure 1f). Finally, we analyzed the effect of coexpression of the different variants together with wild-type calpain 3. This setting reproduces the condition in the patients where the defective and wild-type proteins coexist. The results showed that the presence of wild-type calpain 3 may lead to a decrease in mutant proteins (Figure 1g). The control lysates had no impact on the assay.

TABLE 1 Demographic, clinical, and laboratory findings in the four male carriers of the c.1715G>C variant in CAPN3

Family member	Age at exam (years)	Age at onset (years)	Weakness	Atrophy	Walking aids	Other symptoms	Abnormal fat replacement of muscles on MRI (also see Figure)	Creatine kinase (normal < 250 μ /L)	Muscle biopsy	Calpain 3, % normal
I-1	74	60	Prox limbs	Prox arms Hamstrings, med gast	Crutches since 2009 and walker since 2014	Back pain	Paraspinal, glut max, adductors, med gast, soleus	259	Increased internalized nuclei	7%
II-1	42	28	Prox limbs	Prox arms, Hamstrings, med gast	Walker	Back pain, myalgia	Paraspinal, glut max, adductors, med gast, soleus	719–4,254	Cell necrosis, internalized nuclei, fiber size variability	2%
III-1	20	15	No	No	None	Back pain, Exercise myalgia	Normal	214–2,400	Increased internalized nuclei	4%
III-2	13	8	Shoulder abduction/inward rotation	Shoulder Girdle	None	None	Normal	154–451	ND	ND

Note: Calpain 3 expression as percentage of normal was assessed by western blot. Person designation in the first column refers to the number shown in the pedigree (Figure 1). Abbreviations: glut max, the gluteal maximus muscle; med gast, the medial gastrocnemius muscle; MRI, magnetic resonance imaging; ND, not determined; Prox, proximal.



10 | DISCUSSION

The present study demonstrates that the single, heterozygous c.1715G>C variant in *CAPN3* is responsible for a dominantly inherited form of calpainopathy. This is the first missense variant in *CAPN3* reported to result in a dominant form of LGMD. Molecular analysis indicated loss of calpain 3 expression on western blot analysis (2–7% of normal), by a mechanism which is still uncertain.

Based on the dimeric structure of calpain 3, the possibility of a dominant inheritance of calpainopathy was suggested in 2008 (Beckmann & Spencer, 2008), and demonstrated in 2016 based on one specific variant in *CAPN3* (c.643_663del; Martinez-Thompson et al., 2018; Vissing et al., 2016). Identification of the pathogenic c.1715G>C variant in *CAPN3*, resulting in LGMD, adds to the body of evidence suggesting a dominantly inherited form of calpainopathy. It also suggests that dominant calpainopathy could be found among diagnostically unresolved LGMD cases with calpain 3 deficiency, in whom identification of only one *CAPN3* variant allele is high (Liewluck & Goodman, 2012; Nallamilli et al., 2018).

The c.1715G>C variant is absent from the gnomAD data set, which describes more than 282,000 alleles in the region, but it is recorded in ClinVar and the Leiden databases (LOVD) as reported to be of uncertain significance and pathogenic, respectively. The record in LOVD is based on Piluso et al., who identified a patient solely heterozygous for the variant, in agreement with our findings (Piluso et al., 2005). The symptoms in our family could theoretically be due to a different genetic cause other than the *CAPN3* mutation. However, whole exome sequencing only disclosed c.1715G>C in *CAPN3* as a possible explanation, and in our opinion, the clinical presentation and molecular findings convincingly point to calpainopathy as the cause.

As described for patients carrying the pathogenic c.643_663del variant in *CAPN3*, our patients heterozygous for c.1715G>C were also affected in a milder but similar pattern as that observed in patients affected by LGMD type 2A. Thus, our patients had (a) absence of cardiac involvement (Sveen, Thune, Køber, & Vissing, 2008), (b) early scapular winging in the young son, (c) asymmetric weakness and atrophy, and (d) preferential involvement of posterior thigh muscles (Barp et al., 2020). A prominent feature of our patients was back pain and myalgia, which was also reported at a high frequency in patients with the c.643_663del mutation of *CAPN3* (Vissing et al., 2016). Similar symptoms of myalgia have previously been reported in the recessive form of calpainopathy and other LGMDs and dystrophinopathies (Papadopoulos et al., 2017; Péniisson-Besnier, Richard, Dubas, Beckmann, & Fardeau, 1998).

Calpain 3 has many functions, which among others include sarcomere remodeling and involvement in muscle regeneration (Hauerslev et al., 2012), but what could be the mechanism by which *CAPN3* aberrations result in a dominantly inherited trait? Our results clearly indicate that the variants produce nonfunctional enzymes based on both calpain 3 expression and activity studies. cDNA analysis in the proband showed that both alleles were equally expressed (Duno et al., 2008), indicating that loss of calpain 3 protein happens at the protein level. As the calpain 3 enzyme is a homodimer, it is possible that the mutated protein causes instability of the dimer, which could lead to degradation of both the mutant and the wild-type. While our experiments suggest that this may happen it does not prove that this is the mechanism. It could be argued that an alternative mechanism for the disease in our patients is digenic inheritance of an aggravating allele. However, we consider this possibility highly unlikely because (a) cosegregation of two variants in so many persons and generations is improbable, (b) we found no

FIGURE 1 Clinical affection and biochemical consequences of the c.1715G>C p.(Arg572Pro) variant in *CAPN3* in three generations of a family. (a) Pedigree of the family: Filled symbols designate mutation carriers. Open symbols designate asymptomatic, nonmutation carriers. (b) Western blots of calpain 3 in muscle from the proband, his father, and his oldest son (lanes 2–4), demonstrating a reduction of calpain 3 to 2–7% of normal for the 94 kDa full size protein, comparable to two autosomal recessive patients with calpainopathy (LGMD2A), who were homozygous for a missense c.1699G>T *CAPN3* variant (patient 1) and compound heterozygous for a missense c.1069C>T and nonsense c.2362_2363delinsTCATCT variants in *CAPN3* (patient 2). Protein loading is visualized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (c) Shows the position of the dominantly acting variants c1715G>C p.(Arg572Pro) and c.643_663del, p.(Ser215_Gly221del) in the calcium-free (1KFU) and calcium-bound (3BOW) calpain-2 structure. The site of the deleted region (Ser215_Gly221) is shown in the light green helix in the orange domain of the protease core (PC1). The site of the single amino acid variant (p.Arg572) is shown in pink. (d) Analysis of the calpain 3 proteolytic activity of the patients' biopsies using an artificial substrate, composed on an inactive calpain 3 fused to GFP. Only the biopsy from a healthy control person in lane 1 demonstrates an activity against the substrate, while biopsies from patients with the c.1715G>C and c.643_663del dominantly acting variants as well as from patients with LGMD2A do not. *expected position of calpain 3 band. (e) Detection of autolysis activity in normal and mutated calpain 3 demonstrates that only normal calpain 3 has autolysis activity. (f) Cotransfection experiment of plasmids expressing the different calpain 3 and the substrate. Only the normal calpain 3 can cleave the substrate, indicating the loss of the proteolytic activity of the mutant and confirming the result of the biopsies. (g) Cotransfection experiment of plasmids expressing the different mutated calpain 3 molecules and normal calpain 3. The presence of wild-type calpain 3 leads to disappearance of the mutants. NT = no template control lysate from untransfected HEK293 cells to demonstrate that the cell-line itself does not impact the assay; Mock = control lysate from HEK293-cells transfected with empty plasmid to demonstrate any impact of the empty plasmid on the assay; Calpain-3 = Calpain-3 protein from transfected HEK293 cells; Substrate = Calpain-3 C129A mutant protein from transfected HEK293 cells. (h) MRI of thigh and calf muscles in the proband and father, performed 6 years apart demonstrate affection of hamstring muscles, medial gastrocnemius and soleus in both, with clear progression in most muscle groups after 6 years. Psoas major is preserved and minimal involvement is found in abdominal muscle while lumbar paraspinal muscles are essentially replaced by fat in the father and proband. (i) Thigh, calf, and paraspinal muscles of the two sons of the proband were essentially normal looking muscles on MRI. MRI, magnetic resonance imaging

modifying variant in another genes on exome sequencing, and (c) the genetic background, at least for the 13 families reported with the c.643_633del variant, did not share the same genetic background. Likewise, it could be argued that a deep intronic variant, not picked up by the exome sequencing, could contribute to the disease phenotype. However, this is also very unlikely as such variants typically induce aberrant RNA splicing, which often triggers nonsense mediated mRNA decay, and thus do not result in any protein, which would be a prerequisite for dominant inheritance.

Our findings suggest that dominantly inherited calpainopathy is not unique to the previously reported c.643_663del variant in *CAPN3*, and other single aberrations in *CAPN3*, especially small in-frame deletions/insertions and missense mutations, should be considered as a potential cause of dominantly inherited calpainopathy.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

J. V.: study concept and design, acquisition of data, drafting, and revision of manuscript. I. R. and T. K.: study design, acquisition of data, and revision of manuscript. J. R. D., C. R., J. P., and M. D.: acquisition of data and revision of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. Sequencing and results were reported to LOVD (<https://databases.lovd.nl/shared/individuals/00301694>) and ClinVar databases (<https://www.ncbi.nlm.nih.gov/clinvar/variation/287131/>).

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