Hereditary myopathy with early respiratory failure: occurrence in various populations

Palmio, Johanna; Evila, Anni; Chapon, Francoise; Tasca, Giorgio; Xiang, Fengqing; Brådvik, Björn; Eymard, Bruno; Echaniz-Laguna, Andoni; Laporte, Jocelyn; Karppa, Mikko; Mahjneh, Ibrahim; Quinlivan, Rosaline; Laforet, Pascal; Damian, Maxwell; Berardo, Andres; Taratuto, Ana Lia; Bueri, Jose Antonio; Tommiska, Johanna; Raivio, Taneli; Tuerk, Matthias; Goelitz, Philipp; Chevessier, Frederic; Sewry, Caroline; Norwood, Fiona; Hedberg, Carola; Schroeder, Rolf; Edstrom, Lars; Oldfors, Anders; Hackman, Peter; Udd, Bjarne

Published in:
Journal of Neurology, Neurosurgery and Psychiatry

DOI:
10.1136/jnnp-2013-304965

2014

Hereditary myopathy with early respiratory failure: occurrence in various populations

Johanna Palmio[1]*, Anni Evilä[2]*, Françoise Chapon[3], Giorgio Tasca[4], Fengqing Xiang[5], Björn Brådvik[6], Bruno Eymard[7], Andoni Echaniz-Laguna[8], Jocelyn Laporte[9], Mikko Kärppä[10], Ibrahim Mahjneh[11], Rosaline Quinlivan[12], Pascal Laforêt[7], Maxwell Damian[13], Andres Berardo[14], Ana Lia Taratuto[15], Jose Antonio Bueri[14], Johanna Tommiska[16], Taneli Raivio[16], Matthias Tuerk[17], Philipp Gölitz[18] Frederic Chevessier[19], Caroline Sewry[20], Fiona Norwood[21], Carola Hedberg[22], Rolf Schröder[19], Lars Edström[5], Anders Oldfors[22], Peter Hackman[2], Bjarne Udd[1,2]

* These authors contributed equally to the manuscript

[1]Department of Neurology, Neuromuscular Research Unit, Tampere University and University Hospital, Tampere, Finland

[2]Folkhälsan Institute of Genetics and Department of Medical Genetics, Haartman Institute, University of Helsinki, Finland

[3]CHU de Caen, Neuromuscular Disorders Competence Centre, and University Hospital of Caen, France

[4]Don Carlo Gnocchi Onlus Foundation, Italy

[5]Department of Women´s and Children´s Health, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden

[6]Department of Neurology, Institution of Clinical Sciences, Lund University, Sweden

[7]Institute of Myology, National Reference Center for neuromuscular disorders, University Hospital of Salpêtrière, UPMC, France

[8]Department of Neurology, Hôpitaux Universitaires, Strasbourg, France

[9]Department Dpt of Translational Medecine and Neurogenetics, IGBMC, University Strasbourg, Illkirch, France

[10]Department of Clinical Medicine, Neurology, University of Oulu and Clinical Research Center, Oulu University Hospital, Oulu, Finland

[11]Department of Neurology, University of Oulu and Pietarsaari Hospital, Finland

[12]MRC Centre for Neuromuscular Disease, Institute of Neurology, National Hospital, London, United Kingdom

[13]Cambridge University Hospitals, United Kingdom

[14]Neuromuscular Section, Hospital Universitario Austral (HUA), Buenos Aires, Argentina

[15]Department of Neuropathology, Institute for Neurological Diseases, FLENI, Buenos Aires, Argentina

[16]Institute of Biomedicine/Physiology, University of Helsinki, Finland

Departments of Neurology[17], Neuroradiology[18] and Neuropathology[19], University of Erlangen-Nuremberg, Germany

[20]Wolfson Centre for Inherited Neuromuscular Diseases, RJAH Orthopaedic Hospital, Oswestry, United Kingdom

[21]Department of Neurology, King's College Hospital NHS Foundation Trust, London, United Kingdom

[22]Department of Pathology, University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden

[23]Department of Neurology, Vaasa Central Hospital, Finland

Correspondence to J. Palmio, Department of Neurology, Neuromuscular Research Unit, Tampere University Hospital and University of Tampere, FIN-33014 University of Tampere, Finland

Tel: +358-3-3116111, fax: +358-3-35516164

E-mail: johanna.palmio@uta.fi

Key words: hereditary myopathy; respiratory failure; titin; epidemiology

Word count: 3400

ABSTRACT

Objective Several families with characteristic features of hereditary myopathy with early respiratory failure (HMERF) have remained without genetic cause. This international study was initiated to clarify epidemiology and the genetic underlying cause in these families, and to characterize the phenotype in our large cohort.

Methods DNA samples of all currently known HMERF families without molecular genetic cause were obtained from 12 families in seven different countries. Clinical, histopathological and muscle imaging data were collected and five biopsy samples made available for further immunohistochemical studies. Genotyping, exome sequencing and Sanger sequencing were used to identify and confirm sequence variations.

Results All patients with clinical diagnosis of HMERF were genetically solved by five different titin mutations identified. One mutation has been reported while four are novel, all located exclusively in FN3 119 domain of A-band titin. One of the new mutations showed semi-recessive inheritance pattern with subclinical myopathy in the heterozygous parents. Typical clinical features were respiratory failure at mid-adulthood in an ambulant patient with very variable degree of muscle weakness. Cytoplasmic bodies were retrospectively observed in all muscle biopsy samples and these were reactive for myofibrillar proteins but not for titin.

Conclusions We report an extensive collection of HMERF families with five different mutations in exon 343 of *TTN,* which establishes this exon as the primary target for molecular diagnosis of HMERF. Our relatively large number of new

families and mutations directly implies that HMERF is not extremely rare, not restricted to Northern Europe, and should be considered in undetermined myogenic respiratory failure.

INTRODUCTION

Hereditary myopathy with early respiratory failure (HMERF, OMIM #603689) was described as an autosomal dominant disease characterized by adult onset proximal and/or distal myopathy with respiratory failure typically in ambulant patients. HMERF disease has been associated with two different titin mutations.[1-4] Muscle histopathological features include a combination of cytoplasmic bodies and rimmed vacuoles.[3] A diagnostic pattern of fatty degenerative changes in lower limb muscles on MRI has been identified, showing marked involvement of semitendinosus, obturatorius, sartorius, gracilis, and iliopsoas muscles.[3-6] Several families and patients with clinical, morphological and imaging features compatible with HMERF have remained without molecular genetic cause, including two of the originally reported families[1] and two previously described separate families.[6,7] In the current large international study we identified the recently reported titin A-band mutation [3,4] in six families from various ethnic backgrounds and, moreover, four novel titin mutations in the same A-band domain in six other families indicating that the disease is not extremely rare and maybe underdiagnosed.

Mutations in *TTN* gene, encoding the giant muscle protein titin, are known to cause several different skeletal and/or cardiac myopathies.[8-11] A dominant mutation in the kinase domain of M-line titin leading to HMERF was first described in three families from Sweden.[2] Two recent reports revealed a novel titin A-band mutation in three Swedish and three British families with HMERF.[3,4] Based on

our large international collection of families and new mutations, the range of epidemiological, clinical and mutational spectrum of HMERF disease expands considerably and includes unexpected semi-recessive inheritance with one of the mutations.

METHODS

Study protocol and patients

Patients belonged to 12 unrelated families: one French (A), one Finnish (B), two Swedish (C, D), two British (E, I), two Italian (F, J), one Argentinian (G), one German (H), and two French with Portuguese ancestry (K, L) (figure 1). The collection contains all HMERF families known to the authors without established molecular genetic cause. Among the 31 affected patients, 16 were male and 15 female with a mean age at examination of 49 years (range 29-72 y). All patients had been clinically examined by neurologists including muscle strength evaluation (the Medical Research Council Scale, MRC) and medical and family histories. Pulmonary function tests and echocardiography were performed, as well as electrophysiological examinations consisting of nerve conduction studies and needle electromyogram (EMG), creatine kinase (CK) measurement and muscle imaging by computed tomography (CT) or magnetic resonance imaging (MRI). The diagnosis of HMERF was based on clinical symptoms of respiratory insufficiency with muscle weakness and/or presence of cytoplasmic bodies in muscle biopsy and a typical pattern of muscle involvement on MRI as described

previously.[3,4,6,7] DNA samples of 31 affected patients, as well as 26 healthy family members were provided by the clinicians in the different countries.

Muscle biopsies were obtained from proximal muscle (deltoid or thigh muscle, 19 samples) or from the gastrocnemius muscle (one sample). They were snap frozen and 8-10 μm sections were cut and examined using standard histochemical stainings including haematoxylin and eosin, Gomori trichrome, reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) and ATPase at pH 10.4, pH 4.6, and combined succinate dehydrogenase–cytochrome oxidase (SDH–COX). Four samples were stained for actin with rhodamine-conjugated phalloidin. Sections were also immunostained for different myogenic antigens including myosin heavy chain isoforms (fetal, neonatal, slow and fast MyHC, MHC class I). Five samples were available for additional immunohistochemistry.

Linkage studies

Fluorescently-labeled polymorphic microsatellite markers spanning a region of 7 Mb in TTN locus were used for genotyping the families. Markers used were D2S2314, D2S1244, D2S138, D2S148, D2S2173, D2S300, D2S385, D2S324, D2S2978, D2S2261, D2S384, D2S364, and D2S350. Genotyping was performed using ABI3730xl DNA Analyzer and GeneMapper v4.0 software (Applied Biosystems).

Exome sequencing

Two affected (II-3 and III-1) and one healthy (II-1) member of French family A (figure 1) were exome sequenced at Axeq/Macrogen laboratory in South Korea. The capture method used was Illumina TruSeq™ Exome Enrichment. Captured DNA fragments were sequenced on an Illumina HiSeq2000 platform using 100 bp paired-end reads. Sequence reads were aligned to the human reference genome (UCSC hg19) using the BWA (Burrows-Wheeler Aligner).[12] Variant calling was made with GATK.[13] Variant quality/control data filtering was performed using the analysis and visualization program RikuRator (unpublished), created by Riku Katainen from Lauri Aaltonen's group at the University of Helsinki. To call a variant, the coverage was required to be at least two reads and the mutated allele to be present in at least 20 % of the reads. Only variants that both affected shared were included and filtered against dbSNP132 and one healthy member of the family.

Sanger sequencing

Mutations were confirmed by Sanger sequencing. Sequencing primers were obtained from Genethon and are available on request. PCR was performed with DreamTaq™ DNA Polymerase according to standard protocol (Fermentas). PCR products were sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems), using the Big-Dye Terminator v3.1 kit and analyzed with Sequencher 5.0 software (Gene Codes Corporation).

RESULTS

Clinical characteristics of the patients

Clinical data of 31 patients with HMERF are presented in table 1and pedigrees of the 12 families in figure 1. The presenting symptom was either lower limb weakness (14/22) or respiratory failure (8/22) with a mean age of onset of 36.6 years (range 16-53 y). In family D four patients reportedly had muscle weakness already in childhood but no results of examinations performed in childhood were available for confirmation. Muscle weakness was progressive and usually symmetrical despite occasional asymmetry on imaging (table 2). Disease duration at the time of the latest examination was on average 13 years (range 1-32 y) and the most typical symptoms consisted of both distal and proximal lower limb weakness and respiratory insufficiency that needed invasive or non-invasive ventilation. Neck flexor, abdominal and ankle dorsiflexion weakness was marked. There was no upper limb weakness at onset. In the later course of the disease proximal and distal weakness in the upper extremities was observed in 19 patients. The severity of muscle weakness and its rate of progression varied from mild (no limb weakness in four patients) to loss of ambulation at age 36. One member of family A did not have any weakness in limbs or respiratory muscles at age 38 but did show mild pathognomonic findings in muscle biopsy and definite findings on muscle MRI. Patients in the younger generation of family B and two members of families C (C:IV-4) and D (D:IV-8) did not have respiratory symptoms at ages 32-67 years but had evident findings on muscle biopsy and MRI. Cardiomyopathy was not manifest in any of the patients, based on clinical, electrocardiography, chest X-ray examinations or echocardiography (nine patients). CK activity was normal or

slightly elevated. EMG was myopathic with normal nerve conduction studies in 17 patients. EMG was reported as neurogenic in one patient (B:III-3) and with mixed findings in two (B:III-10, G:II-2).

Table 1. Clinical data retrieved from medical records

TTN A-band g.274367C>G, p.P30068R

French family A[a]

Patient	**Sex/age at exam**	**Age at onset**	**First symptoms**	**Muscle weakness findings at examination (MRC)**	**Respiratory symptoms**	**CK / IU/L**	**EMG findings**
A:I-1	F/72	40	Respiratory failure	Axial Diffuse weakness predominantly proximal UL, LL	Assisted ventilation with tracheostomy	NR	Myopathic
A:II-3	F/45	44	Respiratory failure	Axial Diffuse weakness predominantly proximal UL, LL	Assisted ventilation with tracheostomy	Normal	Myopathic
A:III-1	F/38	-	No symptoms	No abnormal findings	No symptoms	NA	NA

TTN A-band g.274375T>C, p.C30071R							
Finnish family B							
B:III-3	M/54	50	Ankle dorsiflexion weakness	Ankle dorsiflexion	No symptoms	Normal	Neurogenic
B:III-5	M/67	45	Distal lower leg weakness	Proximal UL (4-5), LL (3-4) Distal UL (2-3), LL (1-4, mild asymmetry)	No symptoms	Normal	Myopathic
B:III-10	M/58	45	Gait difficulties	Proximal LL Distal LL	No symptoms	700	Mixed neurogenic, myopathic
B:III-11	M/58	48	Gait difficulties	Proximal UL (4+), LL (3-4) Ankle dorsiflexion	Asthma diagnosed	327	NA
Swedish family C							

C:III-3[b]	M/NR	Adult	NR	Wheelchair bound	Assisted ventilation	NR	NR
C:III-12	F/NR	Adult	NR	Neck flexors Proximal LL (mild) Ankle dorsiflexion	Non-invasive night-time ventilation	NR	NR
C:IV-1	F/45	30	Respiratory symptoms	Proximal UL (mild)	Slightly decreased VC (3.2 L)	NR	NR
C:IV-2	M/NR	Adult	Distal lower limb weakness	Proximal LL Ankle dorsiflexion	Mild respiratory insufficiency	NR	NR
C:IV-3	F/NR	Adult	Respiratory failure	Neck flexors Finger flexors Proximal UL, LL	Non-invasive night-time ventilation	NR	NR
C:IV-4	M/NR	NR	Ankle dorsiflexion weakness	Hand extensors Abdominal	No symptoms	NR	NR

				Proximal LL Ankle dorsiflexion			
C:IV-6	M/NR	NR	NR	Neck flexors Proximal UL, LL Ankle dorsiflexion	Non-invasive night-time ventilation	NR	NR
Swedish family D							
D:III-2	49/F	Child-hood	Clumsiness	Proximal (severe) UL, LL Distal (moderate) UL, LL	Respiratory insufficiency, COPD	Normal	Myopathic
D:III-3	50/F	10	Gait difficulties, proximal LL weakness	Distal UL (3-4), LL (moderate) Proximal LL	Decreased VC (2.6 L)	4,4 (normal value <3,3)	Myopathic, including tongue and throat
D:III-10[b]	43/F	Child-hood	Clumsiness, tendency to fall	Sternocleidomastoid Proximal UL, LL	NA	Slightly elevated	Myopathic

D:III-11	40/M	34	Finger and ankle extension weakness	Neck flexors, sternocleidomastoid Finger extensors Distal LL	Slightly decreased VC in sitting	NR	Myopathic
D:IV-8	32/F	Child-hood	Running difficulties	Proximal UL (mild), LL (moderate)	Normal VC	Normal	Myopathic
British family E							
E:II-1	65/F	35	Difficulties in climbing stairs	Finger flexion contractures of 1st and 2nd fingers, neck contracture limited flexion and extension. Facial muscles (minimal) Neck flexors (4) Proximal UL (4), LL (1-5)	Non-invasive night-time ventilation FVC 26%-18 % (sitting-lying)	Normal	Myopathic
Italian family F							

F:II-1	F/56	30	Lower limb weakness	Neck extensors and flexors Proximal UL, LL Abductor digiti minimi Ankle dorsiflexors	FVC 60%	330	Myopathic
Argentinian family G							
G:II-2	48/M	38	Lower limb weakness-asymmetrical steppage	Neck flexors, Axial weakness, asymmetrical scapular weakness, anterior distal LL (0-5). Subtle calf hypertrophy	Non-invasive night-time ventilation. Severe orthopnea	550-1000	Mixed neurogenic, myopathic
TTN A-band g.274426T>C, p.W30088R							
German family H							
H:II-2	M/ 39	24	NR	Wheelchair bound Proximal UL (0-4), LL (3-4)	CO2-retention	Normal	Myopathic

				Distal UL (1-2), LL (0-2)			
H:III-2	F/29	16 (Child-hood)	Neck flexor weakness	Facial muscles (mild) Neck flexion (2) Neck rotation (4+) Proximal UL (0-4), LL (4) Distal UL (3-4), LL (0-4)	CO2-retention VC 28%	1298	Myopathic
TTN A-band g.274428G>C, p.W30088C							
British family I							
I:II-1	M/58	56	Respiratory failure	No limb weakness	Non-invasive ventilation FVC 29%	Normal	Myopathic
TTN A-band g.274436C>T, p.P30091L							
Italian family J							
J:II-1[c]	M/32	30	Nocturnal	Neck flexors	CO2-retention	Normal	Myopathic

homozygous			hypoventilation, exertional dyspnoea	Proximal LL Ankle dorsiflexion	FVC 39%		
J:I-1 heterozygous	F/56	No	No	Normal	No symptoms	NA	NA
J:I-2 heterozygous	M/59	No	No	Normal	No symptoms	NA	NA
French family K (with Portuguese ancestry)							
K:II-1 homozygous	M/36	27 (Child-hood)	Effort breathlessness	Wheelchair bound at age 36 Proximal UL (2-4), LL (1-3+) Fingers extensors (4) Distal LL (2-3+)	Assisted ventilation with tracheostomy	274	Myopathic
French family L (with Portuguese ancestry)							
L:II-1	M/56	42	Respiratory failure	No limb weakness	Non-invasive	Normal	Myopathic

heterozygous					night-time ventilation FVC 60%		
L:II-2 heterozygous	F/58	53	Hypoventilation	Neck flexors (3) No limb weakness	Non-invasive night-time ventilation VC 45%	Normal	NA

Abbreviations: MRC = the Medical Research Council Scale; NA = not assessed; NR = not retrieved; UL = upper limbs; LL = lower limbs; FVC = forced vital capacity; VC = vital capacity; COPD = chronic obstructive pulmonary disease

[a] French family A first described in ref. 6

[b] DNA not available

[c] The proband of the family J first described in ref

Table 2.	**Muscle imaging findings**																							
Patient	Glma	Glme	Glmi	Ilps	Obt	RF	VL	VI	VM	Sa	Gr	SM	ST	AM	AL	BF	TA	EH	ED	Pr	Gm	Gl	S	TP	FP
A:II-3					3	3	3			3	3		3												
A:III-1	0	0								1	1		2												1
B:III-3	0	0/2*	3	1	3	1	0	0	0	1	1/2	1	3	1	0	1	3	3	3	2/3	0	0/1	0/1	3	3
B:III-5	1	3	3	2	3	1	0	1	0	3	3	2	3	1	1	1	3	3	3	3	1	2	1	2/3	1/2
B:III-10	NA	NA	NA	2	3	0/2	0	0	0/1	3	3	1	3	1/2	2/3	1	1/3	3	0/1	2/3	0	1	1/2	2/3	2/3
B:III-11	2	2	3	3	3	0	0	1	1	3	3	1	3	1/2	0/2	1 BFs 3	1/3	2/3	1	3	0	1	2	3	3
C:IV-1	0	0	0	0	3	0	0	0	0	0	0	1	3	0	0	BFs 1	0	0	0	1	0	0	0	1/2	1
C:IV-2	0	0	0	0	3	0	0	0	0	1	1	0	3	0	0	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
E:II-1	1	2	2	3	3	3	2	3	3	3	3	1	3	3	1	0	2	2	3	1	0	0	0	1	2
F:II-1	2	2	3	3	3	2	1	1	1	3	3	0	3	0	0	0	2	2	2	3	0	0	0	3	#
H:III-2	1/2	1	2/3	1	2	2	1	3	2	1	3	1	3	0	0	BFs 3	3	3	3	3	2/3	2/3	1	2/3	2

I:II-1	NA	NA	NA	NA	NA	0	0	0	0	1	3	1	3	0	0	0	1/0	1/0	1/0	1	0	1/0	1/0	1/2	0
J:II-1	2	2	2	3	3	1	1	1	1	1	1	1	2	2	1	1	1	2	2	2	0	0	1	2	**
J:I-1	1	0	1	0	2	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
J:I-2	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0
K:II-1	3	3	3	3	3	1	1	1	1	3	3	2	3	3	3	3	3	3	3	3	1	1	3	3	3
L:II-2	0	0	0	0	0	0	0	0	0	0	0	1		0	0	1	0	0	0	0	0	0	0	0	0

0 = normal; 1 = mild; 2 = moderate; 3 = severe; NA = not assessed; Glma = gluteus maximus; Glme = gluteus medius; Glmi = gluteus minimus; Ilps = iliopsoas; Obt = obturatorius; RF = rectus femoris; VL = vastus lateralis; VI = vastus intermedius; VM = vastus medialis; Sa = sartorius; Gr = gracilis; SM = semimembranosus; ST = semitendinosus; AM = adductor magnus; AL = adductor longus; BF = biceps femoris; BFs = biceps femoris short head; TA = tibialis anterior; EH = extensor hallucis longus; ED = extensor digitorum longus; Pr = peroneus longus; Gm = gastrocnemius medialis; Gl = gastrocnemius lateralis; S = soleus; TP = tibialis posterior; FP = flexor hallucis and digitorum longus

*Right/left when there are asymmetrical findings; # FP: flexor hallucis longus = 2; flexor digitorum longus = 3; ** FP flexor hallucis longus = 2; flexor digitorum longus = 1

There were ten siblings in the second generation (figure 1, B:II) of family B, of whom six deceased patients had been affected. All four neurologically examined patients (B:II-3, -7, -9, -12) had documented muscle weakness and muscle biopsy obtained in two of them showed rimmed vacuolar myopathy and dystrophic findings. Three of the siblings (B:II-2, -4, -7) had respiratory failure, needed mechanical ventilation and died on average aged 65 years.

Clinical genetics

In Italian family J the proband with homozygous mutation had relatively early adult onset of respiratory failure and the heterozygous parents were reported healthy at ages 52 and 55 years, which is compatible with a recessive mode of inheritance.[7] After the same mutation in heterozygous state was identified in two siblings in family L of Portuguese origin causing the same disease but with considerably later onset, additional studies were performed by MRI in the parents of Italian family J (figure 2). Although subjectively healthy and with no muscle weakness on clinical testing, muscle MRI revealed clear pathology compatible with the known pattern of muscle involvement in HMERF. In French-Portuguese family K, the parents of the homozygous proband were first cousins with no signs of disease on clinical examination at the age of 57 and 61 respectively. Muscle MRI was not performed. The parents of two siblings in family L were reported to be healthy but muscle MRI was not available.

Muscle imaging

The distribution and degree of fatty degenerative changes in muscles of 18 patients were evaluated (table 2). Semitendinosus was moderately to severely affected in all patients and obturatorius, sartorius and gracilis muscles similarly involved in most. Other frequently affected muscles were gluteus minimus, and iliopsoas. Changes in other pelvic and thigh muscles were more variable and quadriceps and biceps femoris were relatively spared. In the lower legs gastrocnemius medialis and lateralis, as well as soleus muscles were relatively preserved, while in all other muscles the changes were moderate to severe. The homozygous proband of family J (J:II-1) had the typical phenotype with pathognomonic findings on imaging. His heterozygous parents without clinical symptoms had mild to moderate fatty degenerative changes on MRI; the father particularly in semitendinosus muscles and the mother in obturatorius muscles (figure 2).

Muscle histopathology and immunohistochemistry

Typical pathological findings were fiber-size variation and increase of internal nuclei (table 3). Cytoplasmic bodies (CBs) were observed in all samples, although in some samples only in a few fibers. Rimmed vacuolar pathology was another constant feature but rimmed vacuoles did not appear in the same fibers with CBs. CBs were present in the sections of four out of the five samples available for additional immunohistochemical evaluation, ranging from one or a few fibers to 10-15 % fibers harboring CBs. They were generally found in subsarcolemmal position, often forming subsarcolemmal rings, and were different in size, but all

displayed similar immunohistochemical features (figure 3). In particular, CBs were positive with anti-myotilin, anti-alpha B-crystallin antibodies, and contained also actin and dystrophin. Desmin was absent from the core of the bodies but sometimes positive in a thin surrounding halo and detectable in areas of myofibrillar disarray. These myofibrillar disruption areas were also present in other fibers in central position, and were positive with anti-myotilin and anti-alpha B-crystallin antibodies. However, CBs were not reactive with anti-titin antibodies, and TDP-43 and p62/SQSTM1 present in rimmed vacuoles were absent from them as well. p62/SQSTM1 also showed a dotted appearance in some hypotrophic fibers, and was positive in the areas of myofibrillar disarray between CBs. CBs did not display affinity for ubiquitin, whereas ubiquitin positivity was sometimes detected at the periphery of the bodies and in abnormal fibers as a diffuse cytoplasmic increase. The autophagosome marker LC3 labeled rimmed vacuoles but was largely absent from CBs.

Table 3	**Muscle biopsy findings**										
Patient	**Muscle**	**Age at Bx**	**Fiber size variation**	**Increase of internal nuclei**	**Fatty change**	**Fibrosis**	**Necrosis**	**Rimmed vacuoles**	**Cytoplasmic bodies**	**Other protein aggregation**	**Other**
A:I-I	Vastus lateralis	72	y	y	n	n	n	y	y	n	
A:II-3	Vastus lateralis	44	y	y	n	n	n	y	y	n	
A:III-1	Vastus lateralis	22	y	n	n	n	n	n	y	n	
B:III-3	Vastus lateralis	55	y	n	n	n	n	y	y	Y	
B:III-5	Vastus lateralis	56	y	y	n	n	n	n	y	n	
B:III-10	Rectus femoris	53	y	y	y	y	n	y	y	n	

B:III-11	Vastus lateralis	57	n	n	n	n	n	n	y (one fibre)	y	irregular NADH staining
C:IV-1	Vastus lateralis	40	y	y	n	n	n	y	y	n	
D:III-2	Vastus lateralis	50	y	y	y	y	n	y	y	n	irregular NADH staining
D:III-10	Vastus lataralis	47	y	y	y	n	n	y	y	dystrophin	Irregular NADH staining
E:II-1	NR	56	y	y	y	y	y	y	y (one fibre)	y	Congo red material, COX-neg fibres,cores phalloidin + ↑ MHC-1, ↑utrophin, groups of atrophic fibres
F:II-1	quadriceps	52	n	n	n	n	n	n	y (one fiber)	n	
G:II-2	deltoid	48	y	n	y	y	n	y	y	n	Autophagic

											vacuoles, irregular NADH staining grouped atrophy, Z-line widening and streaming
H:II-2	NR	24	y	ý	NA	NA	NA	y	y	n	Ring fibers Target fibers
H-III-2	Deltoid	20	y	y	n	y	y	y	y	Desmin, phalloidin, dys	Ring fibers Autophagic vacuoles Irregular NADH and COX staining
I:II-1	NR	57	y	y	n	n	y	y	y	n	
J:II-1	Deltoid	32	y	y	n	n	y	y	y	n	Lack of SDH and COX staining)
K:II-1	Deltoid	27	y	y	n	n	y	y	y	n	Sectorial distribution of morphological lesions

L:II-1	Deltoid	52	y	y	n	n	n	y	y	n	
L:II-2	Deltoid	56	y	y	n	n	n	y	y	EM Several vacuoles, empty or containing myeloid figures	Sectorial distribution of morphological lesions

y = yes; n = no; NR = not retrieved; EM = electron microscopy

Linkage studies

Haplotype segregation using markers at the *TTN* locus 2q31 was identified in all familial materials available. In French family (Family A) all the affected patients shared the same haplotype that was not present in any of the healthy members of the family. Finnish family (B) showed segregation of a different haplotype in the patients, which was also partially present in Swedish (C and D), one British (E), one Italian (F), and one Argentinian (G) families. All patients in these families shared a haplotype including markers D2S300 and D2S385. The shared haplotype was less than 1.3 Mb in size (less than 1.1 cM). Patients in one Italian family (J) and in two French families with Portuguese ancestry (K and L) showed segregation of yet another haplotype which included markers D2S300 and D2S385 and was less than 1.3 Mb in size (less than 1.1 cM). In Italian family J and French (Portuguese) family K both probands showed this identical short haplotype on both chromosomes with extended haplotype marker allele sharing in family K consistent with the parents being first cousins.

Exome sequencing

Exome sequencing was performed on two affected and one healthy member of French family A. When variants shared by both affected patients were filtered against dbSNP132 and the healthy member of the family, only one variant was found within the linked haplotype in the *TTN* gene. The variant g.274367C>G was located in *TTN* exon 343 and it caused one amino acid change, p.P30068R.

Sanger sequencing

Since the new mutation in French family and the previously reported HMERF A-band mutation [3,4] were both in *TTN* exon 343, all other families were screened for exon 343 and four more mutations were identified (table 4). The causative mutation was identified in every HMERF patient in our series. One of these mutations (g.274375T>C, p.C30071R) previously reported in a few Swedish and UK families [3,4] was now identified in one Finnish, one UK, one Italian, one Argentinian, and in the two Swedish families. One of the new mutations (g.274436C>T, p.P30091L) has been observed in an exome sequencing project in one single patient, but without further confirmation of its possible pathogenicity.[14] This mutation was now identified in heterozygosity in two sibs in one French family, and in homozygosity in the proband of the second French family with Portuguese ancestry, as well as in homozygosity in the proband of Italian family J. The German mutation (g.274426T>C, p.W30088R) and the new British mutation (g.274428G>C, p.W30088C) were identified by direct sequencing of the candidate region and could be directly associated because of the identical phenotype and segregation in the affected patients only. Mutations were not present in dbSNP132, 1000 Genomes or NHLBI Exome Sequencing Project databases. *TTN* exon 343 was sequenced from 102 Finnish and 96 Italian healthy controls and none of them had any of these mutations.

Table 4. Mutations identified in the families.

Family	gDNA	Protein
A	g.274367C>G	p.P30068R
B, C, D, E, F, G	g.274375T>C	p.C30071R
I	g.274426T>C	p.W30088R
H	g.274428G>C	p.W30088C
J, K, L	g.274436C>T	p.P30091L

All five mutations are missense mutations changing one amino acid in the protein. The p.P30068R mutation changes a hydrophobic amino acid to a positively charged amino acid, the p.C30071R mutation changes a small neutral amino acid to a large positively charged amino acid, the p.W30088C mutation changes a large amino acid to a small amino acid of which may affect cysteine-cysteine bindings, the p.W30088R to a charged amino acid, and the p.P30091L mutation changes a rigid amino acid to a flexible amino acid. All of the mutations are located in the same FN3 119 domain in A-band titin (figure 4).

DISCUSSION

HMERF disease was first described in single families more than 20 years ago.[6,15] Two of these families showed linkage to chromosome 2q31 locus[16]

and the first titin mutation associated with HMERF was identified in the M-band kinase domain.[2] The genetic cause in the remaining families was elusive until new studies using exome sequencing eventually identified a new dominant titin mutation in three Swedish families and in three UK families in the distal part of A-band titin.[3,4] Since both titin mutations showed the indentical pattern of muscle involvement on MRI [3,4] and histopathology, these parameters were used to re-assess two previously reported families [6,7], two of the original Swedish families without establish genetic diagnosis, and other unreported HMERF families regarding possible titin mutations.

In our larger collection of HMERF patients in 12 unrelated families from different Caucasian populations one previously reported and four novel mutations were identified. Our findings emphasize the geographically wide occurrence, the importance of titin as a causative gene of HMERF disease and particularly the role of exon 343 as a mutational hotspot region. Furthermore, this study considerably expands our understanding of the clinical presentation and provides new insight into molecular pathology.

Previous linkage studies in French family A suggested that the titin gene locus was excluded.[17] New muscle MRI studies lead to reclassification of some of the individuals in the family. Because of the identical pattern of muscle involvement with the other HMERF families, besides exome sequencing, also A-band titin Sanger sequencing was performed resulting in the novel P30068R mutation to be

identified. As expected, after reclassification, new linkage studies showed that the disease was indeed linked to the titin locus.

The C30071R mutation, now identified in six families from five different populations, and previously reported in three Swedish and three British families, [3,4] is the most frequent mutation in HMERF so far. The mutation mediates a dominant effect with full penetrance. The age of onset with this mutation varies from 16 to 53 years being usually after age 30-35. All these families share a genomic region less than 1.3 Mb in size suggesting an ancestral founder. The P30068R, W30088C and W30088R mutations are novel and occur in single families with dominant inheritance and full penetrance. The age of onset and disease severity in patients with these new mutations is within the range of the phenotypes with the C30071R mutation. However, the fifth mutation identified, P30091L, in families J, K and L, shows a different penetrance and is neither completely dominant nor completely recessive. The parents of the probands in families J and K showed no signs of muscle disease and therefore the probands appeared to be sporadic patients. However, because the same mutation occurred in heterozygous state in family L, although with a milder phenotype, additional studies were needed. In families J and K the homozygous P30091L probands (J:II-1 and K:II-1) have a more severe disease with earlier onset and more rapid progression than the heterozygous patients of family L. Furthermore, in family J the heterozygous parents had definite signs of subclinical muscle disease on MRI with fatty degenerative change in HMERF typical muscles. In family L, the

heterozygous patients had respiratory insufficiency although at much later age and without clear limb muscle weakness. Their muscle MRI findings were also milder. Since this novel P30091L mutation may or may not cause clinically manifest disease in heterozygous state, and it causes a clearly more severe phenotype in homozygosity, we prefer to call this mutation semi-recessive or semi-dominant.

The most typical feature of HMERF is respiratory failure at mid-adulthood in an ambulant patient at the first visit. In the case of absent or undiagnosed respiratory symptoms, more specific clues to enable correct diagnosis can be obtained from muscle imaging and histopathology. Our results on muscle imaging confirm the characteristic and so far pathognomonic pattern of muscle involvement in this disease.[3-5,7]

Muscle pathology is another key to diagnosis. CBs, a hallmark of the disease, were observed in all samples. However, in some biopsies they were present in one or a few fibers only and could be easily overlooked in the first reading. Rimmed vacuolar pathology was another consistent finding but these focal degenerative changes did not occur in the same fibers that contained CBs. Myofibrillar changes, Z-disk alterations and CBs that bind phalloidin, a marker for F-actin, and also contain desmin, myotilin, alphaB-crystallin, VCP, and dystrophin have been demonstrated in previous HMERF studies.[1,3,4,7,17,18] Moreover, p62/SQSTM1 containing cytoplasmic inclusions has been observed.[2] In our new series of immunohistochemistry studies including three different mutations (two dominant

and the semi-recessive) we were able to detail the different types of accumulations and aggregations in three different cytoplasmic abnormalities: 1) CBs contained dystrophin, myotilin, actin, and alphaB-crystallin; 2) rimmed vacuoles contained ubiquitin, TDP-43, p62/SQSTM1, and LC3 positive components; and 3) in regions with myofibrillar disarray between cytoplasmic bodies or centrally in some fibers the expression of desmin, ubiquitin and p62/SQSTM1 was increased. Surprisingly, the most compelling finding was that CBs were not reactive for titin.

The spectrum of different human titinopathies is growing but none of the other described forms [8-10,19] show the unique pathology of fibers with multiple CBs apart from rimmed vacuolated fibers, which are both caused by the mutant titin protein. Immunohistochemistry findings indicate that the mutant protein itself is not aggregating and not seeding the CBs but seems to trigger the aggregation of other sarcomeric proteins. The first titin mutation causing HMERF in the kinase domain of M-line titin leads to disruption of the kinase associated protein complex with Nbr1, p62/SQSTM1 and MuRF2.[2] This was shown to lead to the mislocalisation of the multiprotein complex, which is involved in ubiquitin-mediated regulation of transcription, protein turnover via the ubiquitin proteasome system and autophagy-mediated protein turnover via the interaction with ubiquitinated proteins and LC3 of p62/SQSTM1 and nbr1.[20] However, this kinase mutation has later been shown to occur as a variant (rs 140319117) with a frequency of 0.0018 among European Americans that warrants comprehensive new assessment of the mechanism. The A-band region of titin has a central role in

controlling myosin thick filament positioning and function,[21] but the specific links of this particular FN3 119 domain to dysregulated protein re-cycling and autophagy turnover remain to be clarified.

Five different mutations causing HMERF disease in various populations identified in exon 343 of *TTN* makes this a first-step target for molecular diagnosis and the range of mutations indicates that HMERF disease is not extremely rare and not restricted to Scandinavian population.

Acknowledgements

Dr. R. Carlier and Dr. N. Romero, Myology Institute collected data and provided general advice. Pr. D. Hénin, Claude-Bernard Bichat Hospital, Department of Pathology, Genethon and AFM collected data and provided general advice. Dr. E. Bertini, Department of Neurosciences, Unit of Molecular Medicine, Bambino Gesu Children's Hospital collected data and provided general advice. Dr. E Ricci Institute of Neurology, Catholic University School of Medicine, Rome, Italy collected data and provided general advice. Dr. Maria Saccoliti MD, Department of Neuropathology, Institute for Neurological Diseases. FLENI, Buenos Aires, Argentina performed electron microscopy. MSc Maritta Kariniemi, Neuromuscular Research Unit, Tampere Finland assisted with the preparation of Figure 3.

Contributors

Study conception and design: Oldfors, Udd.

Acquisition of data: Palmio, Evilä, Chapon, Tasca, Brådvik, Eymard, Echaniz-Laguna, Laporte, Kärppä, Mahjneh, Quinlivan, Damian, Berardo, Taratuto, Bueri, Tuerk, Gölitz, Chevessier, Norwood, Schröder. Edström, Oldfors, Hackman, Udd.

Analysis and interpretation of data: Palmio, Evilä, Chapon, Tasca, Xiang, Eymard, Laforêt, Tommiska, Raivio, Sewry, Hedberg, Schröder. Edström, Oldfors, Hackman, Udd.

Drafting of the manuscript: Palmio, Evilä, Hackman, Udd

Critical revision of the manuscript for important intellectual content: Chapon, Tasca, Xiang, Brådvik, Eymard, Echaniz-Laguna, Laporte, Kärppä, Mahjneh, Quinlivan, Laforêt, Damian, Berardo, Taratuto, Bueri, Tuerk, Gölitz, Chevessier, Sewry, Norwood, Hedberg, Schröder. Edström, Oldfors, Hackman, Udd.

Obtained funding: Udd.

Administrative, technical, and material support: Hackman, Udd.

Study supervision: Hackman, Udd.

.

Competing interests

Dr. Laforêt has received grants and honorarium from Genzyme Company. He is a member of the Pompe advisory Board for Genzyme. Drs. Tommiska and Raivio have received funding from Academy of Finland. All other authors report no conflicts of interest.

Patient consent

All participants provided appropriate consent according to the Helsinki declaration**.**

Ethics approval

Systemic collection of clinical data and all genetic studies in Finland were approved by the Ethics committee of Tampere University Hospital, Finland.

Funding

This study was supported by the Folkhälsan Research Foundation, the Academy of Finland, the Sigrid Jusélius Foundation, the Liv o Hälsa Foundation, and Tampere University Hospital Research Funds (Dr. Udd)

REFERENCES

1. Edström L, Thornell LE, Albo J, et al. Myopathy with respiratory failure and typical myofibrillar lesions. J Neurol Sci 1990;**96**:211-28.

2. Lange S, Xiang F, Yakovenko A, et al. The kinase domain of titin controls muscle gene expression and protein turnover. Science 2005;**308**:1599-603.

3. Pfeffer G, Elliott HR, Griffin H, et al. Titin mutation segregates with hereditary myopathy with early respiratory failure. Brain 2012;**135**:1695-713.

4. Ohlsson M, Hedberg C, Brådvik B, et al. Hereditary myopathy with early respiratory failure associated with a mutation in A-band titin. Brain 2012;**135**:1682-94.

5. Birchall D, von der Hagen M, Bates D, et al. Subclinical semitendinosus and obturator externus involvement defines an autosomal dominant myopathy with early respiratory failure. Neuromuscul Disord 2005;**15**:595-600.

6. Tasca G, Mirabella M, Broccolini A, et al. An Italian case of hereditary myopathy with early respiratory failure (HMERF) not associated with the titin kinase domain R279W mutation. Neuromuscul Disord 2010;**20**:730-4.

7. Chapon F, Viader F, Fardeau M, et al. Familial myopathy with "cytoplasmic

body" (or "spheroid") type inclusions, disclosed by respiratory insufficiency (in French). Rev Neurol (Paris) 1989;**145**:460-5.

8. Gerull B, Gramlich M, Atherton J, et al. Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. Nat Genet 2002;**30**:201-4.

9. Hackman P, Vihola A, Haravuori H, et al. Tibial muscular dystrophy is a titinopathy caused by mutations in TTN, the gene encoding the giant skeletal-muscle protein titin. Am J Hum Genet 2002;**71**:492-500.

10. Carmignac V, Salih MA, Quijano-Roy S, et al. C-terminal titin deletions cause a novel early-onset myopathy with fatal cardiomyopathy. Ann Neurol 2007;**61**:340-51.

11. Herman DS, Lam L, Taylor MR, et al. Truncations of titin causing dilated cardiomyopathy. N Engl J Med 2012;**366**:619-28.

12. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics 2009;**25**:1754-60.

13. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data.

Genome Res 2010;**20**:1297-303.

14. Vasli N, Bohm J, Le Gras S, et al. Next generation sequencing for molecular diagnosis of neuromuscular diseases. Acta Neuropathol 2012;**124**:273-83.

15. Edström L, Thornell LE, Eriksson A. A new type of hereditary distal myopathy with characteristic sarcoplasmic bodies and intermediate (skeletin) filaments. J Neurol Sci 1980;**47**:171-90.

16. Nicolao P, Xiang F, Gunnarsson LG, et al. Autosomal dominant myopathy with proximal weakness and early respiratory muscle involvement maps to chromosome 2q. Am J Hum Genet 1999;**64**:788-92.

17. Xiang F, Nicolao P, Chapon F, et al. A second locus for autosomal dominant myopathy with proximal muscle weakness and early respiratory muscle involvement: a likely chromosomal locus on 2q21. Neuromuscul Disord 1999;**9**:308-12.

18. Chinnery PF, Johnson MA, Walls TJ, et al. A novel autosomal dominant distal myopathy with early respiratory failure: clinico-pathologic characteristics and exclusion of linkage to candidate genetic loci. Ann Neurol 2001;**49**:443-52.

19. Udd B, Vihola A, Sarparanta J, et al. Titinopathies and extension of the M-line

mutation phenotype beyond distal myopathy and LGMD2J. Neurology 2005;**64**:636-42.

20. Waters S, Marchbank K, Solomon E, et al. Interactions with LC3 and polyubiquitin chains link nbr1 to autophagic protein turnover. FEBS Lett 2009;**583**:1846-52.

21. Bucher RM, Svergun DI, Muhle-Goll C, et al. The structure of the FnIII Tandem A77-A78 points to a periodically conserved architecture in the myosin-binding region of titin. J Mol Biol 2010;**401**:843-53.

Figure 1. Pedigrees of the families.

DNA was collected from individuals marked with an asterisk *. Filled symbols are affected and open symbols unaffected family members. Grey symbols represent members with subclinical manifestations.

Figure 2. Muscle imaging findings of proband and his parents in family J

Muscle imaging of the homozygous proband (J:II-1) shows fatty replacement of iliopsoas, abdominal and obturatorius muscles and all gluteal muscles are moderately involved (A). At the thigh level semitendinosus and adductor magnus are the most involved as are extensor hallucis and digitorum longus, tibialis posterior and peroneus longus muscles on the lower legs (B). MRI in his heterozygous mother shows particular involvement of obturatorius muscle (C) and selective involvement of semitendinosus muscle in his father (D).

Figure 3. Immunohistochemical findings

Immunohistochemical evaluation shows cytoplasmic bodies on Gomori trichrome (A) and hematoxylin and eosin (B), frequently in subsarcolemmal position. Cytoplasmic bodies are reactive for alphaB-crystallin (C), myotilin (D) and actin (E) but not for titin (F). p62/SQSTM1 (G) and desmin (H) are mostly absent from the core of the cytoplasmic bodies but may show increased expression in the surrounding cytoplasm and in other areas of myofibrillar disarray. Antibodies against the following proteins were applied: ubiquitin (DakoCytomation), dystrophin (Novocastra NCL-DYS-2), desmin (Biogenex, USA), myotilin (Novocastra, UK), alpha B-crystallin (Novocastra, UK), actin (Invitrogen, CA, USA), titin (Novocastra, UK), p62/SQSTM1 (Santa Cruz Biotechnology, Inc.) and data in the text for TDP-43 (Proteintech), LC3 (Novus Biologicals). Immunohistochemical stainings were performed on the BenchMark (Roche Tissue Diagnostics/Ventana Medical Systems Inc.) immunostainer using the official protocol of the BenchMark immuno-stainer, visualized with a peroxidase based detection kit. B-H are serial sections.

Figure 4. Titin mutations

Sanger sequencing first confirmed the new French mutation (A). Four other mutations (B-E) were found when all HMERF families were sequenced. All found mutations are located in the same FN3 119 domain of A-band titin (F, G). All substituted amino acids are conserved (H, UCSC genome browser).

Titin references: GenBank: AJ277892, UniProt: Q8WZ42

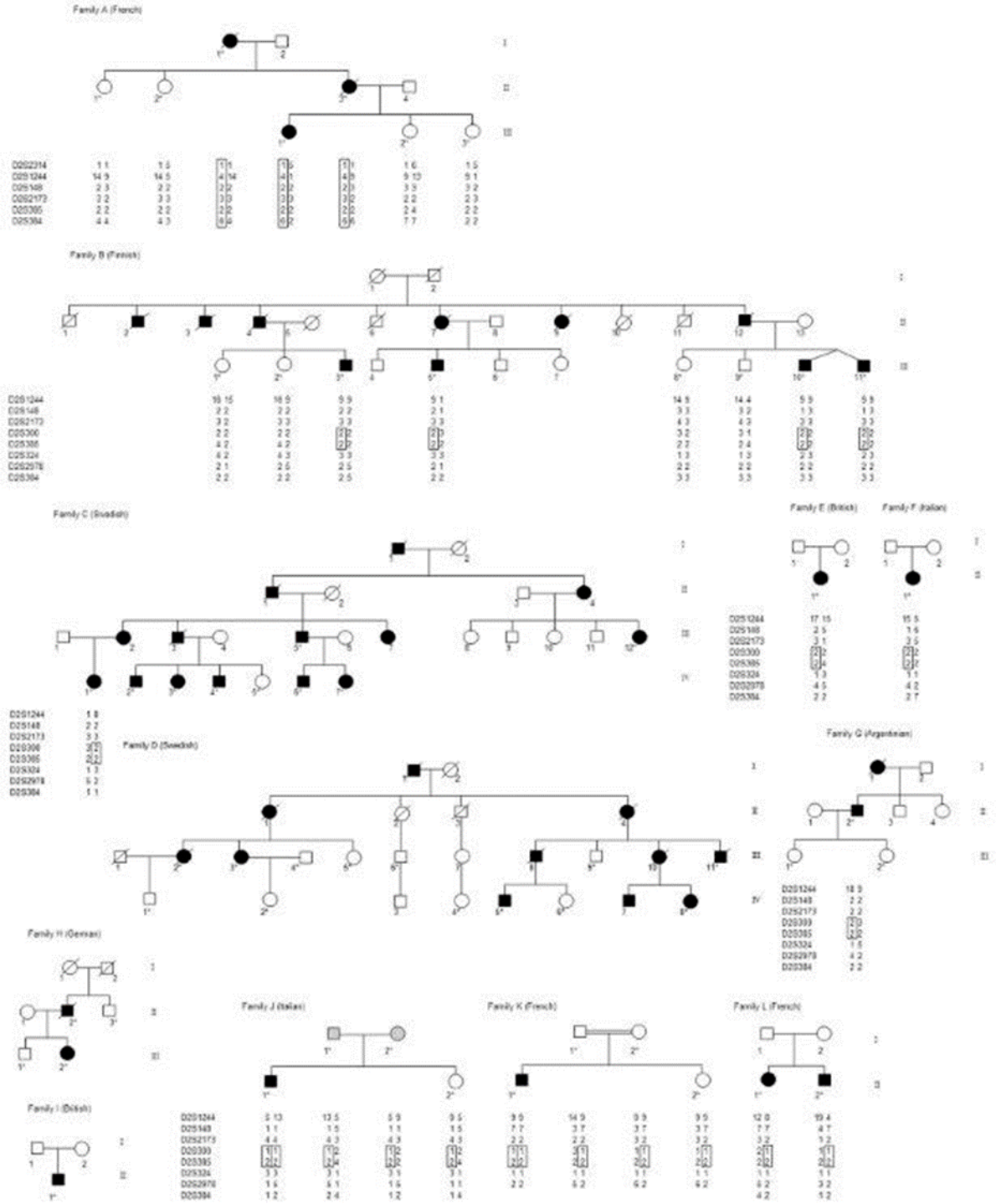
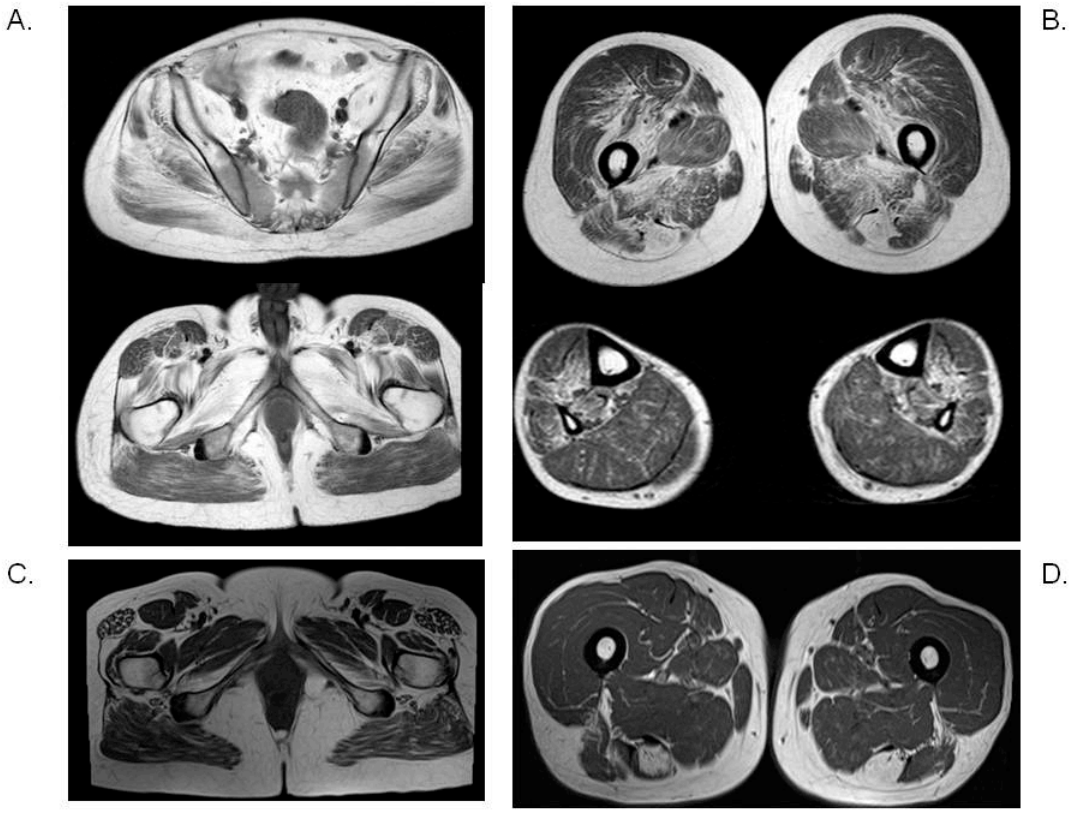
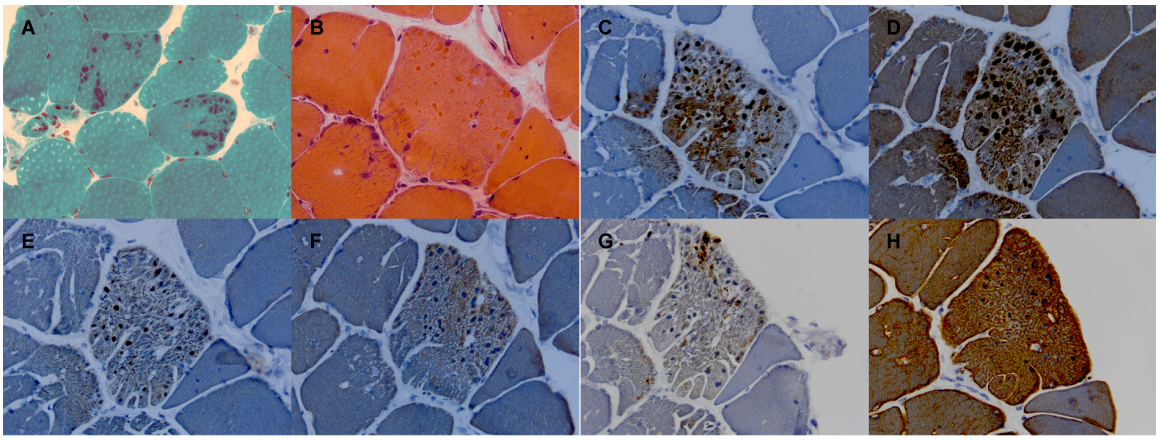
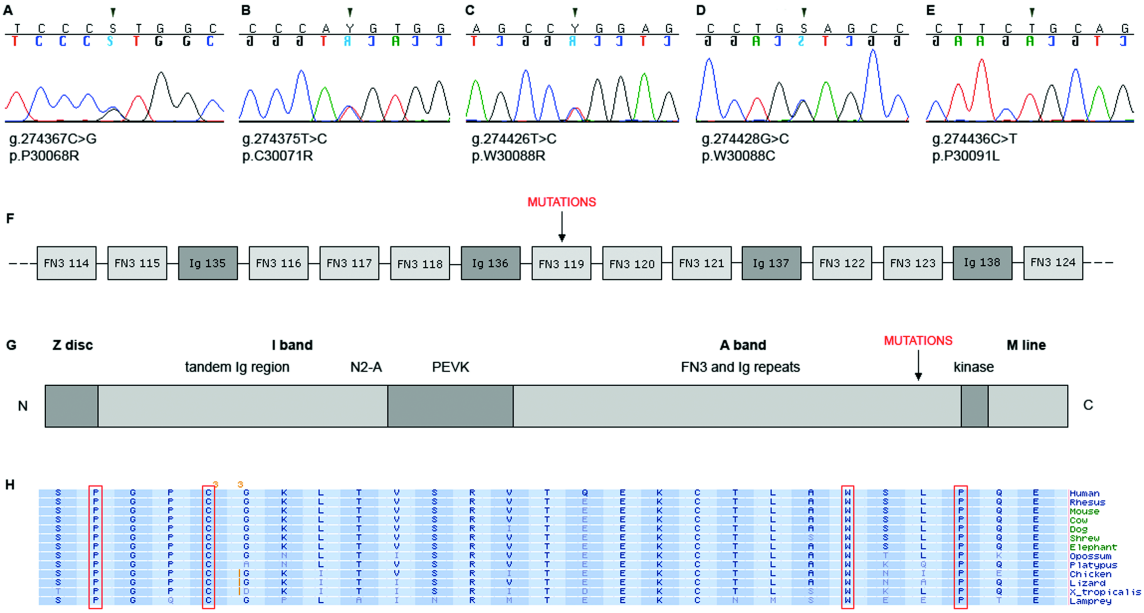

Family A (French)
Family B (Finnish)
Family C (Swedish)
Family D (Swedish)
Family E (British)
Family F (Italian)
Family G (Argentinian)
Family H (German)
Family I (British)
Family J (Italian)
Family K (French)
Family L (French)

Figure 2




A
B
C
D
E
F
G
H


A
g.274367C>G
p.P30068R
B
g.274375T>C
p.C30071R
C
g.274426T>C
p.W30088R
D
g.274428G>C
p.W30088C
E
g.274436C>T
p.P30091L
F
MUTATIONS
FN3 114
FN3 115
Ig 135
FN3 116
FN3 117
FN3 118
Ig 136
FN3 119
FN3 120
FN3 121
Ig 137
FN3 122
FN3 123
Ig 138
FN3 124
G
Z disc
I band
tandem Ig region
N2-A
PEVK
A band
FN3 and Ig repeats
MUTATIONS
kinase
M line
N
C
H
Human
Rhesus
Mouse
Cow
Dog
Shrew
Elephant
Opossum
Platypus
Chicken
Lizard
X_tropicalis
Lamprey