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CAV3 mutations causing exercise intolerance, myalgia and rhabdomyolysis: expanding the phenotypic spectrum of caveolinopathies

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Abstract:

Rhabdomyolysis is often due to a combination of environmental trigger(s) and genetic predisposition; however, the underlying genetic cause remains elusive in many cases. Mutations in *CAV3* lead to various neuromuscular phenotypes with partial overlap, including limb girdle muscular dystrophy type 1C (LGMD1C), rippling muscle disease, distal myopathy and isolated hyperCKemia. Here we present a series of eight patients from seven families presenting with exercise intolerance and rhabdomyolysis caused by mutations in *CAV3* diagnosed by next generation sequencing (NGS) (n=6). Symptoms included myalgia (n=7), exercise intolerance (n=7) and episodes of rhabdomyolysis (n=2). Percussion-induced rapid muscle contractions (PIRCs) were seen in five out of six patients examined. A previously reported heterozygous mutation in *CAV3* (p.T78M) and three novel variants (p.V14I, p.F41S, p.F54V) were identified. Caveolin-3 immunolabeling in muscle was normal in 3/4 patients however, immunoblotting showed more than 50% reduction of caveolin-3 in five patients compared with controls. This case series demonstrates that exercise intolerance, myalgia and rhabdomyolysis may be caused by *CAV3* mutations and broadens the phenotypic spectrum of caveolinopathies. In our series immunoblotting was a more sensitive method to detect reduced caveolin-3 levels than immunohistochemistry in skeletal muscle. Patients presenting with muscle pain, exercise intolerance and rhabdomyolysis should be routinely tested for PIRCs as this may be an important clinical clue for caveolinopathies, even in the absence of other “typical” features. The use of NGS may expand current knowledge concerning inherited diseases, and unexpected/atypical phenotypes may be attributed to well-known human disease genes.

Key Words: *CAV3*; Rhabdomyolysis; Myoglobinuria; Caveolinopathy; Exercise Intolerance; Myalgia

Highlights

- Here we present a series of eight patients with mutations in *CAV3*
- This case series broadens the phenotypic spectrum of caveolinopathies
- Exercise intolerance, myalgia and rhabdomyolysis may be caused by *CAV3* mutations
- Rippling muscle contractions and PIRCs are clinical clues of caveolinopathies
- Immunoblotting may be more sensitive in detecting reduced caveolin-3 levels

Abbreviations:

ATPase: Adenosine triphosphatase

CAV3: Caveolin-3 (M-caveolin) – OMIM # 601253

CK: creatine kinase

COX: cytochrome oxidase

CPT2: Carnitine Palmitoyl Transferase 2

CRP: C-reactive protein

ECC: Excitation-contraction coupling

EM: Electron microscope

ESR: Erythrocyte sedimentation rate

H&E: Hematoxylin and eosin

LGMD1C: limb girdle muscular dystrophy type 1C

MHC: Myosin heavy chain

MRI: Magnetic resonance imaging

NADH-TR: Nicotinamide adenine dinucleotide-tetrazolium reductase

NGS: Next-generation sequencing

nNOS: Neuronal nitric oxidase synthase

PIRCs: percussion-induced rapid muscle contractions

PFK: phosphofructokinase

RM: Acute Rhabdomyolysis

SDH: Succinic dehydrogenase

SR: Sarcoplasmic reticulum

WB: Western blotting

WES: Whole exome sequencing

1 Introduction

Acute rhabdomyolysis (RM) is a serious event often requiring critical care management. Precipitating causes include a range of environmental trigger(s) with and without a known genetic predisposition [1]. In many cases no cause is found. Here we report eight patients who on next-generation sequencing (NGS) were found to carry four heterozygous missense *CAV3* mutations after extensive earlier investigations had been negative. Our findings expand the *CAV3*-related phenotypical spectrum, so far comprising limb girdle muscular dystrophy type 1C (LGMD1C), rippling muscle disease, distal myopathy, isolated hyperCKemia and familial hypertrophic cardiomyopathy [2].

2 Materials and methods:

Eight patients presenting with exercise intolerance, myalgia and/or recurrent RM who remained genetically unresolved despite extensive previous investigations are reported. Six patients (patients 1-5 and 8) were identified from a larger cohort of 225 patients with exercise intolerance, myalgia and/or recurrent RM. DNA from patient 6 (the father of patient 5) was assessed following a genetic diagnosis in his son. Patient 7 was genetically investigated following muscle biopsy analysis. Approval was obtained from the regional ethics committee, and informed consent was obtained from all subjects for genetic studies. Medical notes were reviewed retrospectively and patients were reassessed following the genetic diagnosis, except patients 1,3 and 4 who failed to attend follow up visits, and patient 6, who is deceased. Clinical findings are summarized in Table 1, including: age of onset, age at assessment, presenting symptom, recurrent RM and its triggers, reported rippling muscle contraction, percussion-induced rapid muscle contractions (PIRCs) assessed during physical exam (by a reflex hammer (percussion of a muscle)), muscle pain, muscle weakness assessed

during physical exam, reported exercise intolerance (defined as pain and/or a cramp-like sensation during exercise), fatigue and baseline serum creatine kinase (CK) levels. Histopathological studies were performed as described in Supplementary Material. Polyacrylamide gel electrophoresis and western blotting were performed as previously described [3]. Blots were incubated with 43DAG/8D5 (β-dystroglycan, Leica Biosystems, NCL-b-DG, dil. 1/350) and caveolin-3 (BD Biosciences BD610421, dil. 1/350). Myosin heavy chain staining with Coomassie blue on the post-blotted gel was used as a control for protein loading and quality of the transfer. Bands were visualised with SuperSignal West Pico Chemiluminescent Substrate detection (Life Technology) using AlphaInnotech FluorChemR Q platform and AlphaViewR software v3.0. Densitometric analysis was undertaken using ImageJ v1.47 software with data normalised to the density of the myosin heavy chain band on the Coomassie blue stained post-blotted gel and expressed as a percentage of the control sample.

DNA from six patients was sequenced by a NGS Illumina 'Trusight One' enrichment panel; designed to screen for 60 relevant genes, previously associated or putatively linked with RM (for review [1]). Mutations identified on NGS were confirmed by Sanger sequencing. Patient 7 was evaluated by bi-directional sequence analysis for mutations in *CAV3* and patient 8 by whole exome sequencing (SOLiD^{TM}). Whole exome sequencing was performed as outlined previously [4]. Three μg of DNA was fragmented by sonication and ligated to SOLiD^{TM} system sequencing adaptors. The resulting library was enriched for exomic sequences using the SeqCap EZ Human Exome Library v2.0 exome capture system (Nimblegen, Roche Diagnostics) and sequenced using a 5500XL Genetic Analyser (Life Technologies). After sequencing and alignment, average coverage was 56-fold with 73% of the exome covered to 20-fold or greater. Variant calling was performed using LifeScopeTM 2.5 (Life Technologies) and the resulting variants were filtered using ANNOVAR. The *CAV3*

mutations were confirmation by bi-directional Sanger sequencing. Mutations were described using the single letter nomenclature to describe non-synonymous variants.

3 Results:

The clinical history from each patient is outlined below and key findings are summarized in Table 1. Patient 1 presented with fatigue, muscle pain, and recurrent episodes of myoglobinuria (highest CK: 28,000 IU/L) without apparent trigger. Inflammatory markers (CRP and ESR), HIV testing and auto-antibodies for auto-immune myositis were negative. Plasma acylcarnitine profile, urine organic acids, fatty acid oxidation flux and CPT2 activity in skin fibroblasts were all normal. Patient 2 presented with a longstanding history of muscle pain and tenderness exacerbated by mild physical activities and exercise that interfered with normal daily activities. Examination was unremarkable except for muscle pain evoked by muscle palpation. Routine biochemistry was normal, apart from raised CK. Inflammatory markers (CRP and ESR) and autoantibodies including ANA, GAD, Anti-DNA, Rheumatoid Factor, Anti-Hu, Anti-Yo, Anti-Ri, were negative. Patient 3 had exercise intolerance throughout adult life. RM occurred at age 37 following a few hours of moderate intensity swimming. At the time he was also taking antibiotics for an infection. Severe pain and acute muscle weakness were accompanied by myoglobinuria. A second episode was associated with exercise (swimming) in conjunction with fever. A third episode occurred spontaneously with no apparent precipitant. Patient 4 presented with exercise-related muscle cramps and stiffness since childhood. Examination was unremarkable. He had hypoglycaemic seizures in the neonatal period. Genetic testing for *GLUT1*, *HADH* and *LPIN1* were normal. Patient 5 had muscle symptoms from childhood. He had mild muscle weakness, and could not perform endurance activities. Paroxysmal weakness lasting 2-3 hours occurred after strenuous exercise. Post exercise muscle pain was also a feature. Hypertrophic cardiomyopathy was

diagnosed in his 40s during a routine health check. Extensive genetic investigations were negative (including full mutation screening of *VCP*, *DES*, *MYOT*, *CRYAB*, *ZASP*, *TTN*, targeted sequencing of *POLG1* and *PEO1*, plus testing for large scale rearrangements and full sequencing of muscle-extracted mtDNA). Muscle biopsy slides were not available for review. Patient 6, the father of patient 5, presented with progressive muscle weakness from the fourth decade and died aged 85. Muscle weakness initially involved the anterior thighs with progression to the distal upper limbs later in life. He became wheelchair-dependent in his late 60s. He had no swallowing or respiratory difficulties and no facial or axial weakness, no ptosis and a full range of eye movements. A muscle biopsy performed at 67 years was reported as showing dystrophic features (muscle biopsy slides not available for review). Genetic investigations for *FKRP*, *VCP* and dystrophin gene mutations and FSHD testing were all negative. Patient 7 presented with exercise intolerance and muscle pain relieved by rest. He occasionally experienced muscle cramps post exertion with muscle rippling. He had mild proximal weakness (MRC 4+/5) with a modified Gowers' manoeuvre. He walked with a mild wide-based gait with everted flat feet and had mild tightness of the tendon achilles (-5 degrees bilaterally) and a mild lumbar lordosis. Muscle MRI showed mild fatty infiltration on the rectus femoris, sartorius, biceps femoris, gastrocnaemius and semitendinosus muscles. Serum lactate, acylcarnitine profile and routine biochemistry were normal. Patient 8 was an elite athlete, but her high level training was complicated by severe exercise-induced myalgia. On examination she had muscle hypertrophy especially marked in the lower limbs without muscle weakness.

Four heterozygous *CAV3* mutations were found: a previously described p.T78M substitution (exon 2, c.233C>T) (Patients 3-6) located in the central hydrophobic trans-membrane domain of the protein, and three novel substitutions: p.V14I (exon 1, c.40G>A) (Patients 1 and 2) located in the N-terminal domain of the protein, p.F41S (exon 2,

c.122C>T) (Patient 7) and p.F54V (exon 2, c.160T>G) (Patient 8), both within the oligomerization domain.

Muscle biopsies (n=5) showed non-specific changes as seen in Fig 1 (Supplementary Table 1). To investigate the effect of the mutations, caveolin-3 protein expression was assessed by WB in five patients (Patients 1,2,4,7,8). Levels in four patients (Patients 1,2,4,8) were reduced by more than 50% compared to controls (Fig 2). β-dystroglycan, used as a loading control, also had reduced levels in three of the samples (Patients 1,2,4). Caveolin-3 was markedly reduced on muscle tissue sections from Patient 7 and absent on WB on the same tissue (Supplementary Fig 1).

4 Discussion

We present eight patients with myalgia (n=7), exercise intolerance (n=7) and recurrent RM (n=2) who were found to carry mutations in *CAV3*; a few of them had been screened for selected metabolic myopathies before NGS was performed. The use of NGS is rapidly expanding the phenotypic spectrum of many neuromuscular disease genes, as sequencing of disease genes is done in an unbiased approach, influencing future classification of disease phenotypes and inherited disorders. This case series illustrates that a “metabolic phenotype”, so far more frequently recognized in association with disorders of muscle metabolism (e.g.: glycogen storage disorders and disorders of fatty acid metabolism), may also be part of the phenotypic spectrum of proteins without immediately apparent primary metabolic link such as caveolin-3. As opposed to disorders of muscle metabolism, there was no clear provoking trigger for RM episodes in the reported cases. Patient 1 had no clear trigger for several episodes of RM. It is unclear if exercise, infection or the combination of both contributed to the RM episodes in patient 3, who also developed a third episode with no apparent trigger.

CAV3 encodes caveolin-3, a muscle specific plasma membrane protein involved in several processes related to the formation of caveolae, invaginations of the plasma membrane. Autosomal dominant – and, less frequently, recessive – *CAV3* mutations have been implicated in hyperCKaemia, rippling muscle disease and LGMD1C [2]. Recurrent RM has been previously described in only one patient with a *CAV3* mutation, in whom NGS had not been performed to exclude other genetic causes of RM [5]. Our findings suggest that *CAV3* mutations are a more frequent cause of myalgia, exertion intolerance and RM than previously thought, and ought to be considered in patients presenting with such features. The pathogenicity of the *CAV3* variants identified and emerging genotype-phenotype correlations are supported on several levels: 1) Exclusion of other genetic conditions predisposing to similar symptoms by NGS; 2) the apparent association of *CAV3*-related symptoms with the recurrent p.V14I and p.T78M substitutions; 3) and reduced caveolin-3 protein levels on WB.

Although myalgia, exercise intolerance and/or recurrent RM were the most prominent features leading to referral for further assessment, other features – hyperCKaemia, reported muscle rippling, PIRCs, muscle weakness and hypertrophic cardiomyopathy – were either noted at presentation or evolved over time, emphasizing the previously observed wide and overlapping phenotypical spectrum associated with *CAV3* mutations. Along similar lines, there was also marked intrafamilial variability, with one affected son (Patient 5) having a different clinical picture to his father (Patient 6). PIRCs, a clinical hallmark of caveolinopathies, were seen in five out of six patients examined, but were only assessed after the genetic diagnosis had been established in 2 patients (patient 1 and 2). Four out of six patients reported rippling muscle contraction. We believe PIRCs and rippling muscle contraction are important clinical clues for caveolinopathies and should be assessed for in patients presenting with exercise intolerance, myalgia and RM, even if other features suggestive of a caveolinopathy are absent. Additional features in our series, which may or

may not be related, included a history of neonatal hypoglycaemic seizures, previously reported in one patient with *CAV3* mutations [5], and paroxysmal muscle weakness, a novel association.

Primary protein defects and absence of caveolin-3 staining on immunohistochemistry is well documented in patients with caveolinopathies [2]. The key histopathological finding in patient 7 was marked reduction of immunostaining for caveolin-3; in combination with an absent band on WB, supporting the pathogenicity of the novel p.F41S substitution. Almost complete absence of caveolin-3 on immunoblotting of patient 8 (p.F54V) supported pathogenicity. These variants were absent from or expressed at a low frequency (0.0008%) in ExAC, respectively. The three muscle biopsies from patients harbouring the p.V14I and p.T78M substitutions showed only minor non-specific histochemical findings and normal caveolin-3 immunostaining. However, reduced caveolin-3 levels on WB, supported the pathogenicity of these mutations and suggested that WB may be the more sensitive method to detect caveolin-3 deficiency. Reijneveld *et al.* also identified normal caveolin-3 immunostaining in two patients with hyperCKaemia, one with the p.T78M substitution [6]. Although these and our own observations indicate sufficient caveolin-3 expression to generate a normal immunostaining pattern, it is currently uncertain if the p.V14I and p.T78M substitutions exert their pathogenic effect through relative reduction or abnormal protein function, or both.

Bioinformatics analysis (Table 2) predicts that p.T78M is damaging and it has been described in association with long QT syndrome and sudden death [7, 8], isolated hyperCKaemia [6], and rippling muscle disease with proximal myopathy in a patient carrying a D4Z4 FSHD-sized allele [9]. Bioinformatic programs also predicted the p.F41S and p.F54V substitutions to be pathogenic, while the p.V14I substitution was predicted to be benign by all programs.

The interaction between caveolin-3 and the skeletal muscle ryanodine receptor (RyR1), involved in excitation-contraction coupling (ECC) and also implicated in virally- and exercise induced myalgia and rhabdomyolysis [10, 11], could be of potential relevance to the phenotype observed in our patients. Both caveolin-3 and RyR1 co-localize at the T-tubule and sarcoplasmic reticulum (SR), and a critical role for caveolin-3 has been suggested in the correct localization of RyR1 to the SR and as a modifier of ECC [12, 13].

The significance of β-dystroglycan reduction in three WB samples is uncertain, even though β-dystroglycan, is a known interactor of caveolins [14, 15]. Myosin heavy chain was also used as a protein loading control, ensuring that this observation is not due to uneven loading of skeletal muscle protein.

A limitation of this study was the inability to perform co-segregation studies in other family members to strengthen the pathogenicity of the *CAV3* variants and to evaluate their penetrance. In addition, future more detailed functional characterization may clarify the precise pathogenic effect of the new *CAV3* variants and their role in genetic counselling, and additional genetic modifiers with potential synergistic effects may be identified.

5 Conclusions

In summary, myalgia, exertion intolerance and recurrent RM are features associated with *CAV3* mutations, highlighting the broad and expanding spectrum of caveolinopathies. Rippling muscle contraction and PIRCs may be important clinical clues indicative of caveolinopathies and should be assessed in patients who present with exertion related symptoms. Non-specific changes in muscle biopsy do not exclude *CAV3* mutations, and WB and/or specific genetic testing should be performed if a caveolinopathy is strongly suspected on clinical grounds.

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Legends:

Table 1: Key findings in patients with *CAV3* mutations. Het: heterozygous; RM: rhabdomyolysis; NA: not applicable; ND: no data; Rippling (reported): rippling muscle contraction reported by the patient; PIRCs: percussion-induced rapid muscle contractions;

LL: lower limbs; UL: upper limbs; CK: creatine kinase; ECG; electrocardiogram; NCS-EMG: neurophysiology evaluation; CTS: carpal tunnel syndrome; MRI: magnetic resonance imaging; Family history: Family history for neuromuscular symptoms.

Table 2: Frequency of the *CAV3* mutations in ExAC and the *in silico* predictions for each substitution.

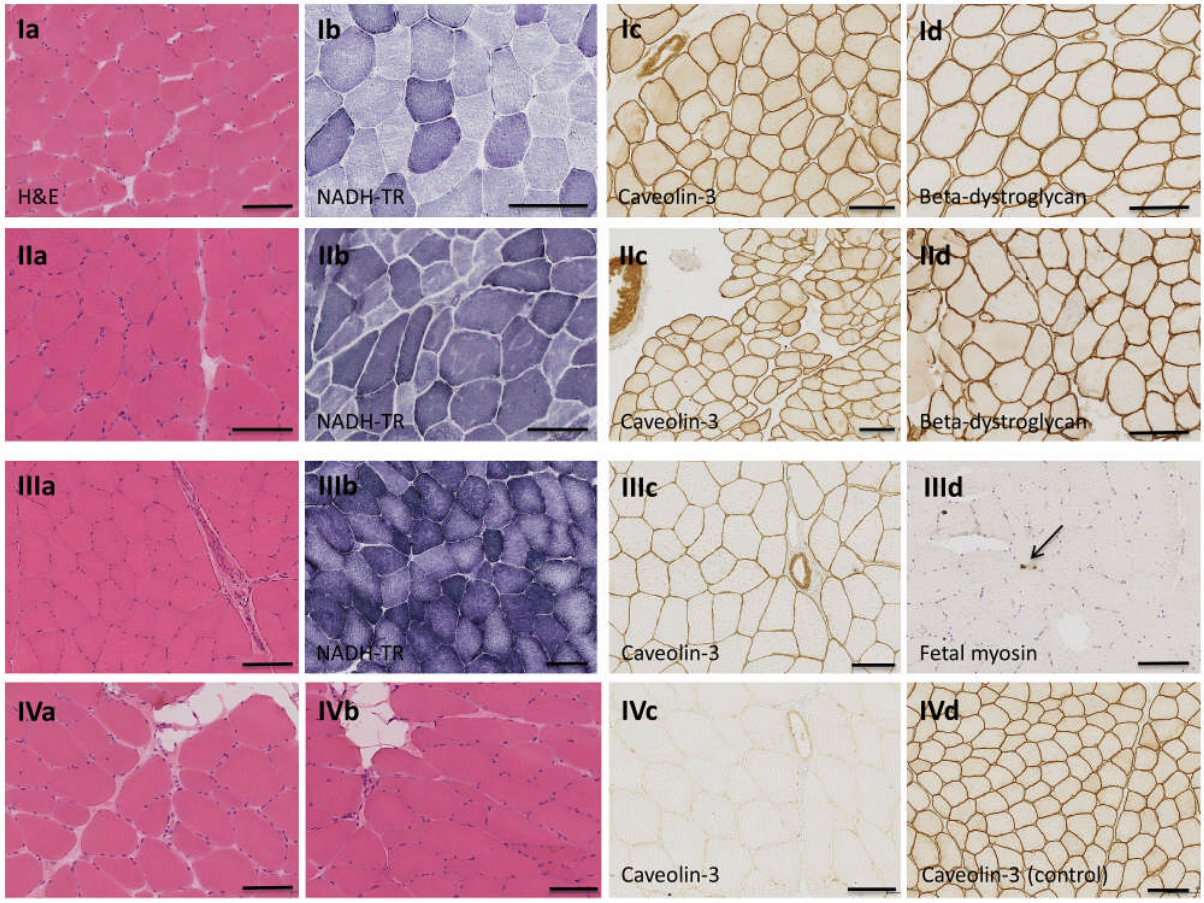

Figure 1: Quadriceps muscle biopsies were available from four patients; patients 1 (I) & 2 (II) were biopsied as adults, while 4 (III) and 7 (IV) were biopsied at 14 years. The adults (Ia, IIa; HE) and one child (IIIa; HE) showed minimal non-specific changes including occasional small fibres. The second child (IVa; HE) showed mild myopathic changes with increased fibre size variation, few small granular basophilic fibres, mild internal nucleation and focal perimysial fatty infiltrate. Fibre typing was preserved in all cases with mild oxidative abnormalities ranging from slight unevenness of stain to presence of a few mini-cores (Ib, IIB, IIIB; NADH-TR). Occasional fibres expressed fetal myosin (IIId). Protein immunoanalysis revealed normal sarcolemmal/basal lamina expression of the DAG complex including caveolin-3 (Ic, IIc, IIIc) and beta-dystroglycan (Id, IId) except in patient 7 (IV),

where a marked reduction was identifiable in sections (IVc). Caveolin-3 labeling in a non-disease control (IVd). The bar in the bottom right represents 100 microns in length.

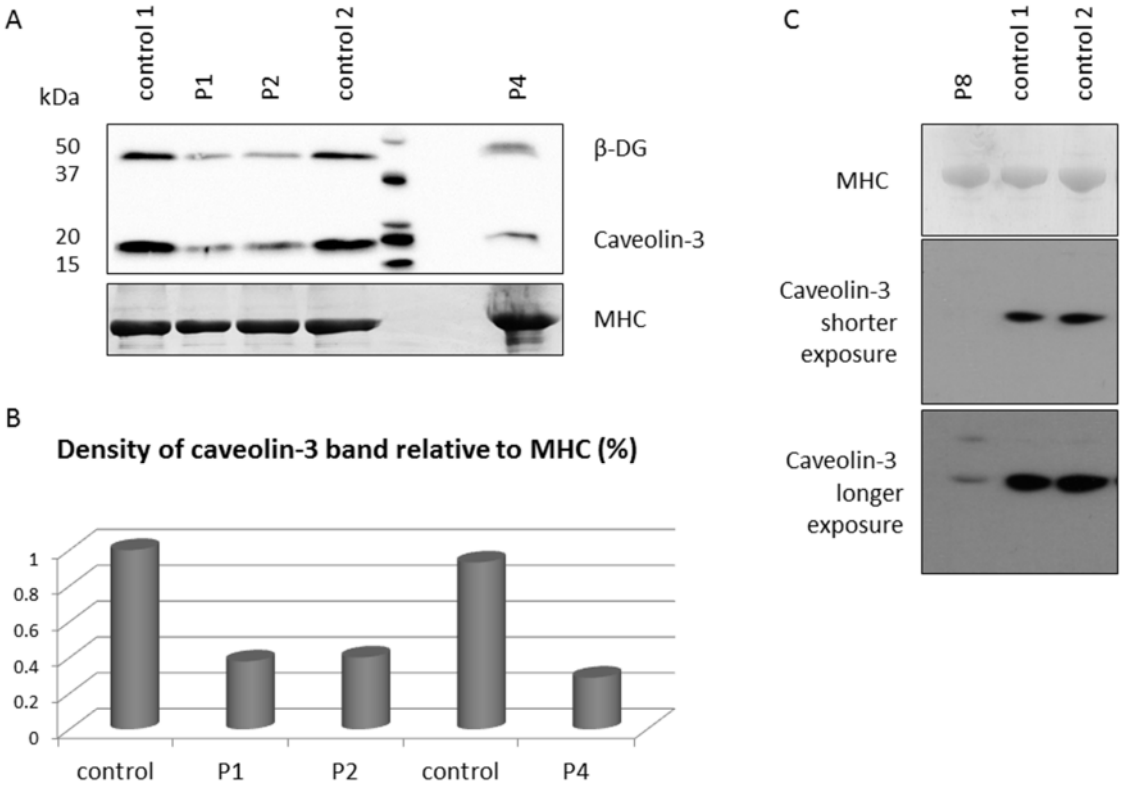

Figure 2: Western blots showed reduced levels of caveolin-3 and β-dystroglycan (β-DG) in patients with CAV3 mutations (A,B,C). P1: patient 1; P2: patient 2; P4: patient 4; P8: patient 8. Levels of caveolin-3 in patient 8 were calculated to be 0.12 of controls (using the longer exposure blot). Myosin heavy chain (MHC) was used as a protein loading control.

Table 1:

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	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
DNA analysis *CAV3*	V14I Het	V14I Het	T78M Het	T78M Het	T78M Het	T78M Het	F41S Het	F54V Het
Ethnicity	African	Afro-Caribbean	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Gender	M	F	M	M	M	M	M	F
Age of onset / age at assessment	20s / 33	30s / 57	30s / 43	9 / 16	Childhood / 58	30s / 85	7 / 12	Late teens / 26
Presenting symptom	Severe fatigue, myalgia	Calf pain	Myalgia following physical activity	Myalgia, muscle cramps	Myalgia, Muscle weakness, difficulty to keeping up with peers	Muscle weakness	Myalgia on exertion, fatigue, difficulty to keeping up with peers	Severe exercise-induced myalgia
Recurrent RM	Yes	No	Yes	No	No	ND	No	No
Trigger RM	ND	NA	Exertion, Antibiotics/infection, Unknown	NA	NA	ND	NA	NA
Rippling (reported) / PIRCs	Yes / Yes	Yes / Yes	ND / ND	No / Yes	Yes / Yes	ND / ND	Yes / No	No / Yes
Myalgia	Yes	Yes	Yes	Yes	Yes	ND	Yes	Yes
Weakness	Upper limbs	Proximal lower limbs	No	No	Handgrip – mild	Proximal (LL) Distal (UL) Handgrip	Proximal – mild	No
Muscle atrophy	ND	Yes (Thigh)	No	No	No	Yes	No	No
Exercise intolerance	Yes	Yes	Yes	Yes	Yes	ND	Yes	Yes
Fatigue	Yes	Yes	No	No	Yes	ND	Yes	Yes
Basal CK	500	300 – 600	126	600 - 4000	142	280	300-685	217
ECG	SR with biphasic T waves V4 to V6	Normal	ND	Normal	Abnormal	ND	Normal	Normal
Echo cardiogram	Normal	ND	ND	Normal	Abnormal	ND	Normal	Normal
NCS-EMG	Normal	CTS	ND	Normal	Normal	Myopathic changes	Normal	Normal
Lower limb muscle MRI	Normal	Normal	ND	Normal	ND	ND	Abnormal	ND
Family history	Negative	Negative	Negative	Negative	Affected father (P6), mother (enlarged heart), sister (fatigue), son (exercise related myalgia)	Affected son (P5)	Father (difficulty with mobility)	Negative

Table 2:

Substitution	Novel	Freq ExAC (%)	MutationTaster	Polyphen-2	SIFT	Provean	MutationAssessor (Fx impact)	cDNA change	Genomic coordinates
p.V14I	yes	0.0519	Polymorphism	Benign	Tolerated	Neutral	Neutral	c.40G>A	3:8775602 G / A (rs121909281)
p.F41S	yes	not present	Disease-causing	Probably damaging	Damaging	Deleterious	Medium	c.122T>C	3:8787219 T / C
p.F54V	yes	0.0008	Disease-causing	Benign	Tolerated	Deleterious	Medium	c.160T>G	3:8787257 T / G
p.T78M	no	0.3038	Disease-causing	Probably damaging	Tolerated	Neutral	Low	c.233C>T	3:8787330 C / T (rs72546668)

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