AUTHOR	Manu Jokela, Giorgio Tasca, Anna Vihola, Eugenio Mercuri, Per-Harald Jonson, Sara Lehtinen, Salla Välipakka, Marika Pane, Maria Donati, Mridul Johari, Marco Savarese, Sanna Huovinen, Pirjo Isohanni, Johanna Palmio, Päivi Hartikainen, Bjarne Udd
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An unusual ryanodine receptor 1 (RYR1)- phenotype: mild, calf-predominant myopathy

Running head: RYR1 calf myopathy

Jokela M, MD, PhD, Tasca G, MD, PhD, Vihola A, PhD Mercuri E, MD, prof, Jonson PH, PhD, Lehtinen S, MSc, Välipakka S, MSc, Pane M, MD, PhD, Donati M, MD, PhD, Johari M, Savarese M, PhD, Huovinen S, MD, Isohanni P, MD, PhD, Palmio J, MD, PhD, Hartikainen P, MD, PhD, Udd B, MD, prof

Corresponding author: Jokela Manu, MD, PhD. Neuromuscular Research Center, Department of Neurology, University Hospital and University of Tampere, Finland and Division of Clinical Neurosciences, Turku University Hospital, and University of Turku, Turku, Finland and Kiinamyllynkatu 4-8,20520 Turku. Telephone number: 041 547 7211. E-mail: mejoke@utu.fi

Tasca Giorgio, MD, PhD. Unità Operativa Complessa di Neurologia, Dipartimento di Scienze dell'Invecchiamento, Neurologiche, Ortopediche e della Testa-Collo, Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma, Italia

Vihola Anna, PhD. Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

Mercuri Eugenio, MD, prof. Institute of Pediatric Neurology, Catholic University School of Medicine, Rome, Italy

Jonson Per-Harald, PhD. Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

Huovinen Sanna, MD. Department of Pathology, Fimlab Laboratories, Tampere University Hospital, Tampere, Finland

Lehtinen Sara, MSc. Neuromuscular Research Center, Tampere University and University Hospital, Tampere, Finland

Välipakka Salla, MSc. Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

Pane Marika, MD, PhD. Institute of Pediatric Neurology, Catholic University School of Medicine, Rome, Italy

Donati Maria, MD, PhD. Metabolic and Neuromuscular Unit, Meyer Hospital, Florence, Italy

Johari Mridul, MSc. Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

Savarese, Marco, PhD Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

Isohanni Pirjo, MD, PhD. Department of Pediatric Neurology, Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Hartikainen Päivi, MD, PhD. Department of Neurology, Kuopio University Hospital and University of Eastern Finland, Kuopio, Finland

Palmio Johanna, MD, PhD. Neuromuscular Research Center, Department of Neurology, University Hospital and University of Tampere, Finland.

Udd Bjarne, MD, Prof. Neuromuscular Research Center, Department of Neurology, University Hospital and University of Tampere, Finland. Department of Neurology, Vasa Central Hospital, Vasa, Finland

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Marco Savarese reports no disclosures relevant to the manuscript
Sanna Huovinen reports no disclosures relevant to the manuscript
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Glossary:

RYR1= Ryanodine receptor 1
EMG/NCS= electromyography/ nerve conduction studies
MRI= magnetic resonance imaging
MH= malignant hyperthermia

CCD= congenital central core disease

DHPR= dihydropyridine receptor

WES= whole exome sequencing

CK= creatine kinase

An unusual ryanodine receptor 1 (RYR1)- phenotype: mild ,calf-predominant myopathy

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Abstract

OBJECTIVE: To identify the genetic defect causing a distal calf myopathy with cores.

METHODS:
Families with a genetically undetermined calf-predominant myopathy underwent detailed clinical evaluation, including EMG/NCS studies, muscle biopsy, laboratory investigations and muscle MRI. Next-generation sequencing and/or targeted Sanger sequencing were utilized to identify the causative genetic defect in each family.

RESULTS:
A novel deletion-insertion mutation in *RYR1* was found in the proband of the index family and segregated with the disease in six affected relatives. Subsequently, we found two more families with a similar calf-predominant myopathy segregating with unique *RYR1*-mutated alleles. All patients showed a very slowly progressive myopathy without episodes of malignant hyperthermia or rhabdomyolysis. Muscle biopsy showed cores or core-like changes in all families.

CONCLUSIONS:
Our findings expand the spectrum of *RYR1*-related disorders to include a calf-predominant myopathy with core pathology and autosomal dominant inheritance. Two families had unique and previously unreported *RYR1* mutations, while affected persons in the third family carried two previously known mutations in the same dominant allele.

Introduction

Autosomal dominant mutations in the *RYR1* gene encoding for skeletal muscle ryanodine receptor (RyR1 protein) were recognized as the cause of malignant hyperthermia (MH) susceptibility and congenital central core disease (CCD) over 20 years ago [1-3]. Other *RYR1*-related phenotypes have since emerged, including centronuclear myopathy [4], multiminicore myopathy [5,6], congenital fibre type disproportion [8], axial myopathy [9], King-Denborough syndrome [10], atypical periodic paralysis [7, 11] and exertional rhabdomyolysis/myalgia [12]. Both autosomal dominant and recessive inheritance patterns have been described. Heterozygous recessive mutations have also been suggested to cause disease due to epigenetic silencing of the wild type allele, ie. without an additional mutation in the other allele [19].

RYR1 is a calcium release channel of the sarcoplasmic reticulum, which together with sarcolemmal voltage-gated calcium channels (DHPR), is required for the triggering of muscle contraction following sarcolemmal depolarization and subsequent calcium release (excitation-contraction coupling). Molecular disease pathomechanisms of *RYR1*-related disorders have not been completely elucidated, but may involve leaky or hypersensitive RYR1 channels, depletion of sarcoplasmic reticulum calcium stores and excitation/contraction uncoupling [20].

We describe an unusual phenotype of *RYR1*-myopathy: a mild, calf-predominant myopathy segregating as an autosomal dominant disease in two unrelated Finnish and one Italian family.

Methods

Patients

Two Finnish (F1 and F2) and one Italian family (F3) with previously unclarified distal calf myopathies were re-examined by clinical and molecular genetic investigations, including laboratory, muscle imaging, histopathological and molecular pathology studies.

Genetic investigations

Targeted next-generation sequencing

Probands (F1:II-5, F2:II-1, and F3:II-1) were genetically analyzed using our targeted next-generation sequencing (NGS) assay, MyoCap (21), from NimbleGen (Roche Nimblegen, Madison, WI, USA). MyoCap is targeted towards the coding exons and UTRs of confirmed or putative myopathy causing genes. The proband of F1 was analyzed with a version of MyoCap (v2) that targeted 236 genes. Probands of F2 and F3 were analyzed with a version of MyoCap (v3) that had been expanded to target 265 genes. The list of genes for both versions of MyoCap are available upon request. For enrichment of the targeted region NimbleGen SeqCap EZ Choice Library protocol (Roche Nimblegen) was used. The enriched libraries were paired-end sequenced to 75 base pair (bp) read length. Library preparations, enrichment and next-generation sequencing were performed either at Biomedicum Functional Genomics Unit (FuGU, Helsinki, FIN) using Illumina NextSeq500 Sequencer or at Oxford Genomics Centre (OGC, Wellcome Trust Centre for Human Genetics, Oxford, UK) using Illumina HiSeq4000 Sequencer. The proband of F1 was processed at FuGu and probands of F2 and F3 at OGC. Sequence read alignment, variant calling, quality and frequency filtering were done using an in-house developed pipeline [21]. We required a minimum of 94 % of the target region to have a coverage of at least 20X.

Whole exome sequencing

To identify possible disease-causing mutation that would have escaped detection with MyoCap assay, a whole exome sequencing (WES) was performed for a trio of affected family members of F1 (F1:II-5, F1:II-7 and F1:III-5). WES was performed at Institute for Molecular Medicine Finland (FIMM, Helsinki, FIN) using KAPA Hyper library preparation Kit (Kapa Biosystems, Wilmington, MA, USA) and SeqCap EZ MedExome assay (Roche Nimblegen) for target enrichment. Paired-end sequencing to 100 bp read length was performed using Illumina HiSeq2500 Sequencer. Trimmed sequence reads were aligned to GRCh37 reference genome with the Burrows-Wheeler Aligner. PCR duplicates were removed using Picard MarkDuplicates and GATK IndelRealigner was used for local realignment of indel sites. The mpileup function from the SAMtools package was used for variant calling. ANNOVAR was used to annotate the detected variants. For a variant to be considered dominant disease-causing mutation, we required it to be private or have minor allele frequency (MAF) $\leq$ 0.0001% (ExAC_All database).

Sanger sequencing

Segregation analyses were performed using Sanger sequencing. The regions of interest were amplified by PCR (2X PCR Master Mix; ThermoFisher Scientific, Waltham, MA, USA) and sequenced using Big-Dye Terminator v3.1 Kit on an ABI3130xl automatic Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences and PCR conditions are available upon request. Sequence analysis was performed with Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Clinical, laboratory and imaging investigations

All patients were clinically examined by one of the authors and/or results from previous investigations were retrospectively obtained from medical records. Nine members of the index family (F1) gave blood samples for genetic studies, six of whom were affected, while three family members were asymptomatic. In the other Finnish family (F2), the proband was affected while the parents reported no symptoms. Further clinical investigations, however, revealed a mild distal myopathy also in the father (F2: I-1). In the Italian family (F3), two affected and three unaffected family members underwent genetic analyses (see fig 1 for pedigrees of the three families).

Serum creatine kinase (CK) values were measured in all symptomatic family members and nerve conduction/EMG studies were performed in eight patients (see table). MRI scans with axial sections of the lower limb muscles and using T1-weighted and short TI inversion recovery sequences were evaluated in 8 patients (F1: II-1, II-5, III-1 and III-5; F2: I-1 and II-1; F3: II-1 and III-1). Only coronal sections and axial scout images of the lower legs were available in 1 patient (F1-II-7). Also the upper girdle muscles were imaged in three patients (F1: II-5, F1:III-1 and F3:II-1). Frozen muscle samples were obtained from 6 patients. Biopsies were stained with hematoxylin and eosin (H&E), gomori trichrome, NADH-TR, COX-SDH, MyHC-neonatal, myotilin and MyHC slow and MyHC fast doublestaining or ATPases. Sarcoplasmic reticulum and T-tubule associated calcium channels (RyR1, calsequestrin, DHPR and SERCA1/2) were assessed by immunostaining and western blotting.

Muscle biopsies were subjected to subcellular fractionation using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Merck KGaA, Darmstadt, Germany) to obtain membrane protein fractions, and the samples for western blotting were prepared with a membrane protein

compatible method, as described earlier [22]. SDS-PAGE and western blotting were performed using standard methods. The PVDF filters were incubated in primary antibody overnight at +8°C, with gentle agitation, and detected with ECL using the ChemiDoc reader (Bio-Rad Laboratories, CA, USA). The Image Lab software (Bio-Rad) was used to calculate the relative quantities of the detected proteins. The mouse monoclonal primary antibodies used were RYR1 clone 34C (ab2868, Abcam, Cambridge, UK), DHPR clone A1 (ab2862, Abcam), SERCA1 clone VE121G9 (Research Diagnostics Inc., NJ, USA), SERCA2 clone IID8 (ab2817, Abcam).

Immunofluorescent (IF) stainings were performed on 8 µm thick frozen muscle sections after fixing with 4% PFA, using conventional methods. The following primary antibodies were used: rabbit polyclonal anti-RYR1 antibody (HPA056416, Atlas Antibodies) and mouse monoclonal anti-DHPR antibody (clone 1A, ab2862, Abcam). Alexa 488-conjugated anti-rabbit and Alexa 546-conjugated anti-mouse secondary antibodies (Invitrogen) were used for detection.

Standard Protocol Approvals, Registrations, and Patient Consents,

Local ethics committee approval and informed consent from the subjects were obtained for this study.

Data availability statement

Any anonymized data not published within the article will be shared by request from any qualified investigator.

Results

Summarized clinical data of the 10 affected family members from families F1, F2 and F3 is presented in table 1.

Genetic investigations

The proband of F1 and her sister had previously been investigated because of a calf myopathy of unknown cause. Based on the finding of core pathology on oxidative enzyme stainings, *RYR1* was considered to be a strong candidate gene, but an earlier analysis of selected exons had not identified any mutations. A genetic re-evaluation of the proband was performed using our targeted myopathy gene panel, MyoCap, which disclosed a previously unreported heterozygous mutation c.11710_11712delACAinsTGTCCGTCTGTGTCCTGTCTGTGT p.R3903_Q3905delTinsCPSVSCLC in exon 85 of *RYR1* gene (annotation based on transcript NM_000540). The same mutation was later confirmed in all affected members of family F1 by Sanger sequencing. In addition, two asymptomatic family members who were younger (20 and 23 years, respectively) than the typical age of onset in other family members, were found to carry the *RYR1* mutation. The proband's oldest child (F1:III-2) was also asymptomatic and had not inherited the familial mutation.

Three family members of F1 (F1:II-5, F1:II-7 and F1:III-5) were analyzed with WES. In all samples, WES yielded 20X coverage for over 90 % of the target region. The studied trio shared 47 variants with MAF ≤ 1%. In addition to the *RYR1* variant, three of the shared variants met our frequency requirements for a possibly dominant disease-causing mutation. These private variants were detected in genes *MRPS9*, *PARP14* and *NOS2.* The reported functions of the corresponding proteins (mitochondrial translation (MRPS9) or immunological processes (NOS2, PARP14)) makes them unlikely candidates for a non-inflammatory and non-mitochondrial myopathy.

In the proband of F2, the MyoCap gene panel identified two previously reported mutations in *RYR1:* c.7063C>T p.R2355W in exon 44 and c.13513G>C p.D4505H in exon 92 (with MAF = 0.000026 and MAF = 0.006, respectively). Both mutations have been associated with malignant hyperthermia susceptibility [23, 24]. Additionally, p.D4505H mutation has been reported to cause late-onset axial myopathy [9]. However, segregation analysis using parental samples unexpectedly showed both mutations to be in the same allele (*in cis*) and inherited from the asymptomatic father.

In family F3, the MyoCap gene panel identified a previously unreported heterozygous missense mutation c.13670C>T p.S4557F in exon 94 of *RYR1*. The mutation co-segregated in the family with the phenotype.

Clinical investigations

Family 1

The proband of family 1 (F1:II-5) was initially investigated because of CK elevation (up to 2500 IU/l) while on statin medication at the age of 50 years. Retrospectively, she recalled calf myalgias and a tripping tendency since age 40 presumably attributable to Achilles tendon tightness. Her sister (F1:II-7) had similar symptoms also after age 40 and had noted atrophy of calf muscles. Both F1:II-5 and F1:II-7 had marked myopathic EMG changes in calf muscles, but myopathic motor unit potentials were also detected in gluteal, thigh and upper girdle muscles. At age 56, F1:II-5 had developed mild proximal muscle weakness in upper and lower limbs and getting up from a squat required effort. Gross motor development in childhood had been normal although they were never fast runners. Their two brothers (F1:II-1 and F1:II-3) became symptomatic at about age 50 years, when they developed calf pain and fatigue after running or fast walking. Both brothers had physically demanding jobs and the older brother ran marathons until about age 50 years. The daughters of F1:II-5 and F1:II-1 (F1:III-5, F1:III-1) were originally examined because of toe walking beginning in their teenage years. At age 40 years, F1:III-1 walked with a slight limp due to asymmetric heel cord tightness and was unable to walk on her heels, but was otherwise asymptomatic.

Symptoms in all affected family members of F1 were very slowly progressive and the patients did not have signs of respiratory or cardiac involvement (apart from coronary artery disease in F1:II-3) or required walking aids. None of the patients had experienced episodes of malignant hyperthermia or rhabdomyolysis. However, F1:II-5 reported having unexplained fever (> 38 °C) after two separate surgical operations,that had been performed under general anesthesia. Cardiac ultrasound and spirometry values were normal in patient F1:II-3 at age 55.
STIR edema and/or fatty infiltration in the calf muscles was detected in 5/5 patients on MRI, particularly in medial gastrocnemius muscles (see fig 2). F1:II-5 had fibro-fatty infiltration also in the medial extensor spinae muscles at lumbar level.

Family 2

The proband of family F2 (F2:I-2) presented with childhood-onset toe walking due to tight Achilles tendons, but progressive weakness or disability has not developed over the last ten years. CK was elevated at 1500 IU/l. Muscle MRI of the lower limbs did not reveal obvious abnormalities, just somewhat prominent medial gastrocnemius muscles. EMG showed myopathic changes in calf muscles. The father (F2:I-1) carried the same *RYR1* mutations as the proband, and therefore also underwent a clinical examination. He had no muscle weakness or atrophy, but CK was elevated (1000 IU/l) and mild fatty degenerative changes were detected in the right gastrocnemius medialis (figure 2). Muscle biopsy was not performed.

Family 3

The proband of family 3 (F3:II-1) presented with diffuse muscle pain after effort, and easy fatigability after age 40. On physical examination, he was found to have a slightly waddling gait, mild rigid spine and Achilles tendon contractures, mildly high arched palate but no muscle weakness. Other features suggestive of King-Denborough syndrome, such as short stature or a history of undescended testes, were not present. Lower limb MRI findings are detailed in Figure 2. MRI of the upper girdle muscles did not disclose signs of fatty degeneration.

His son (F3:III-1) was examined at age 11 because of similar symptoms of diffuse muscle pain after effort and at night. He had a diagnosis of attention deficit hyperactivity disorder (ADHD). His CK was elevated as well (2-6 X), while neurological examination and a lower limb MRI were normal.

Findings on muscle histopathology and western blotting

Family F1: Biopsies obtained from gastrocnemius medialis (GM) muscle of patient F1: III-5 and tibialis anterior (TA) muscles of patients F1: II-5 and F1: III-1 showed myopathic changes (FIG. 3: A, C, D). On oxidative enzyme stainings fibers with core and multicore or moth-eaten areas were seen in both fiber types (FIG. 3: B, D, F). On ultrastructural studies unstructured core and multicore lesions were found (FIG. 3: K, I). Immunohistochemistry for sarcoplasmic reticulum associated

calcium-handling proteins including RyR1 and calsequestrin showed enhanced immunolabeling at the periphery of the cores and reduced RyR1-staining in the center of some of the cores (FIG. 3: H-L, Fig 4 C-E). GM biopsy from F1: II-7 showed end-stage pathology.

Family F2: In GM muscle biopsy from F2: II-1 increased fiber size variation in both fiber types, occasional fibers with internal nuclei, focal necrosis and poorly defined multicore-like and occasional typical core lesions on oxidative enzyme stainings were seen.

Family F3: Muscle biopsy of right soleus muscle of F3: II-1 showed increase in endomysial connective tissue and scattered necrotic fibers. Marked increase in fiber size variability, fibers with internal nuclei and fiber splitting were also observed. On oxidative enzyme stainings few fibers with core-like areas devoid of stain were evident.

Western blot analysis of RYR1 was performed on muscle biopsies from patients F1 (II-5 and F1:III-5) and F2 (F2:II-1), and 5 control muscles free of neuromuscular disease.
The results showed that RYR1 content in the muscle membrane fraction was reduced in F1 (F1:II-5 and III-5) to approximately 10% and 20%, respectively, and to 10% in patient F2 (II-1) (Figure 4).

Discussion

This study provides clinical, genetic and pathologic evidence that *RYR1* mutations may cause an unusual phenotype of calf-predominant myopathy with autosomal dominant inheritance. We detected a similar phenotype in two unrelated Finnish and one Italian family, and in all three families affected members were found to carry previously unreported mutations or mutation combinations in the *RYR1* gene.

Several lines of evidence suggest that the *RYR1* variants identified in our patients are disease-causing. First, the mutations were found in all affected family members and in two generations. Second, cores or core-like pathology were detected on muscle biopsy, which is a typical, even

though not specific, abnormality in *RYR1*-related myopathies. Third, RYR1 protein levels were clearly reduced on western blot in families F1 and F2, which supports a dominant negative pathogenic effect of the mutation on the wild-type protein. Although two asymptomatic family members in F1 were confirmed to carry the mutation, they were two decades younger than the typical age of disease onset in family F1 and thus presymptomatic.

The classical clinical manifestations caused by *RYR1* mutations are CCD and susceptibility to MH. Patients with MH susceptibility usually do not have a clinical myopathy. However, since the MH trait is most commonly detected in patients before they reach adulthood, the possibility that some MH patients might develop a mild or subclinical myopathy at a later age cannot be excluded. Indeed, axial myopathy has been suggested to be a late manifestation in some carriers of malignant hyperthermia-associated mutations [9]. Because malignant hyperthermia reactions have been reported also in patients with core myopathies [4], all carriers of pathogenic *RYR1* mutations should preferentially avoid MH-triggering anesthetic agents (volatile anaesthetics, succinylcholine), regardless of the phenotype unless an in vitro contracture (IVCT) test has been performed. In addition to CCD and MH susceptibility, *RYR1* mutations have recently been found to cause a variety of muscle disorders presenting with phenotypes ranging from asymptomatic hyperCKemia to severe congenital multiminicore myopathy [24]. *RYR1*- related myopathies usually commence in early childhood and adult-onset cases have only rarely been reported [17]. In our patients, clinical disease onset was later than 40 years in half of the patients. Toe-walking and histopathological changes in gastrocnemius muscles were detected already during teenage years in three patients, but it seems that fatty-degenerative changes visible on MRI may develop later, as they were found only in adult patients in our families.

Weakness in *RYR1*-related myopathies is usually predominantly proximal and axial [24], and contractures or hyperlaxity of joints as well as spinal deformities are common [11, 24]. CK levels may be normal or mildly elevated. Ophthalmoplegia may be observed in recessive cases. *RYR1* is not expressed in cardiac muscle, but rare patients with cardiac involvement have been described and it has been speculated that this may be due to vascular smooth muscle dysfunction [11]. Excessive sweating, increased bleeding tendency as well as bladder or bowel dysfunction may occur with some potentially MH-associated mutations, but were not detected in any of our families. Extraocular muscles were also normal on clinical examination.

The weakness distribution and the imaging findings in the families described here were different from those usually observed in RYR1-related myopathies. At lower leg level, gastrocnemius medialis was preferentially affected in our patients on MRI, whereas in *RYR1*-related congenital myopathies dystrophic changes predominate in the soleus and gastrocnemius lateralis [25]. Muscle MRI in some of our patients showed mild paravertebral (F1:II-5) and hamstring muscle involvement (F3:II-1), which is more similar to that reported in patients with potentially MH-associated RYR1 mutations[9] rather than that seen in early-onset, RYR1-related central core congenital myopathies.

Typical central cores on muscle biopsy are highly suggestive of *RYR1*-mutated central core myopathy [26], but core pathology and other defects on oxidative enzyme staining may be mild or absent in some *RYR1*-related disorders [24]. In addition, the histological abnormalities may evolve over time from central cores to multiminicores even in the same patient [5]. Type I fibre predominance and internalized or central nuclei are common findings, and nemaline rods may be encountered occasionally [27,28]. Our patients showed histopathological findings compatible with a *RYR*1 defect, including increased internal nuclei and core pathology.

We postulate that all *RYR1* mutations identified in this study are disease-causing due to a dominant-negative effect. The mutation in family F1 should lead to an extended protein product, but to the best of our knowledge, a similar mutation has not been previously described in autosomal dominant pedigrees. The reason for the reduced RYR1 protein content in the sarcoplasmic reticulum as shown by western blot analysis is not directly explained by the in-frame mutations found in the families F1 and F2, but a negative effect of the mutant protein on wild type RyR1 monomers is the most plausible explanation.

Members in family F2 carried previously reported malignant hyperthermia-associated mutations R2355W and D4505H in cis. Interestingly, the D4505H mutation in combination with another mutation (R3983C) on the same allele has been reported to result in a more severe defect in RyR1 channel function in vitro, than what was observed with RyR1 channels carrying only the D4505H amino acid change [29]. Whether the R2355W and D4505H mutations may cause similar synergistic effects on RYR1 channel function warrants further study. The mutation in family F3 (p.S4557F) is previously unreported, although mutation in the adjacent codon 4558 has been associated with

recessive congenital central core disease [13]. Substitution of serine 4557 with phenylalanine should prevent hydrogen bond formation of this residue with neighbouring amino acids and would therefore probably destabilize the alpha-helix.

RYR1 is a large gene and mutations in different domains give rise to several different clinical phenotypes. Private variants in *RYR1* are not uncommon and their clinical significance may be difficult to determine, especially in small families with unusual phenotypes. We have shown that *RYR1* mutations may also cause a calf-predominant myopathy, which further expands the wide phenotypic spectrum associated with *RYR1* mutations.

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Appendix 1: authors

Name	Location	Role	Contribution
Jokela Manu, MD, PhD	Jokela Manu, MD, PhD. Division of Clinical Neurosciences, Turku University Hospital and Neuromuscular Research Center, Tampere University Hospital	Author	Design and conceptualization of the study; analysis of the data; drafting of the manuscript for intellectual content
Tasca Giorgio, MD, PhD	Unità Operativa Complessa di Neurologia, Dipartimento di Scienze dell'Invecchiamento, Neurologiche, Ortopediche e della	Author	Acquisition, analysis and interpretation od data, drafting of the manuscript

	Testa-Collo, Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma, Italia		
Vihola Anna, PhD.	Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute and Neuromuscular Research Center, Tampere University Hospital	Author	Acquisition, analysis and interpretation od data, drafting of the manuscript
Mercuri Eugenio, MD, prof.	Institute of Pediatric Neurology, Catholic University School of Medicine, Rome, Italy	Author	Acquisition, analysis and interpretation od data
Jonson Per-Harald, PhD.	Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland	Author	Acquisition, analysis and interpretation od data
Huovinen Sanna, MD.	Department of Pathology, Fimlab Laboratories, Tampere University Hospital, Tampere, Finland	Author	Acquisition, analysis and interpretation od data, drafting of the manuscript
Lehtinen Sara, MSc.	Neuromuscular Research Center, Tampere University and University Hospital, Tampere, Finland	Author	Acquisition, analysis and interpretation od data, drafting of the manuscript
Välipakka Salla, MSc.	Folkhälsan Institute of	Author	Acquisition, analysis and

	Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland		interpretation od data, drafting of the manuscript
Pane Marika, MD, PhD.	Institute of Pediatric Neurology, Catholic University School of Medicine, Rome, Italy	Author	Acquisition, analysis and interpretation od data
Donati Maria, MD, PhD.	Metabolic and Neuromuscular Unit, Meyer Hospital, Florence, Italy	Author	Acquisition, analysis and interpretation od data
Johari Mridul, MSc.	Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland	Author	Acquisition, analysis and interpretation od data
Savarese, Marco, PhD	Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland	Author	Acquisition, analysis and interpretation od data
Isohanni Pirjo, MD, PhD.	Department of Pediatric Neurology, Children's Hospital, University of Helsinki and Helsinki University Hospital	Author	Acquisition, analysis and interpretation od data
Hartikainen Päivi, MD, PhD.	Department of Neurology, Kuopio University Hospital and	Author	Acquisition, analysis and interpretation od data

	University of Eastern Finland		
Palmio Johanna, MD, PhD.	Neuromuscular Research Center, Tampere University Hospital, Tampere, Finland	Author	Acquisition, analysis and interpretation od data
Udd Bjarne, MD, Prof..	Neuromuscular Research Center, Tampere University Hospital, Tampere, Finland	Author	Study concept and design; acquisition, analysis and interpretation od data; drafting of the manuscript; study supervision

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Figure legends

Figure 1. Title: Pedigrees of the three families.
Legend: Filled-in symbols indicate affected family members. Individuals marked with (+) are carriers of the disease mutation and those marked with (++) have two mutations in cis. Individuals marked with (-) do not carry the disease mutation.

Figure 2. Title: Muscle biopsy findings from patients with *RYR1*-related calf-predominant myopathy with cores.
Legend: Gastrocnemius medialis (GM) biopsy from F1:III-5 (A) shows mild endomysial fibrosis, fibers with multiple internal nuclei, marked fiber size variation and slightly basophilic fibers with whorled internal structure (haematoxylin and eosin, H&E). Tibialis anterior (TA) biopsies from F1:II-5 (C) and F1:III-1 (E) show mild myopathic changes with fiber size variation and increased internal nuclei (H&E). Many fibers with cores and moth-eaten uneven distribution of stain are present in the GM biopsy from F1: III-5 (B), and well defined cores and multicore-like unevenness of stain are seen in the TA biopsies from F1:II-5 (D) and F1:III-1 (F) (Nicotinamide adenine

dinucleotide, NADH). RyR1 and calsequestrin immunohistochemical stainings showing abnormal immunolabeling of cores in the GM biopsy from F1:III-5 (G, H) and in the TA biopsy from F1:III-1 (I, J). RyR1 and calsequestrin show protein accumulation mainly around the cores (G-J), and depletion of RyR1-immunolabeling is seen in the center of some of the cores (arrows, G). Electron micrographs of the GM biopsy from F1:III-5 (K) and the TA biopsy from F1:II-5 (L), show large (K) and small areas (L) with severe disruption of myofibrillar structure with excess of Z-disk material and some accumulation of sarcoplasmic reticulum or T-tubule structures corresponding to the unstructured core and multicore lesions. Scale bar = 100 μm (A-J), scale bar = 2 μm (K-L).

Figure 3. Title: Lower limb MRI findings
Legend: Lower limb MRI of patients F1: II-1 (A, B); F1: III-1 (C, D) ; F1: II-5 (E, F); F2: II-1 (G, H) ; F3: II-1 (I, J) at thigh and calf levels. All patients except F2: II-1 show severe fatty degeneration bilaterally in gastrocnemius medialis muscles. In patient F2: II-1 there is moderate, asymmetric involvement of the right gastrocnemius medialis (blue arrow) and of the left soleus in F3: II-1. Thigh muscles are spared in all patients except F3:II-1, who has minor changes in the left semimembranosus and long head of the right biceps femoris. Very mild, diffuse fatty streaks present in the thigh muscles of patients F1:III-1 and F1:II-5 probably represent normal, age-related changes.

Figure 4. Title: RYR1 Western blotting and RYR1/DHPR double immunofluorescence (IF).
Legend: RYR1 Western blotting shows reduced amounts of RYR1 in patients F1:II-5, F1:III-5 and F2: II-1, when muscle biopsy membrane fractions were analysed. In a representative western blot image (A) the patients and three control samples are shown. The analysis was performed in triplicate, with five controls, and RYR1 was normalized to SERCA1,2 expression on the same blots after stripping the filters. Averages of RYR1/SERCA1,2 ratios from three experiments were calculated to obtain graphical presentation (B), where average of control samples has been normalized to value 1. RYR1-DHPR double IF staining shows abnormal mislocation of these sarcoplasmic reticulum/T-tubule interface- associated calcium channels that seems to be restricted to the disrupted myofibrillar core areas in the abormal fibers, besides the overall increased intensity of staining in mildly atrophic fibers. RYR1 (C), DHPR (D), merge (E).

Table legend

Table 1. Clinical features of the three families with *RYR1*-related calf myopathy. N/A= not available. CK= creatine kinase. EMG/NCS= electromyography and nerve conduction studies.

Patient	Age and symptoms at onset	Age at study	Ankle contracture	Walking ability	EMG/NCS abnormalities	Proximal weakness	Toe/heel walking	CK
F1:II-5	40 (Ankle weakness, tripping)	56	Achilles tendon tightness	Normal	Myopathic	Mild proximal weakness in upper and lower limbs	Normal/difficult	2-10x
F1:II-7	40 (asymmetric calf atrophy)	52	Achilles tendon tightness	Normal	Myopathic	No weakness	Normal/normal	2x
F1:II-3	50 (calf myalgias after exercise)	63	Achilles tendon tightness (right>left)	< 1km in one stretch at age 65	Previous L5 radiculopathy, gastrocnemius medialis: subacute neurogenic/ myopathic changes	Mild proximal lower limb weakness	Difficult/ difficult	2-5x
F1:II-1	50 (calf myalgias after exercise)	67	Achilles tendon tightness (left >right)	Normal at age 67	Myopathic	No weakness	Normal/normal	2-5x
F1: III-5	14 (toe walking)	16	Toe walking	Normal	Myopathic	No weakness	Normal/unable	5x
F1:III-1	15	40	Aymmetric achilles tendon tightness	Normal	Myopathic	No weakness	Normal/unable	1,5x
F2: I-1	No symptoms	48	No symptoms	Normal	N/A	No weakness	Normal/normal	7x
F2: II-1	Childhood	15	Toe walking	Normal	Myopathic	No weakness	Normal/unable	5x
F3: II-I	40 (easy fatigability and myalgias)	56	Achilles tendon tightness	Normal	Myopathic	Mild proximal lower limb weakness	Normal/normal	3-10x
F3: III-1	10 (myalgias)	11	No	Normal	Not performed	No weakness	Normal/normal	2-6 X

Table 1.