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JĀNIS STĀVUSIS

MOLECULAR MECHANISMS UNDERLYING RARE NEUROMUSCULAR DISEASES

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Supervisor: Dr. med. Baiba Lāce

Reviewers:

1) Asoc. prof., Dr. biol. Jānis Kloviņš, Biomedical Research and Study Centre; University of Latvia, Riga, Latvia
2) Asist. prof., Dr. med. Viktorija Ķēniņa, Riga Stradiņš University, Riga, Latvia
3) Asoc. prof., Dr. biol. Julien Ochala, University of Copenhagen, Copenhagen, Denmark

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Chairman of the Doctoral Committee ____________/Kaspars Tārs/

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ABSTRACT

The term neuromuscular disorders refers to a wide range of diseases, caused by deficits in the motor unit. The focus of this work is the neuromuscular disorder subgroup associated with defects in muscle fibers themselves and arising from genetic causes - muscular dystrophies. They are a group of genetically inherited degenerative disorders of muscle, sharing clinical features of progressive muscle weakness and dystrophic pathological appearance on muscle biopsy. First described as a separate entity in 1852, muscular dystrophies in total are estimated to affect 1:5000 newborns.

The complexity and variability of muscular dystrophy clinical manifestation as well as underlying molecular mechanisms has represented a challenge for accurately diagnosing patients since their discovery, and still does. In the era of molecular genetic research, a large number of genes associated with muscular dystrophies have been identified, with more very rare variants being identified every year. However, it is crucial for patients to receive precise molecular diagnosis, as this allows for an individual-tailored treatment that increases life quality and expectancy, with a potential of having curative treatment in the future.

This work was initiated with the aim to aid and advance neuromuscular disorder diagnostics in Latvia, through research.

Initial data showed that for caveolinopathies the frequency of mutations in the patient group is in the expected 1-2% range, with variant frequencies in the general population not differing from data available in public databases. Interestingly, we found a person possibly suffering from HyperCK-emia in the general population. Our data did not indicate involvement of *CAV3* variants in the group of cardiomyopathy patients, leading to the hypothesis that in our target population cardiomyopathy is a rare phenotypic trait of *CAV3* pathogenic variants.

Several diagnostic methods have been developed and used over the period of more than ten years. Data from these confirmed the theory that calpainopathies seem to be the most common group of rare muscle diseases in Latvia, even though the variant believed to be the most common in Eastern Europe - c.550delA is not common in our population, it is, however, the most common in Lithuania. General population screening for carriers of this variant showed similar results. Furthermore, none of the variants in *ANO5* or any of the sarcoglycan genes that have high allele frequencies in other European countries were found in our population.

The Sanger sequencing of certain disease associated gene regions as well as massive parallel genotyping that were initially developed have been discontinued in favor of the much more cost and result efficient next generation sequencing. The main issue that the previous technologies faced was the high level of heterogeneity in regards to both the neuromuscular

disorders and our population, leading to decreased diagnostic yield when testing only certain genes and variants.

In the last years we have introduced research-level next generation sequencing in neuromuscular disorder diagnostics, and by doing so have increased the yield of positive diagnosis considerably. The full data analysis process for these cases can be very time consuming, and sometimes additional factors, like genes with no known function or variants with many conflicting interpretations make it even more difficult. According to the American College of Medical Genetics and Genomics guidelines, a variant in the *COL6A3* gene c.7447A>G, (p.Lys2483Glu) was reclassified as pathogenic, based on criteria, like reduced COL6 secretion and intracellular retention, association with phenotype and the typical muscle magnetic resonance imaging results, associated with COL6 related myopathy. We described the patients we found and concluded that homozygous carriers of this variant exhibit a mild collagen VI-related myopathy phenotype.

A new phenotype, associated with domain-specific mutations in the *MYBPC1* gene was described. The phenotype is characterized by mild myopathy, skeletal deformities and tremor. The tremor was shown to be lacking a central nervous system related cause and therefore hypothesized to be of myogenic origin. Based on molecular modeling and functional testing, the possible disease-causing mechanism was described. Additional cases have now been described, further confirming our hypothesis, with more functional research ongoing, aimed at better describing the underlying molecular mechanism. The phenotype has now been termed myotrem.

We have continually aided rare neuromuscular disease diagnostics in Latvia through research, gradually evolving, to the point where now we have brought the field up to a world-class standard.

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LIST OF ABBREVIATIONS

ACMG	American College of Medical Genetics and Genomics
ATP	adenosine triphosphate
Ca^{2+}	calcium ions
CAPN3	calpain 3
Cav3 / CAV3	caveolin 3
CK	creatine phosphokinase
CMT	Charcot-Marie-Tooth disease
Col6	collagen type 6
COL6A3	collagen type 6 alpha 3 chain
COOH	carboxyl group
DA	distal arthrogryposis
DCM	dilated cardiomyopathy
DM	distal myopathy
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DUX4	double homeobox 4 protein
DYSF	dysferlin
EMG	electromyography
ERDF	European Regional Development Fund
FHL1	four and a half LIM domains 1 protein
FKRP	fukutin-related protein
Fn-III	fibronectin-III
FSHD	Facioscapulohumeral muscular dystrophy
FVC	forced vital capacity
GATK	Genome analysis toolkit
HCM	hypertrophic cardiomyopathy
HMM	heavy meromyosin
Ig	immunoglobulin
LGMD	limb-girdle muscular dystrophy
LMM	light meromyosin
LMNA	lamin A/C
MPTP	mitochondrial permeability transition pore protein
MRI	magnetic resonance imaging

mRNA	messenger ribonucleic acid
MyBP-C / MYBPC	myosin binding protein C
NGS	next-generation sequencing
NH_2	amino group
NIH	National Institutes of Health
NMD	neuromuscular disorders
nNOS	neuronal nitric oxide synthase
PCR	polymerase chain reaction
POMT1	protein O-mannosyltransferase 1
RMD	rippling muscle disease
ROS	reactive oxygen species
SCARMD	severe childhood autosomal recessive muscular dystrophy
SMA	spinal muscular atrophy
sMyBP-c / MYBPC1	myosin binding protein C1
SNP	single nucleotide polymorphism
TH	triple helical domain
TMEM43	transmembrane protein 43
TRIM72	tripartite motif containing protein 72
VEP	Variant effect predictor
VQSR	variant quality score recalibration
VWA	Von Willebrand factor type A domain

INTRODUCTION

In the modern era, when we have a huge, constantly growing pool of reference data and the world uses next-generation sequencing (NGS) as a first-line diagnostic tool, due to the constant rate of technological development, it is sometimes hard to remember the time not that long ago, when the chain termination sequencing was seen as the go-to tool when dealing with rare neuromuscular disease (NMD) diagnostics and research.

The aim of this work was to aid and advance neuromuscular disorder diagnostics in Latvia, through research. In order to reach this aim, the following tasks were set:

- To determine the frequency and distribution of the many various forms of neuromuscular disorders in the population of Latvia, as well as the most common forms of the disease and the associated pathogenic variants.
- Using the results of scientific research, to guide and develop rational diagnostic testing for patients suffering from neuromuscular disorders in Latvia.
- To determine the pathogenic potential of possible disease associated variants, based on available information, variant segregation, molecular testing and modeling.
- To characterize a previously unknown neuromuscular disease phenotype and elucidate the underlying molecular mechanism, thus gaining information on genotype-phenotype correlations.

When the initiative to improve neuromuscular disorder diagnostics in Latvia began in 2010, with the European Regional Development Fund (ERDF) project "New tests for the diagnosis of inherited neuromuscular diseases", the main tool that was readily available was the Sanger sequencing of full genes or specific regions of interest within a certain gene, also the real-time quantitative PCR. It was and still is a robust and fairly cheap method for detection of known or common disease-causing variants. It is due to this that it is still considered a standard in many labs for validation of relevant NGS findings. The project was mainly focused on the muscular dystrophy part of NMDs. The success of finding a variation at this point most often correlated with the ability of medical doctors to precisely diagnose patients and predict the exact form of NMD, allowing us to choose and test only known disease associated genes. This resulted in the unsurprisingly low percentage of cases with confirmed genetic cause in Latvia. Between the years 2002 and 2009 there were 25 confirmed cases of Spinal muscular atrophy (SMA), 11 cases of Charcot-Marie-Tooth disease (CMT) 1A, 9 cases of Duchenne or Becker muscular dystrophy, 3 cases of Facioscapulohumeral muscular dystrophy (FSHD), 3 cases of Limb-girdle muscular dystrophy (LGMD) and a few cases of various other muscular dystrophies (unpublished data).

Not long after the first initiative, in 2012, one of the first massive parallel genotyping platforms – Illumina's Golden Gate was introduced and first used in diagnostics of NMDs for LGMD patients from Latvia and Lithuania. This collaboration with specialists from our neighboring country resulted from attempts to pool patient samples, which would allow us to look at a larger patient population, and to optimize resources. The testing was done in the Latvian Biomedical Research and Study Centre, as a part of the same ERDF project ("New tests for the diagnosis of inherited neuromuscular diseases"). The platform enabled genotyping of a large number of known sequence variants in genes *DYSF*, *FKRP*, *ANO5*, *CAPN3*, *SGCA*, *SGCB*, *SGCD*, *SGCG* and *CLCN1*, for a large number of patients (60 patients from Latvia and Lithuania, as well as more than 200 controls) in a short period of time. In conjunction with custom made genotyping panels this seemed to be a good alternative or an addition to previous methods. However, this method also had its inherent flaws. It works on a probe-like principle, detecting only previously determined variants, which is why for our needs the most common variants associated with LGMD were chosen. Furthermore, this approach meant that only variants with a certain genetic distance between them could be included, therefore not allowing detection of several variants that are located next to each other, for example, in mutation hotspots. In addition, library preparation was tedious and error-prone and the results were not always uniform or of good quality. Unfortunately, in a few years' time this technology was already outdated.

The onset of the NGS era changed things drastically both worldwide and in Latvia as well. The possibility to sequence all coding regions or even the whole genome came with the promise of an increased probability of detecting rare disease-causing variants on an unprecedented scale. This instantly rendered the genotyping technology we used previously inferior due to costs and the amount of data gathered from a single person.

We, however, were not able to start massive sequencing at that point, mostly due to lack of local practical knowledge and resources. For a number of years data gathering and analysis was done in collaboration with other institutes or in the framework of international projects, like the NIH research laboratories in the USA and the MYO-SEQ project from England. This meant that each year only a few selected patient samples could be sent for this new sequencing. However, over time local capacity has been built up to allow patient analysis and research, using the entire power granted by the next-generation sequencing.

Currently geneticists in Latvia have the possibility to send NMD patient samples to one of the private laboratories that provide international services for exome or panel sequencing, if deemed necessary. Alternatively, it is possible to enroll them in local projects, aimed at diagnostics and research of rare diseases with unknown underlying genetic cause, within the

framework of ERDF projects “The determination of rare inherited diseases' causative mechanisms using whole genome sequencing approach” and “Functional and genetic research of rare unidentified neuromuscular disorders”. Sanger sequencing, however, is still used for result validation or small-scale testing, for example, carrier status confirmation for the extended family members.

The current range of diagnostic tools has allowed many more rare neuromuscular disease patients to get a precise diagnosis, when compared to the beginning of the initiative. Even so, there is still a notable percentage of cases where the underlying cause is not identified, with data analysis and variant annotation being the biggest hurdles. During the last years we have, however, been able to move even further, starting to shift from purely sequencing and DNA level research to more functional testing of found variants with unknown or conflicting pathogenicity data.

1. LITERATURE OVERVIEW

The term neuromuscular disorders (NMD) refers to a wide range of diseases, caused by deficits in the motor unit, that consists of motor neuron, nerve root, peripheral nerve, myoneural junction and muscles (Reed 2002). These diseases can be both inherited and acquired, with the classical way of classifying them being by the affected part of the motor unit. The first group is associated with the involvement of the peripheral motor neuron (infantile spinal amyotrophy, enterovirus infections (poliomyelitis)), the second - with involvement of nerve roots and peripheral nerves (hereditary motor-sensitive polyneuropathies (Charcot-Marie-Tooth type I and Déjerine-Sottas type III), Guillain-Barré syndrome) and the third - with involvement of the myoneural junction (congenital myasthenic syndrome, severe myasthenia and botulism). The fourth and final group and the focus of this work is associated with defects in muscle fibers themselves. The same as all the others, this group includes disorders arising from both genetic (congenital muscular dystrophy, progressive muscular dystrophy, myotonic dystrophy, congenital myopathies and metabolic myopathies) and acquired causes (myositis) (Rowland and McLeod 1994; Reed 2002).

1.1. Muscular dystrophies

Muscular dystrophies are a group of genetically inherited degenerative disorders of muscle, sharing clinical features of progressive muscle weakness and dystrophic pathological appearance on muscle biopsy (Emery 2002). Muscular dystrophy as a separate entity was first described in 1852 (Meryon 1852; Kirschner and Lochmüller 2011). In 1861 Duchenne in the second volume of his book "Paraplégie Hypertrophique de l'enfance de cause cérébrale" described a boy with a muscular dystrophy that now carries his name. This began a ripple in the medical community and eventually led to many new discoveries (Longo et al. 2011). Approximately 40 years later F. Batten described the first congenital muscular dystrophy (Kirschner and Bönnemann 2004). In the era of molecular genetic research, more than 40 genes associated with muscular dystrophies have been identified, with more very rare variants being identified every year (Kaplan and Hamroun 2015, 2016). The disorders have been classified in two groups - conditions with early onset (congenital muscular dystrophies, affecting patients at birth or even before birth) and later-onset conditions (limb girdle muscular dystrophies), in reference to acquisition of ambulation. Most common variants are Duchenne muscular dystrophy, Becker muscular dystrophy, Emery–Dreifuss muscular dystrophy, and facioscapulohumeral muscular dystrophy, all of them being recognized and described long before other muscular dystrophies, due to their unique clinical features or high incidence, or

both (Mercuri et al. 2019). Most of the patients mention muscle weakness as the main symptom, however decreased functionality of one or more organ systems, including cardiac, digestive and respiratory, can also adversely affect the quality of life (Zivkovic and Clemens 2015). The average incidence of all muscular dystrophies is predicted to be 1:5000 newborns (Theadom et al. 2014).

The main protein classes associated with muscular dystrophies are extracellular matrix and basement membrane proteins, sarcolemma-associated proteins, enzymes or proteins with putative enzymatic function, nuclear membrane proteins, sarcomeric proteins, endoplasmic reticulum proteins, as well as others (Mercuri et al. 2019).

Congenital muscular dystrophies are most commonly associated with mutations in proteins in the extracellular matrix, external membrane proteins or enzymes involved in their post-translational modification (Mercuri et al. 2019). The most common of them being pathogenic variants in collagen 6, laminin 211 (formerly named merosin) and the cellular receptor α-dystroglycan (Urciuolo et al. 2013).

The major part of sarcolemma-associated proteins is composed by the dystrophin-associated glycoprotein complex (dystrophin, sarcoglycans and dystroglycan) (Figure 1). Mutations in these give rise to the Duchenne and Becker muscular dystrophies (dystrophin) and limb girdle muscular dystrophies known as sarcoglycanopathies (four sarcoglycan genes) (Mercuri et al. 2019). The dystrophin-associated glycoprotein complex has a shock-absorbing function between the cytoskeleton and the extracellular matrix, providing stabilization of the muscle fibers against the mechanical forces of muscle contraction (Mercuri et al. 2019). A functionally related group of sarcolemmal proteins are dysferlin and anoctamin 5, involved in the repair process of the sarcolemma (Han and Campbell 2007; Bolduc et al. 2010; Sharma et al. 2010; Han 2011; Griffin et al. 2016). Dysferlin interacts at the sarcolemma with caveolin 3, part of the caveolar formation mechanism, all three being associated with their respective forms of limb girdle muscular dystrophies (Hernández-Deviez et al. 2008; Pradhan and Prószyński 2020).

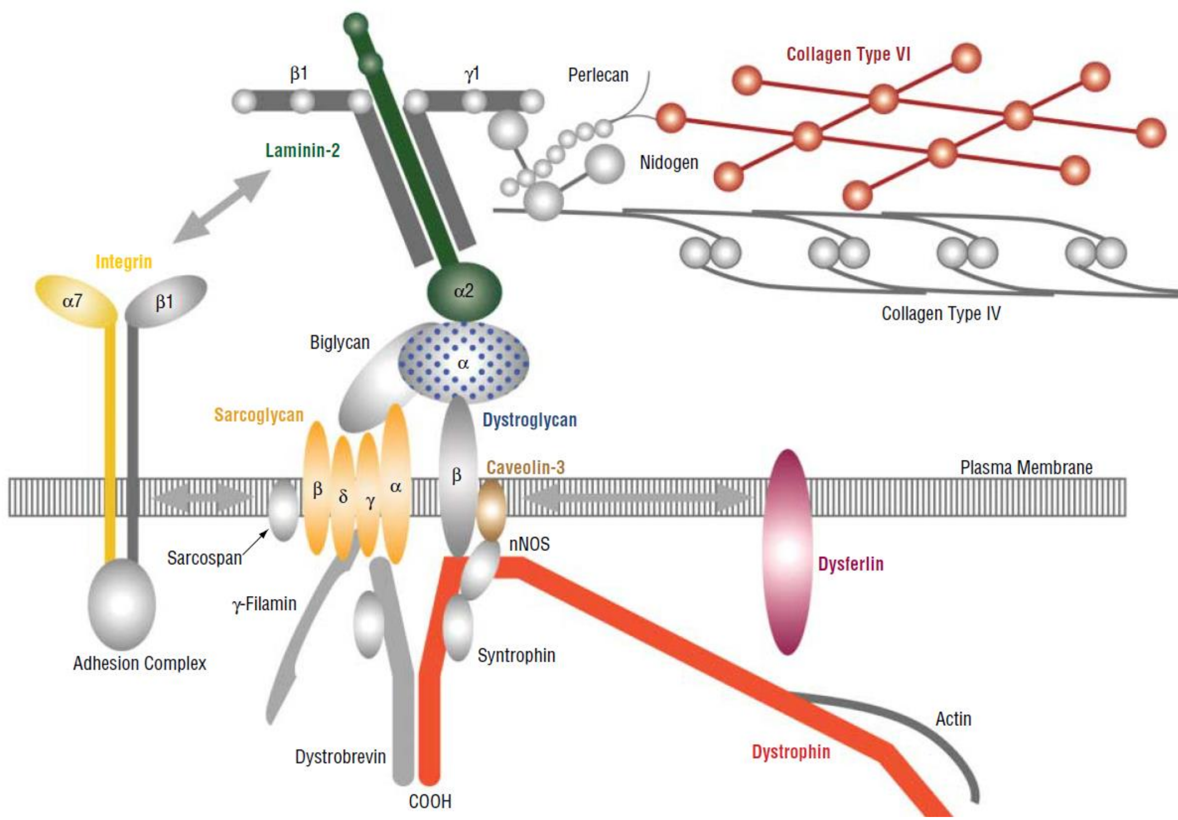

Figure 1. Muscular dystrophy relevant proteins (known MD associated proteins are shown in color). Figure adapted from Kirschner et al. 2004.

From proteins associated with muscular dystrophies, that are enzymes or have enzymatic function, ones responsible for proper glycosylation of α-dystroglycan are the most well-known ones, resulting in conditions called dystroglycanopathies (Muntoni et al. 2002, 2011). Currently there are 18 such genes known, mutations in them resulting in dystroglycanopathy. The disease can manifest with a range of pathological features, ranging from limb girdle muscular dystrophy phenotypes to congenital muscular dystrophy with structural brain involvement (Godfrey et al. 2007a, 2011; Mercuri et al. 2009). Another protein not associated with α-dystroglycan but having an enzymatic function and a role in the development of LGMD is the Ca^{2+}-activated neutral protease calpain 3 (Richard et al. 1995).

Nuclear envelope protein (lamin A/C, emerin, nesprin 1 and 2, and TMEM43 (LUMA)) mutations are resulting in a progressive muscular dystrophy phenotype known as Emery–Dreifuss muscular dystrophy (Bonne et al. 1999, 2000). Interestingly, mutations in these genes can give rise to a great variety of phenotypes, suggesting specific roles for specific protein domains. For example, pathogenic variants in lamin A/C are currently associated with eleven different phenotypes: dilated cardiomyopathy 1A; Charcot-Marie-Tooth disease (type 2B1); Emery-Dreifuss muscular dystrophy (types 2 and 3); heart-hand syndrome (Slovenian type); Hutchinson-Gilford progeria; lipodystrophy (type 2); Malouf syndrome; mandibuloacral

dysplasia; congenital muscular dystrophy and lethal restrictive dermopathy (Amberger et al. 2019).

Even though several disorders stemming from mutations in sarcomeric proteins are classified as non-dystrophic congenital myopathies, recent data has shown sarcomeric protein involvement in dystrophic muscle disorders, examples being titin and various myosin proteins. It is interesting to note that in this case the associated disease gives rise to distal distribution of muscle weakness (Udd 2011, 2012).

There are also other proteins, associated with muscular dystrophies, that do not fit in any of the previous categories - transport proteins and proteins expressed in both endoplasmic reticulum and the sarcolemma (Bögershausen et al. 2013; Schindler et al. 2016). Furthermore, one of the most common autosomal dominant adult muscular dystrophies - facioscapulohumeral muscular dystrophy is associated with dysregulation of transcriptional factor DUX4 (Lemmers et al. 2010).

The last decade has seen a rapid increase of research into different cures for muscular dystrophies, with few already available for patients carrying specific variants. However, the vast majority of them are still in pre-clinical research. Additionally, researchers and medics are trying to move from a variant specific approach to developing more generalized therapies. It also needs to be taken into consideration that for some patients there are disease associated pathological changes that are for the time being irreversible. Because of this, precise molecular diagnosis and specialized medical management is of paramount importance for all muscular dystrophy patients.

1.2. Diagnostics

Historically the complexity and variability of muscular dystrophy clinical manifestation has represented a challenge for accurately diagnosing patients, and it still does. A complete diagnosis would involve clinical examination, patient’s medical history, blood test (creatine kinase), electromyography, genetic testing and muscle biopsy (Rosalki 1989; Carbone et al. 2000; Fardeau and Desguerre 2013).

The role of muscle biopsy in the diagnosis of muscular dystrophies, considered by many to be the gold standard of testing (using light microscopy, histochemistry and immunohistochemistry methods, as well as electron microscopy), has lessened, nonetheless it still provides information essential for diagnosis. When considering biopsy results, it is necessary to be mindful of cases when there is a secondary protein defect or deficiency that is masking the primary problem, an example being a secondary reduction of calpain in cases of LGMD R2, R9 and R10 (Anderson et al. 2000; Haravuori et al. 2001; Sáenz et al. 2005).

Western blotting can also be used to determine the level of protein expression as well as the length of the protein (Longo et al. 2011).

In the last decade the value of magnetic resonance imaging (MRI), in order to identify patterns of muscle involvement in genetically distinct muscle disorders, has been recognized time and again, as identifying a specific muscle involvement pattern can give valuable information and guide genetic testing, sometimes even pinpoint an exact disease form (Mercuri et al. 2007; Bönnemann 2011a; Quijano-Roy et al. 2012; Tasca et al. 2012, 2018; Barp et al. 2017, 2020). Furthermore, a tight correlation of muscle MRI data with functional performance has been shown, further highlighting its diagnostic value (Carlier et al. 2016).

With the development of technology in the recent years, techniques like protein analysis and molecular genetic testing have become increasingly more important in diagnosis of muscular dystrophies, however classic diagnostic tools, like clinical observations, family history, biochemical tests, serum creatine kinase testing and the aforementioned muscle biopsy are still crucial first-line options in order to facilitate accurate diagnosis (Gaina et al. 2019). Many believe that the next generation sequencing has the potential to be one of the most powerful tools to date for diagnostics of muscular dystrophies, especially the rare variants. There has already been research showing the benefits of using next generation sequencing, when working with undiagnosed muscular dystrophy patients. The results vary between laboratories though, and the rate of success varies between 15% and 65% (Wu et al. 2018).

When looking only at clinical criteria, recessive muscular dystrophies are most difficult to differentiate, due to the heterogeneity of phenotypic traits, age of onset and rate of progression (Ginjaar et al. 2000; Amato and Griggs 2011; Romitti et al. 2015; Ciafaloni et al. 2016). With progressive muscle weakness being the most common feature of different types of muscular dystrophies, it is the other variable features, like age of onset, the affected muscle groups and the rate of disease progression, that may point towards a specific type of muscular dystrophy (Muntoni et al. 2003; Amato and Griggs 2011).

A lot of effort has been put into trying to improve the diagnostics of muscular dystrophies. An example of this are the inter-laboratory collaborations and the international consortiums, where research institutions with funds and expertise try to help other specialists lacking either of the two. One such consortium is the MYO-SEQ project, based in Newcastle, UK, established with the goal of aiding the work-ups of muscle disease patients and to better understand the underlying disease etiology. The project offered exome sequencing for undiagnosed patients from all over the world, examining variants in genes associated with muscle conditions (Töpf et al. 2020).

1.3. Limb-girdle muscular dystrophies

The term limb-girdle muscular dystrophy itself appeared in the middle of the 20th century, when it became apparent that there is a large group of muscular dystrophies, that differ from the X-linked Duchenne-Becker and the autosomal dominant fascioscapular dystrophy forms (Kirschner and Bönnemann 2004). Currently LGMD has become an important subgroup of muscular dystrophies, that is classified by certain traits - a clinical phenotype of a progressing muscle weakness, affecting predominantly limb-girdle muscles and a histologically confirmed dystrophin-positive muscular dystrophy (Kirschner and Bönnemann 2004). There is an equal chance for both men and women to get LGMD. In addition to the weakness of the skeletal muscles, respiratory deficiency, caused by the weakness of the diaphragm, as well as cardiomyopathy has been observed in LGMD patients (Longo et al. 2011). In the past LGMD was attributed only to cases where weakness manifested in the teen or early adult age, and more severe forms, with manifestation in childhood, were termed severe childhood autosomal recessive muscular dystrophy (SCARMD). Now it is classified as one of the LGMD subtypes (Pegoraro and Hoffman 2012).

LGMD autosomal dominant forms constitute the first group, classification wise, with recessive forms making up the second group. Roughly 10% of all LGMDs are dominant forms, while the rest 90% are recessive (Angelini et al. 2018). In terms of incidence, autosomal recessive forms are approximately ten times more common than dominant forms (Sáenz et al. 2005). Within the groups each form gets assigned a consecutive alphabet letter, based on the chronological discovery of the associated locus. In theory this was meant to be constantly expanded (Longo et al. 2011). The problem this system ran into was that with the rise of large-scale sequencing, many new recessive forms were discovered, and so the end of the alphabet for recessive forms was reached. This was made worse by the fact that there were diseases initially added to this classification that are not classified as LGMDs by modern standards, and therefore gave rise to inconsistent presentation among the various subtypes (Straub et al. 2018). In 2017 a workshop was organized that proposed a new definition for what is classified as LGMD and created a new LGMD naming system. According to the new system, LGMD is now followed by a “D” or an “R” for dominant and recessive types respectively. This is followed by a number denouncing the order in which the diseases were discovered and the name of the protein that is associated with the disease. To give an example, the disease previously classified as LGMD2A is now known as LGMD R1 Calpain3-related (see supplemental Table 1). It was decided though that for the near future both naming conventions can be used in order to ease professionals and especially patients in the new system (Straub et al. 2018).

The incidence of LGMDs is relatively rare, ranging from 4-7 per 100 000 newborns, however there are some geographical and ethnic exceptions, due to founder effects and consanguineous marriages, and the numbers vary slightly (Angelini et al. 2018). It is difficult to determine the exact incidence of all the different LGMD forms, however, based on the data from clinical populations and variant databases, estimations have been made. The more common forms are: calpainopathies (LGMD2A/LGMD R1 calpain3-related) with an average estimate of 8.4 in all populations, and with reported estimates of 9.5 in northeastern Italy and 6.0 in northern England; dysferlinopathies (LGMD2B/LGMD R2 dysferlin-related) with an average estimate of 7.5, and a reported estimate of 1.3 in northern England and FKRP related muscular dystrophies (LGMD2I/LGMD R9 FKRP-related) with an average estimate of 4.5, and a reported estimate of 4.3 in northeastern Italy. According to estimates the most common LGMD however is the more recent form LGMD2L/LGMD R12 anoctamin5-related, with an average estimate of 17.6, and with reported estimates of 2.7 in northern England, 20 in Finland and 10 in Denmark (all prevalences are estimated per one million individuals) (Fanin et al. 2005; Norwood et al. 2009; Hicks et al. 2011; Penttilä et al. 2012; Witting et al. 2013; Mazzucato et al. 2014; Liu et al. 2019).

The main affected proteins usually are the sarcolemmal proteins, though there are cases when the cytoskeletal proteins or muscle specific enzymes are affected (Brockington et al. 2001a). Even though LGMDs by definition are usually characterized by the classical clinical phenotype that includes the predominantly proximal muscle weakness, there exists considerable genetic heterogeneity, and a variety of clinical features, progression of symptoms and types of heredity. Due to this reason, the phenotypic spectrum of this group of diseases has been considerably broadened to include non-typical cases and cases with distal involvement. A broad approach to muscle examination can help alleviate the precise determination of dystrophy type. Different age ranges have been described for the many forms of LGMD. It is assumed that the dominant forms manifest after the second decade of life and usually have slow progression (Guglieri and Bushby 2011).

One of the most common and cheapest types of non-invasive testing for muscular dystrophies is the creatine phosphokinase (CK) level determination in peripheral blood. In case of the disease it is increased, with much higher levels in case of recessive forms, when compared to dominant ones. What makes this testing more difficult is the fact that there are some forms of LGMD that do not present with an increased CK level. Furthermore, in all LGMD cases the CK level naturally drops with age, so for older patients this measurement can be normal or only slightly elevated (Guglieri and Bushby 2011). It might however also be an additional tool in differentiating between different LGMD forms, with CK normal or slightly elevated in

transportinopathies, moderately raised in telethoninopathies or dystroglycanopathies and greatly increased in dysferlinopathies, calpainopathies and sarcoglycanopathies. A new blood biomarker that is being investigated is the expression of serum myomiRNA, with dysregulated profiles pointing at dystrophies (Angelini et al. 2018).

With a few rare exceptions electromyography (EMG) can be used to exclude neurogenic causes of muscle weakness (Angelini et al. 2018).

Muscle magnetic resonance imaging (MRI) is being used more and more to diagnose LGMD and for follow-ups. This is due to the fact that the extent and localization of muscle abnormalities can provide valuable information. In some cases, the pattern of muscle involvement can be almost pathognomonic and help guide genetic testing or choose the optimal muscle for biopsy (Straub et al. 2012). It is muscle biopsy that allows confirmation of dystrophic changes in muscle fibers and also differentiate LGMD from other possible causes of progressive proximal muscle weakness (Kirschner and Bönnemann 2004). However, in LGMD dystrophic features, like increased fiber size variability, increased central nuclei, endomysial fibrosis, necrosis, and regeneration can be widespread and the degree can be variable and not always correlate well with disease severity (Angelini et al. 2018). Immunohistochemistry testing of the biopsied material may sometimes pinpoint an exact form of LGMD, especially in cases of sarcoglycanopathy, dysferlinopathy, telethoninopathy and plectinopathy. It appears that in these cases severely reduced or absent labeling for the respective protein is a highly specific disease marker (Pegoraro and Hoffman 2012; Angelini et al. 2018). Unfortunately, in Latvia the field of muscle biopsy testing for neuromuscular disorders is very underdeveloped, and therefore we have to rely mostly on biochemical blood tests and molecular diagnostics.

The most precise method in differentiating all the forms of LGMD is however the detection of the causative genetic variant, using molecular analysis, usually done after or in parallel with determining the exact protein that is affected. It is worth remembering though that while there are some LGMD forms where the causative variants are well known and researched, there are also forms where the causative genes are large enough to cause possible difficulties when sequencing, and there can also be certain gene structure peculiarities. The diagnostic situation is improving in the last few years with the rise of next-generation sequencing, which allows simultaneous testing of larger portions of the genetic code. Furthermore, lately the whole exome sequencing is often being replaced with whole genome sequencing, also in our project, thus enabling much better splice region and deep intronic variant analysis, and helping to fill in the missing pieces. Interestingly, in 2013 the American Academy of Neurology guidelines for LGMD suggested using NGS as a last resort examination, however the stage has changed so

much in the years since, that nowadays NGS is often considered as a first-line approach to LGMD testing, as positive findings often require no further laboratory work-up (Giza et al. 2013; Angelini et al. 2018).

In the early 2000s, there still existed around 25% of familial cases that were not associated with any of the known disease genes and around 40% of isolated cases with severe or intermediate LGMD phenotype that did not have pathogenic variants in any of the LGMD related genes identified (Aurino et al. 2008). More recent approximations put the figures of LGMD cases with determined diagnosis at 30-40%, using traditional genetic, biochemical and histopathological methods and at 40-45% using exome sequencing, with this percentage going up to even 70% in certain populations with founder effect mutations (Reddy et al. 2017; Valencia et al. 2017; Angelini et al. 2018).

After having their molecular diagnosis established, the patients require a consultation from a geneticist. In cases of autosomal dominant forms, there is the 50% chance of passing on the disease to the next generation, which can be influenced, however, by various factors, like incomplete penetrance or gonadal mosaicism. The carriers of recessive diseases have a relatively low chance to meet another carrier, in order to have an affected child. Nonetheless this chance is markedly increased in populations where a founder effect for a certain mutation is present, for example, the *CAPN3* variants - c.550delA in Croatia and c.2051-1G>T as well as c.2338G>C in the Agarwal population in India and Asia (Canki-Klain et al. 2004; Khadilkar et al. 2016). Because of these reasons, in familial cases it is important to find out as much about the family history as possible. However, due to phenotypic variability and the *de-novo* pathogenic variants, dystrophy cannot be truly ruled out in cases where the disease is seemingly absent from a family (Guglieri and Bushby 2011).

LGMD often present themselves as multisystemic disorders. The presence or absence of various phenotypic traits, for example, contractions or circulatory and respiratory system problems helps in determination of the exact diagnosis. More importantly, identification of possible risks for a patient helps in creating a customized prevention strategy. In sarcoglycanopathies cardiac involvement is one of the main clinical features, able to manifest as several forms (conduction defects, dilated cardiomyopathy or hypertrophic cardiomyopathy) (Barresi et al. 2000; Politano et al. 2001; Meznaric-Petrusa et al. 2009; Angelini et al. 2018). Cardiac involvement is also typically present in FKRP related myopathies, where, interestingly, it may not correlate with the severity of skeletal muscle involvement (Angelini et al. 2018). Respiratory involvement is more frequent for sarcoglycan-related LGMD forms as well as the FKRP, titin and POMT1 related LGMD forms, furthermore respiratory involvement is in general a more common phenotypic feature of LGMDs (Angelini et al. 2018). Working with

patients and a timely diagnosis is paramount to have precise genetic consultation and maximum possible risk reduction. It is especially important to avert problems and treat complications as soon as possible in cases where patients have circulatory and respiratory system involvement, as this helps to increase life quality and expectancy. In very severe cases there is a high risk of infections, hypoventilation and respiratory arrest, in addition regular heart monitoring is recommended for LGMD patients (Guglieri and Bushby 2011; Angelini et al. 2018).

The treatment of the majority of LGMD forms is still based on palliative and supportive therapy. As LGMD pathomechanisms are still not well understood, true curative treatment does not exist yet (Angelini et al. 2018). In cases of alpha-sarcoglycanopathy Deflazacort has been used and has shown improved performance (Angelini et al. 1998). Another possible treatment might be the use of antisense oligonucleotides, able to induce exon skipping, generating smaller transcripts that result in functional proteins. This approach has been shown to work in cells from an alpha sarcoglycanopathy patient with an in-frame deletion as well as in a mouse model of LGMD R5 γ -sarcoglycan-related exhibiting a frameshift variant (Shimo et al. 2018; Demonbreun et al. 2020). Research into stem cell application is ongoing, but has yet to yield any meaningful results. Another of the possible cures might be the virus-mediated gene transfer, being tried in various muscle dystrophy animal models. Promising results have been shown in LGMD R3 α-sarcoglycan-related, LGMD R1 calpain3-related and LGMD R2 dysferlin-related mouse models (Guha et al. 2019; Griffin et al. 2021). Sarepta Therapeutics already have three gene therapy products in clinical phase testing - two for sarcoglycan related LGMD and one for dysferlin-related LGMD. However, there is still work to be done for these methods to be approved for wider clinical use.

The optimal patient care should be provided by an interdisciplinary team of doctors, which, in addition to a neuromuscular disease specialist, would include also an orthopediatrician, a pulmonologist, a cardiologist, a social worker and various therapists, tasked with maintaining the independence of movement for as long as possible (Kirschner and Bönnemann 2004). The patient care should be tailored to each individual, based on the exact type of LGMD. The general care, in order to increase the longevity and quality of life, includes weight control, physical therapy and stretching exercises to maintain mobility and avoid contractures. Many cases require mechanical aids or surgery to ensure mobility of patients (Pegoraro and Hoffman 2012). The diagnosis of the exact form of LGMD can considerably improve the quality of life of patients and prolong survival, furthermore helping us gain knowledge for the future, when more advanced gene and protein specific therapies might be available (Guglieri and Bushby 2011).

1.3.1. Caveolinopathies

Caveolinopathies are a group of muscle disorders, associated with defects and the subsequent decrease in the levels of the membrane protein caveolin-3 (Galbiati et al. 2001b; Williams and Lisanti 2004; Woodman et al. 2004). The five main phenotypes associated with *CAV3* pathogenic variants used to be limb-girdle muscular dystrophy autosomal dominant type C (LGMD 1C), isolated hyperCKemia, rippling muscle disease (RMD), hypertrophic cardiomyopathy (HCM), and distal myopathy (DM), although a few other rarer phenotypes had been identified over the years (Aboumousa et al. 2008; Gazzerro et al. 2010). During the re-classification however, it was determined that the main phenotypical features of caveolinopathies are rippling muscle disease and myalgia, with muscle weakness being a secondary phenotypic feature, and therefore it is no longer considered an LGMD (Straub et al. 2018). Before reclassification caveolinopathies were found to be the most common dominant form of LGMD in Italy, with a frequency of 63.5% (Magri et al. 2017). The patients are described to have the disease onset in the first decade of life (Gazzerro et al. 2011a).

The hypertrophic cardiomyopathy phenotype has been associated with *CAV3* variants in humans in only one family, however the mice models have shown a possible role of caveolin-3 in the pathogenesis of cardiac disorders (Hayashi et al. 2004). The protein is expressed in cardiomyocytes and it is hypothesized that caveolae function as a control center of cellular signaling in these cells (Kikuchi et al. 2005). Cav-3 knockout mice display convincing cardiomyopathy phenotype (myocardium hypertrophy, dilation, and reduced fractional shortening), thus lending credence to these claims, however no definite mechanisms have been found so far (Woodman et al. 2002; Gazzerro et al. 2010).

The gene encoding caveolin-3, *CAV3*, is the only known locus associated with caveolinopathies, with pathogenic variants identified in 99% of all caveolinopathy cases (Gazzerro et al. 2011b). *CAV3* itself is located on chromosome 3p25.3 and has two coding exons (Fulizio et al. 2005). *CAV3* expression has been detected in all muscle cells, as well as in glial cells and early postnatal peripheral nerves. It is a highly evolutionarily conserved gene, suggesting a vital function; therefore, frequent sequence variations at this locus are not expected (Gazzerro et al. 2010).

The protein caveolin-3 is a muscle specific member of the caveolin family of proteins that form flask-shaped invaginations (caveolae) on the cytoplasmic surface of sarcolemmal membranes (Figure 2.) (Cohen et al. 2004; Gazzerro et al. 2010). It interacts with a number of proteins, including beta dystroglycan, dysferlin, nNOS, phosphatidylinositol 3-kinase, Src-kinase and phosphofructokinase (Hayashi et al. 2004; Couchoux et al. 2011). The main known functions of caveolae include organization of specific microdomains at the plasma membrane,

the regulation of signaling, endocytosis, exocytosis, and mechano-transduction (Shaul and Anderson 1998; Stahlhut et al. 2000; Dewulf et al. 2019).

The protein is formed by the C'- and N'-terminal portions that are exposed to the cytoplasm and a hydrophobic middle part that forms a single loop, embedded in the lipid bilayer, without crossing it (Schlegel et al. 1999; Schlegel and Lisanti 2000). The near-membrane part of the N'-terminal tail contains the caveolin scaffolding domain - a region that is believed to allow interactions with many signaling proteins (Figure 2.). This region is located within the oligomerization motif, which allows nine caveolin monomers to assemble into a toroidal nonamer. These structures can further assemble into even larger polyhedral-like structures, which trigger the membrane invagination (Whiteley et al. 2012; Pradhan and Prószyński 2020). This process is controlled by a family of proteins, called cavins, that interact with caveolin (Pradhan and Prószyński 2020). At the cell surface caveolins can be scaffolded by actin and microtubules, thus regulating their stability (Head et al. 2006; Richter et al. 2008).

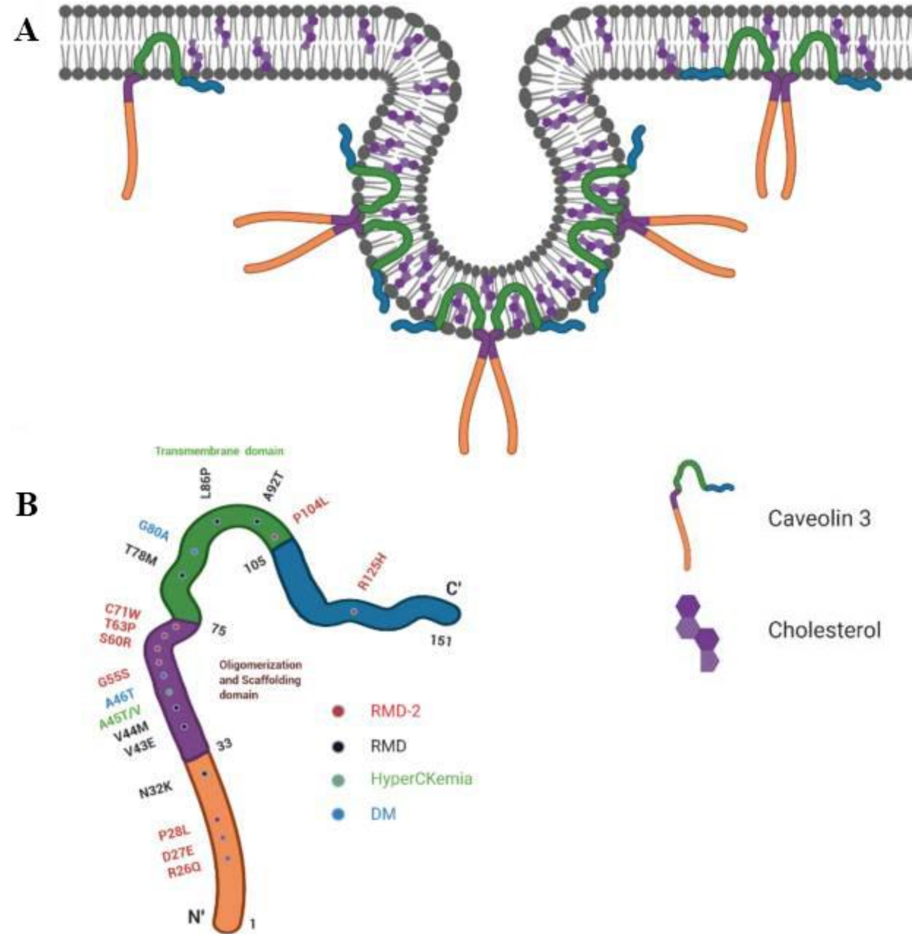

Figure 2. Caveolin-3 in the caveolae and its domain organization. A. Schematic representation of caveolae and the associated caveolin-3 proteins. B. Pathogenic variants in caveolin-3, associated with caveolinopathies. RMD – rippling muscle disease, CK – creatine phosphokinase, DM – distal myopathy. Figure adapted from Pradhan *et al.* 2020.

In vitro functional research has shown that caveolin-3 is an important regulator of myoblast differentiation and efficient fusion (Volonte et al. 2003; Quach et al. 2009; Stoppani et al. 2011; Madaro et al. 2011). Furthermore, not only is caveolin known to play a part in cell membrane repair, its retention in the Golgi apparatus due to pathogenic variants, causes mislocalization of TRIM72 and DYSF proteins, both known to be involved in muscle repair (Hernández-Deviez et al. 2008; Cai et al. 2009a, b; Corrotte et al. 2013). Another process where caveolin plays a role is the formation of T-tubules, which themselves are crucial for proper muscle contraction. Cav3 is enriched in T-tubules and it has been shown in Cav3 knock-out animal model that the absence of the protein leads to disorganization of T-tubules system in both skeletal and cardiac muscles (Parton et al. 1997; Galbiati et al. 2001a; Minetti et al. 2002; Bryant et al. 2018). The protein is also known to associate with the dystrophin-associated glycoprotein complex, which plays a crucial role in muscle fiber integrity by linking the

extracellular matrix with cytoskeleton. Cav3 binds to the same site of β-dystroglycan as dystrophin, thus over-abundance of caveolin-3 might cause instability of the entire glycoprotein complex. Interestingly, elevated caveolin-3 expression has been observed in Duchenne muscular dystrophy patients, where the formation of this complex is affected (Repetto et al. 1999; Pradhan and Prószyński 2020). It has also been described that increased level of Cav3 expression in skeletal muscle of transgenic mice causes muscular dystrophy, which is more similar to Duchenne muscular dystrophy (Galbiati et al. 2000).

Muscle biopsies usually show varying muscle fiber size, muscle fiber degeneration and regeneration, associated with the elevated serum CK levels, as well as increased number of central nuclei. The decrease in Cav-3 expression can be up to 80% of norm (Gazzerro et al. 2010).

It is possible for people from one family with the same variant to have different phenotypes, overlapping disease phenotypes being described in some of the families with caveolinopathies; no certain phenotype–genotype correlations have been observed (Catteruccia et al. 2009). It has been proposed that the multiple pathways that caveolin-3 is a part of, might explain some of the phenotypic variability. Serum creatine kinase (CK) levels increase steadily over time, but do not correlate with the progress of disease (Gazzerro et al. 2010).

1.3.2. Calpainopathies

Calpainopathies were the first form of LGMDs, associated with lack of a non-structural protein. The degenerative changes are caused by pathogenic variants in the *CAPN3* gene that codes the muscle specific enzyme calpain-3, associated with LGMD type 2A, currently known as R1 (Sáenz et al. 2005). It is believed that this type constitutes 15-40% of all LGMD cases, and has the average frequency of 1:100 000 people, with some regional exceptions. This frequency is close to the estimated prevalence in all populations of 8.4 per one million individuals. The disease has high prevalence in Eastern Europe, Czech Republic, Spain, Italy, the Netherlands, northern England, and Brazil (Duno et al. 2008; Norwood et al. 2009; Chu and Moran 2018; Liu et al. 2019). The gene is located on chromosome 15 q1.5.1 to q1.5.3. It is a large gene, consisting of 24 coding exons, which makes classical mutation testing time and resource consuming. Around 500 unique pathogenic variants in *CAPN3* have been associated with LGMD R1, in addition to apparently neutral or unclassified variants (Landrum et al. 2018; Barp et al. 2020; Howe et al. 2021).

Calpain-3 is a part of the calpain superfamily of Ca^{2+}-dependent non-lysosomal cysteine proteases that are heterodimers composed of two distinct subunits. The protein is activated by intracellular Ca^{2+} and cleaves specific substrates (Carafoli and Molinari 1998). One of the

peculiarities of CAPN3 is its high autolytic activity, which is being suppressed in skeletal muscles through its binding to connectin/titin (Sorimachi et al. 1993; Ono et al. 2006). Several mechanisms have been shown to depend on the proteolytic function of the protein, such as mechanosensory transduction and sarcomere remodeling after exercise (Kramerova et al. 2005; Ono et al. 2016). The non-proteolytic functions of CAPN3 allows it to contribute to the maintenance of Ca^{2+} homeostasis, by stabilizing critical Ca^{2+} handling proteins. The main functions of CAPN3 are thought to be regulation of muscle contraction and sarcomere stability, with suggestions of it playing a role as a sensor of sarcomere integrity (Ojima et al. 2007; Ono et al. 2010; Lasa-Elgarresta et al. 2019). The protein has been found in most parts of the muscle fiber - sarcomere, membrane fraction, sarcoplasmic reticulum, cytosol, and the nucleus (Ojima et al. 2011; Lasa-Elgarresta et al. 2019).

The age of onset for calpainopathies may vary from 2 up to 40 years of age, with 14 being the most common age of onset. The average age when patients lose ambulation is around 32 years of age, with no apparent correlation between the age of onset and the progression in loss of ambulation, or between the sex of patient and the possibility of losing ambulation (Sáenz et al. 2005). The possible disease phenotypes range from mild to severe, and include the more common pelvifemoral LGMD (muscle weakness first in the pelvic girdle and then in the shoulder girdle, with toe-walking as an early childhood sign, which might manifest before muscle weakness), the less common and less severe scapulohumeral LGMD (muscle weakness first in the shoulder girdle and then in the pelvic girdle), hyperCKemia and eosinophilic myositis, which may be an early and transient feature (Brown and Amato 2006; Kramerova et al. 2007; Mori-Yoshimura et al. 2017; Lasa-Elgarresta et al. 2019; Barp et al. 2020). Even though all of the pathogenic mechanisms, involving CAPN3, are not entirely understood, it is fairly certain that it is a multifunctional protein. Different studies have shown the following mechanisms to be associated with calpainopathies: oxidative damage, Ca^{2+} dysregulation, sarcomere disorganization, mitochondrial abnormalities, abnormal muscle adaptation, and impaired muscle regeneration (Kramerova et al. 2004, 2009, 2012, 2016; Rajakumar et al. 2013; Nilsson et al. 2014; Yalvac et al. 2017; Toral-Ojeda et al. 2018; El-Khoury et al. 2019; Lasa-Elgarresta et al. 2019). All of these together might lead to the characteristic LGMD R1 features (inflammation, necrosis, fibrosis, atrophy, and progressive muscle degeneration) (Lasa-Elgarresta et al. 2019). Same as in most dystrophies, no definite genotype-phenotype correlations have been found (Fanin et al. 2009; Lasa-Elgarresta et al. 2019). The initial phenotypic traits of calpainopathies usually are pelvic and shoulder muscle weakness and atrophy, which often allows for comparatively easy diagnosis (Duno et al. 2008).

In general, calpainopathies present with either a severe form that has a rapid course or an adult-onset form with loss of ambulation in the late adulthood, they are a more atrophic muscular dystrophy type, with a comparatively narrow affected muscle profile and an early appearance of contractures. The contractures can be so extensive that they start to resemble Emery-Dreifuss muscular dystrophy. Affected individuals usually do not have cardiac involvement but respiratory dysfunction is somewhat common, even though it is usually mild and develops into a serious problem only in the late stages of the disease (Di Ri K et al. 2001; Groen et al. 2007; Peters et al. 2009; Mori-Yoshimura et al. 2017). The disease is progressive, however, with proper care the life expectancy of patients is close to normal (Kirschner and Bönnemann 2004; Angelini et al. 2018). Muscle biopsy western blotting in the case of calpainopathies may be useful to differentiate secondary calpain reduction, seen in autoimmune disorders (Mammen 2017). However, muscle biopsy data shows that around 20-30% of LGMD R1 patients have normal calpain-3 expression on Western blot. These patients often carry missense variants, leading to impaired autolytic activity (Nascimbeni et al. 2010). It must be noted though that it is difficult to predict the clinical outcome of these variants, as they have been associated with varying degrees of disease severity and protein amount (Barp et al. 2020). Various groups have reported specific patterns of muscle involvement seen by MRI or computer tomography that may help in differentiating LGMD R1 from other myopathies that are characterized by early contractures (Fardeau et al. 1996; Richard et al. 2016; Feng et al. 2018; Barp et al. 2020).

In the last few years, a number of publications have put forward the concept of an autosomal dominant form of calpainopathy. In 2016, 10 families (36 patients) were reported carrying a recurrent variant (c.643_663del21) that was associated with calpainopathy and a seemingly dominant pattern of inheritance (Vissing et al. 2016). This was followed by reports of three families harboring the same variant, showing an autosomal dominant mode of transmission, and 17 additional index cases with the same variant (Martinez-Thompson et al. 2018; Nallamilli et al. 2018). Following this, the classification of calpainopaties was reassessed and the dominant LGMD D4 calpain3-related form was introduced (Straub et al. 2018). Another heterozygous in-frame deletion (c.598_621del15) was reported as associated with LGMD in 16 unrelated individuals, and later reported in an affected family as well (Nallamilli et al. 2018; Cerino et al. 2020a). Recently, a missense variant - c.1333G>A p.(Gly445Arg) has been reported to be associated with LGMD D4 in four families (14 patients) from France, based on familial segregation analysis. In these cases, patients presented with a mild, late onset form of muscular dystrophy (Cerino et al. 2020b). Around the same time another missense variant c.1715G> C p.(Arg572Pro) has also been reported in a family with mild autosomal dominant

calpainopathy (Vissing et al. 2020). González-Mera *et al.* have also reported c.1333G>A and another four variants (c.1661A> C, c.1706T> C, c.1327T> C and c.700G> A) in heterozygous state in seven families (21 patients). In five of these, the respective variant segregates with autosomal dominant calpainopathy, and in the remaining two the index patient was the only carrier in the family; however, the clinical phenotype and muscle imaging was highly similar to the previously described LGMD D4 cases (González-Mera et al. 2021). In all cases protein studies demonstrated reduced calpain-3 level and protein modeling predicted these variants to disrupt either calmodulin binding, which affects proteolytic activation, or β-sheet formation, which affects protein stability (González-Mera et al. 2021). All patients described exhibit slowly progressive proximal myopathy, elevated CK levels and a generally milder phenotype, when compared to the recessive form (Vissing et al. 2020; Cerino et al. 2020b; González-Mera et al. 2021). Interestingly, we have also identified a family in Latvia, carrying the c.1333G>A variant, and presenting with a mild autosomal dominant calpainopathy phenotype (unpublished data). Identical to the report by González-Mera *et al.* we found one individual in this family presenting with a more severe phenotype, and further testing revealed another variant in *CAPN3* (c.1043delG), confirming that carriers of two trans-compound variants exhibit the recessive calpainopathy phenotype (González-Mera et al. 2021).

1.3.3. Collagen 6-related myopathies

Historically, mutations in one of the three collagen VI genes—*COL6A1*, *COL6A2* and *COL6A3* are associated with Bethlem myopathy 1 and the more severe Ullrich congenital muscular dystrophy 1. Nowadays, however, the two diseases are often seen as the opposite extremes of a spectrum of collagen VI-related myopathies (Briñas et al. 2010; Bönnemann 2011a). Bethlem myopathy was first described in 1976 by Bethlem and Wijngaarden, with patients having a milder and relatively stable myopathy that affects both sexes equally. Autosomal dominant inheritance is predominant, but autosomal recessive cases have been described as well, with the disease usually starting in early infancy and progressing slowly (Bethlem and Wijngaarden 1976; Schmalbruch et al. 1987; Foley et al. 2009; Gualandi et al. 2009; Deconinck et al. 2015; Sframeli et al. 2017; Caria et al. 2019). The trunk and limb muscles are most affected, with involvement of proximal muscles more than distal, and extensors more than flexors. Patients exhibit moderate weakness and atrophy. Nearly all patients eventually develop flexion contractures of the fingers, wrists, elbows, and ankles, which could be considered a hallmark of the Bethlem myopathy (Lampe and Bushby 2005). A lot of affected patients become wheelchair bound by their fifth decade of life, with some dying of respiratory failure (Haq et al. 1999). The collagen VI-related myopathies present with a distinct

combination of phenotypic traits (hyperlaxity, contractures and weakness), which usually makes them rather easily recognizable (Bönnemann 2011a). The muscle biopsy findings are variable, with samples from an early disease stage often having no overt dystrophic features but samples from advanced stages showing dystrophic features (rare degenerating and regenerating fibers and a build-up of fibrous tissue in the muscle) (Schessl et al. 2008; Bönnemann 2011a). Most cases don't have elevated CK levels, even though cases with elevated creatine kinase have also been reported (Schmalbruch et al. 1987). Immunostaining biopsy material for collagen VI seems to be a useful diagnostic tool, as recessive variant cases often exhibit greatly reduced or absent collagen VI staining (Higuchi et al. 2001; Vanegas et al. 2001; Peat et al. 2007; Bönnemann 2011a; Zamurs et al. 2015). MRI imaging can be another useful diagnostic tool, as the characteristic pattern allows better recognition of collagen VI-related myopathy, while also allowing to differentiate it from the LMNA related Emery–Dreifuss muscular dystrophy and other myopathies with similar phenotypic traits (Mercuri et al. 2010; Deconinck et al. 2010; Bönnemann 2011a). Although precise information is still scarce, there is compelling evidence that collagen VI-related myopathies are among the most common congenital muscular dystrophies worldwide (Okada et al. 2007; Peat et al. 2008; Norwood et al. 2009; Sframeli et al. 2017).

Genetic heterogeneity is observable all throughout collagen VI-related myopathy cases (Panadés-de Oliveira et al. 2019). This does not make the job of their classification any easier, but it has led scientists to the hypothesis that these myopathies comprise a spectrum of conditions, with variable severity, that is based on a number of factors (Gualandi et al. 2009). To muddle things further, it has been shown that haploinsufficiency is not enough to cause myopathy. This suggests that the previously reported autosomal dominant patients are either carrying an additional undiagnosed variant or there is some still unknown additional regulatory mechanism (Gualandi et al. 2009). Brinas *et al.* and Bonnemann *et al.* have proposed two systems that classify collagen IV-related muscular dystrophies into three or four classes, respectively, based on the severity of the phenotype (Briñas et al. 2010; Bönnemann 2011a). In addition to these, there are two other, more rare phenotypes: a limb-girdle muscular dystrophy (LGMD) like presentation and myosclerosis (Bönnemann 2011b).

The collagen VI is a beaded microfibril collagen that is expressed in the extracellular matrix of nearly all tissues. It is important in anchoring the basement membrane to the extracellular matrix (Timpl and Chu 1994; Kuo et al. 1997; Cescon et al. 2015). The three genes involved, each code one of the three alpha chains of type VI collagen. The alpha chains form heterotrimeric monomers, these are the basic components of most collagen VI, and are then secreted into the extracellular matrix where they form collagen VI microfibrils (Engvall et al.

1986). Each chain contains a triple helical (TH) domain with repeating Gly-X-Y subunits, that is flanked by globular Von Willebrand factor type A (VWA) domains (Figure 3) (Chu et al. 1989; CHU et al. 1990). Missense variants in the conserved glycine residue, located in the Gly-X-Y motif constitute around 30% of known pathogenic variants in the collagen IV genes (Briñas et al. 2010). Such variants or exon skipping in the N-terminal end of the TH domain seems not to disrupt the formation of triple helical monomers, however it does disrupt higher order assembly. Furthermore, variants in a critical region (Gly-X-Y triplets 10-15 in the TH) are associated with a more severe disruption and accordingly increased clinical severity (Lamandé et al. 2002; Pace et al. 2008). Similarly, mutations that cause a complete lack of one of the three chains, cause a complete lack of collagen VI and extracellular microfibrils, resulting in a more severe disease (Higuchi et al. 2001; Vanegas et al. 2001; Demir et al. 2002; Giusti et al. 2005; Peat et al. 2007).

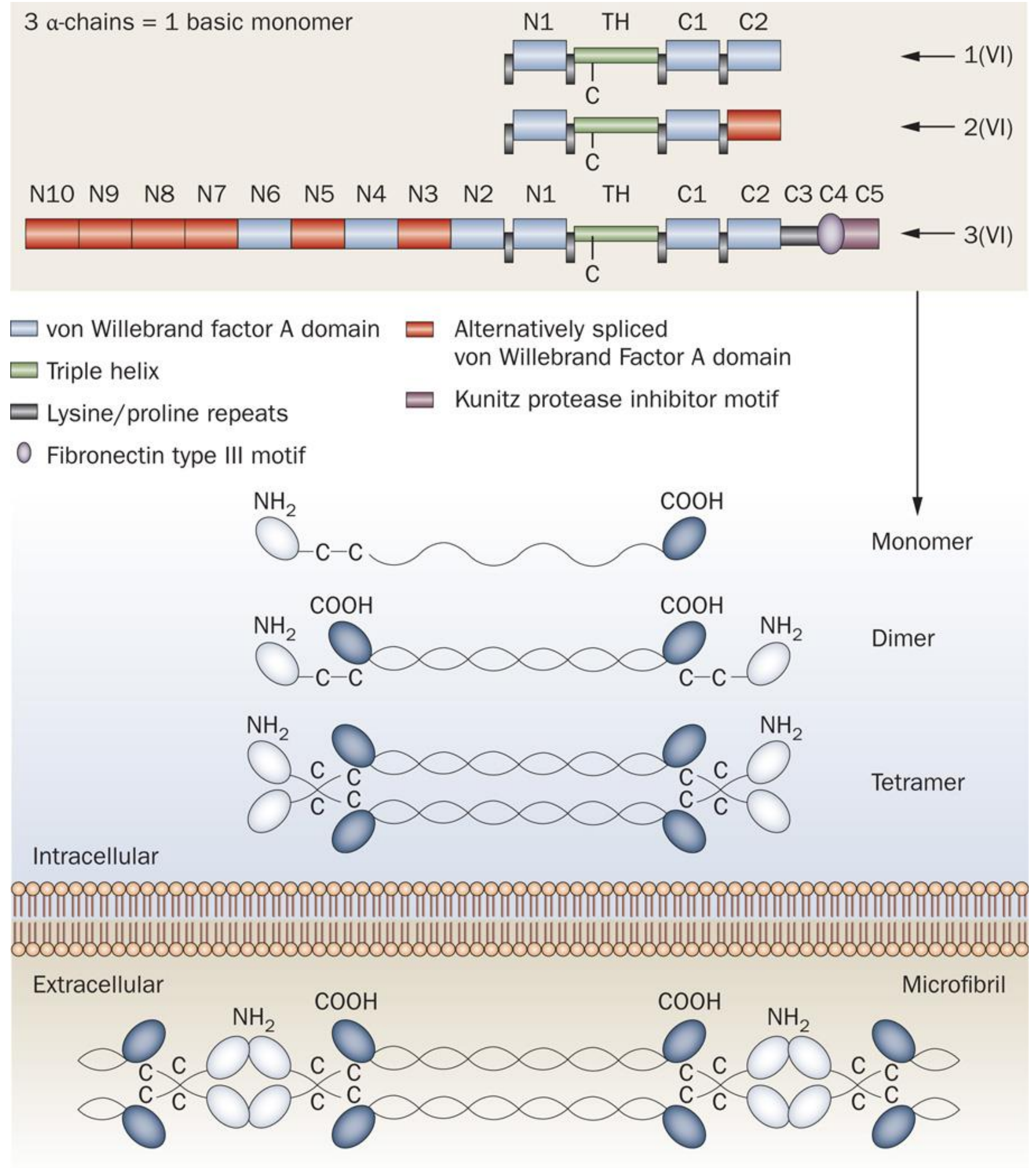

Figure 3. Schematic of the three collagen 6 alpha chain protein domain structures and the formation of microfibrillar structures. Figure adapted from Bönnemann 2011.

Even though collagen VI is expressed in many tissue types, collagen VI-related myopathy pathogenic mechanism research focuses on effects on muscles and tendons. It has been shown that defective or absent collagen VI can affect the formation of collagen I fibrils in tendons, leading to irregular fibrils that are on average smaller and often large and irregular in shape (Sardone et al. 2016; Lamandé and Bateman 2018). Similar process is observable in knock-out and mutant mouse models, such changes in extracellular matrix could explain the biomechanical deficiencies observed in disease (Izu et al. 2011; Pan et al. 2013, 2014). Abnormal mitochondria, dilated sarcoplasmic reticulum and abundance of apoptotic nuclei are characteristic observations in the knock-out mouse model, thus pointing at the fact that collagen VI has cytoprotective properties (Irwin et al. 2003; Lamandé and Bateman 2018). Similar

features can be observed in biopsy samples from collagen VI-related myopathy patients (Tagliavini et al. 2013). A latent mitochondrial dysfunction was found in the knock-out mice myofibers, and it revealed the mitochondrial permeability transition pore (MPTP), an important protein in cell death, as a contributor to the phenotype. This dysfunction could be shown by using an inhibitor of mitochondrial ATP synthase (oligomycin), and normalized by a MPTP inhibitor (Irwin et al. 2003). This mechanism works by blocking the MPTP regulator - mitochondrial cyclophilin D (Palma et al. 2009; Giorgio et al. 2010). Therefore, drugs that reproduce the cyclophilin D blocking activity, without causing immunosuppression (a side-effect of the original cyclophilin D blocker) could have possible therapeutic application. Two such drugs have shown effectiveness, when tested in the Col6 knockout mouse model (Tiepolo et al. 2009; Zulian et al. 2014). Oxidative stress is believed to contribute to the disease phenotype through the aforementioned mitochondrial dysfunction, as MPTP activation increases reactive oxygen species (ROS) release. Consistently, ROS production and ROS producing protein levels are higher in Col6 knock-out muscle (Menazza et al. 2010; Zorov et al. 2014). Oxidation and inappropriate interactions between myofibrillar proteins are possible pathogenic mechanisms underlying muscle function defects observed in collagen VI-related myopathies (Lamandé and Bateman 2018). It has been shown that the abnormal mitochondria in these disorders also lead to defective autophagy, by negatively affecting its activation. The reversal of this, by increased autophagy activation, improves the phenotype (Grumati et al. 2010; Lamandé and Bateman 2018). The final mechanism, possibly contributing to the disease phenotype, has to do with satellite cells, the adult muscle stem cells, crucial for muscle regeneration. These cells are in close contact with collagen VI and express collagen VI mRNA (Urciuolo et al. 2013; Lamandé and Bateman 2018). The Col6 knockout mouse muscles show more satellite cells, but also greatly reduced regeneration and increased depletion of these cells (Urciuolo et al. 2013). These are the possible molecular mechanisms, underlying the collagen VI-related myopathy phenotype. However, it must be noted that many aspects of these are still unknown.

1.4. Myosin Binding Protein C1

The muscle sarcomere regulatory and mechanical mechanisms consist of many proteins with fine-tuned properties and tasks. A mutation in any one of those can result in a severe phenotype. With the onset of the genetic era and molecular testing it has been shown that different variants in different domains of the same sarcomeric protein can give rise to very distinct clinical manifestations. These can range in onset - from congenital to late, and result in either muscle weakness or stiffness, based on the specific function of the protein domain that

has been affected, whether its changes in calcium sensitivity or target binding. For genes *TPM2*, *TPM3*, and *ACTA1* pathogenic variants causing muscle stiffness have been described in addition to ones associated with the more classical phenotype of muscle weakness and congenital myopathy (Ravenscroft et al. 2015). This diversity highlights the central role of the sarcomere in muscles and the need to elucidate the associated molecular mechanisms and the effects of these mutations on them.

One of such sarcomeric proteins is the myosin binding protein C (MyBP-C) (Figure 4.). MyBP-C was originally discovered in 1971 as a contaminant of skeletal muscle myosin samples, and was later characterized as a myosin binding protein (Starr and Offer 1971; Offer et al. 1973). The protein comprises a family of sarcomeric accessory proteins and includes three isoforms: cardiac (c), fast skeletal (f), and slow skeletal (s) (Flashman et al. 2004). The main functions associated with this family of proteins are stabilization of thick filaments and regulation of actin-myosin cross-bridge cycling (Oakley et al. 2007; Ackermann and Kontrogianni-Konstantopoulos 2010; Rybakova et al. 2011). Normal levels of MyBP-C is required for proper assembly of synthetic myosin filaments, in regards to thickness, length, formation of bare zone, and distribution of myosin heads (Winegrad 1999). Muscles lacking MyBP-C are shown to develop sarcomeric misalignments (McConnell et al. 1999; Harris et al. 2002; Palmer et al. 2004; McGrath et al. 2006). The first time the regulatory properties of the protein were noticed, was when it was observed that adding skeletal MyBP-C to skinned myofibers slows down the shortening velocity of actomyosin cross-bridges (Hofmann et al. 1991). It was later shown, using cMyBP-C, that the ~29kDa NH_2- terminus region (including the first 17 amino acids of the M-motif) of the protein can inhibit the sliding velocity of thin filaments along thick filaments, when high Ca^{2+} concentrations are present (Previs et al. 2014). Interestingly, it has also been reported that while cMyBP-C does inhibit the maximum sliding velocity at high Ca^{2+} concentrations, it does activate actomyosin force generation and thus filament sliding at low Ca^{2+} concentrations (Razumova et al. 2006; Previs et al. 2012). This has led people to hypothesize that MyBP-C may act as sort of a “throttle”, by regulating the actomyosin cross-bridges in dependence of Ca^{2+} levels. Furthermore, presence or lack of cMyBP-C leads to dramatic changes in Ca^{2+} sensitivity, and knock-out model muscles show much higher Ca^{2+} sensitivity than wild type ones (Hofmann et al. 1991; Harris et al. 2002; Kulikovskaya et al. 2003; Palmer et al. 2004; Cazorla et al. 2006; Chen et al. 2010; Marston et al. 2012). There is, however, another function associated with MyBP-C. It was shown that cMyBP-C increases the ATPase activity of cardiac myosin in the presence of actin, independently of ionic strength, which is the concentration of Ca^{2+} in the surrounding environment. This affects only the actin-activated myosin ATPase activity (Yamamoto 1986;

Winegrad 1999). Interestingly, the effect of skeletal MyBP-C is affected by ionic strength - it is a strong inhibitor at low ionic strength, but a moderate stimulator at high ionic strength (Yamamoto 1986). It has also been proposed that at least one of the members of this family has a certain signaling function (Sadayappan and de Tombe 2012). It must be noted though that most of what we know about these proteins comes from work done on the cardiac form, not sMyBP-C. However, the proteins within the family are very similar, and that lends to thinking that their mechanisms might be highly similar. The slow skeletal form of the protein (sMyBP-C) is encoded by the *MYBPC1* gene located on chromosome 12 q23.2 (Weber et al. 1993). One thing that sets this member of the family apart, is the alternative splicing of this gene, not seen in other MyBP-C forms, that gives rise to at least 14 different variants, regulated through phosphorylation, that are co-expressed in different combinations and amounts in both slow- and fast-twitch muscles, however there is no single muscle that expresses all known sMyBP-C variants, hinting at their distinct roles (Ackermann et al. 2009, 2013; Ackermann and Kontrogianni-Konstantopoulos 2010, 2011a, b, 2013).

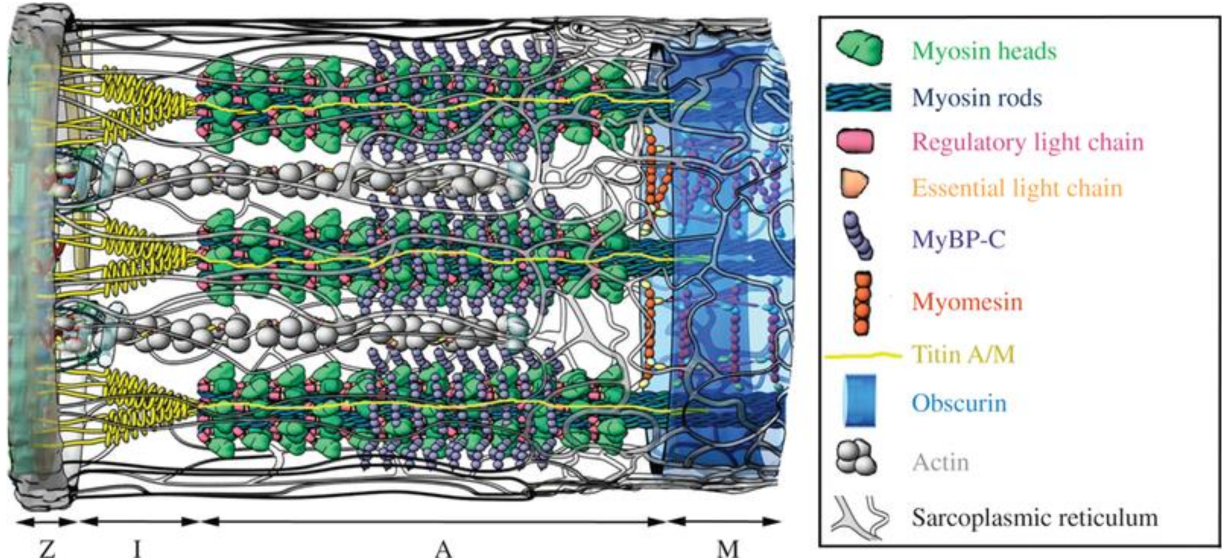

Figure 4. Schematic representation of a half sarcomere depicting the position of the Z-disk, I-band, A-band, and M-band. Figure adapted from Wang *et al.* 2018.

The protein is located in the thick filament, in the C-zone of the A-band of the sarcomere, present in 7 to 9 transverse stripes (Figure 4.) (Craig and Offer 1976). It is noteworthy that the distance between these stripes is about 43nm - equal to the spacing of the myosin helix repeat (Luther et al. 2008). There are also some sMyBP-C variants that have a unique 26bp insertion in the COOH- terminus and therefore localize to the periphery of the M-band (Ackermann et al. 2009). MYBPC1 is anchored tightly through its interactions with myosin and titin, while its other end modulates the formation and cycling of actomyosin cross-bridges, through its interaction with both actin and myosin (Offer et al. 1973; Okagaki et al. 1993; Freiburg and

Gautel 1996; Harris et al. 2011). Experiments on cultured human skeletal muscle cells showed that MYBPC1 is expressed in the initial stages of muscle cell differentiation; in mouse models its expression was observed during embryonic development. In both cases it succeeds the expression of titin and sarcomeric myosin. Due to this, it has been hypothesized that the protein has an essential role during myofibrillogenesis and it may be essential for sarcomeric assembly and maintenance (Gautel et al. 1998; Abdul-Hussein et al. 2012). The protein itself is modular and consists of ten distinct domains - seven immunoglobulin (Ig) and three fibronectin-III (Fn-III) repeats, numbered C1 to C10. The NH_2- terminus of the protein contains the Pro/Ala–rich motif, followed by the Ig domain C1 and the MyBP-C specific motif, termed M-motif - the region of interest in our project, that is expressed in all splice variants (Figure 1., page 38) (Einheber and Fischman 1990; Ackermann and Kontrogianni-Konstantopoulos 2010). Another feature that sets the sMyBP-C apart from both other forms is that it is lacking a linker region between Ig domains C4 and C5 (Flashman et al. 2008). Most of the variably spliced regions are located in the NH_2- and the COOH- termini, and are believed to attribute to specific binding properties, as the NH_2- terminus of sMyBP-C, including the Pro/Ala–rich motif, C1, and the M-motif, supports binding to actin and myosin subfragment-2 (S2) in a variant-specific manner (Ackermann and Kontrogianni-Konstantopoulos 2013). Meanwhile the COOH- terminus binds directly to light meromyosin (LMM) and supports binding to titin, obscurin, four and a half LIM domains 1 (FHL1) protein, and creatine kinase (Okagaki et al. 1993; Freiburg and Gautel 1996; McGrath et al. 2006; Ackermann et al. 2009; Chen et al. 2011). The NH_2- terminus is also where the phosphorylation sites are located for the sMyBP-C - three sites have been identified in the Pro/Ala rich region, and one in the M-motif. Not all sites are present in all of the protein variants (Ackermann and Kontrogianni-Konstantopoulos 2011b, 2013). The effects of sMyBP-C phosphorylation are still being studied; however, it has been shown that phosphorylation levels are altered in response to stresses. They are significantly reduced in both slow and fast twitch muscles in the processes of ageing, dystrophy, and distal arthrogryposis, but they are increased in slow twitch muscles in response to fatigue (Ackermann et al. 2015).

Interestingly, while for another member of the MYBPC family - the cardiac form, more than 500 pathogenic variants have been described and associated with hypertrophic or dilated cardiomyopathy, first pathogenic variants for sMyBP-C have been reported only relatively recently (Landrum et al. 2018; Wang et al. 2018). The first reported pathogenic variants in the *MYBPC1* gene, and the majority of variants reported now are associated with autosomal dominant types of congenital distal arthrogryposis (DA, types 1B and 2) (Gurnett et al. 2010; Li et al. 2015). Additionally, recessive variants have been associated with Lethal Congenital Contracture Syndrome 4 and Arthrogryposis Multiplex Congenita (Markus et al. 2012;

Ekhilevitch et al. 2016). A zebrafish model of the human DA associated variants exhibited features similar to human patients - impaired motor unit excitation, disorganized myofibrils, reduced number of sarcomeres and severe body curvature (Ha et al. 2013). Our work, as part of the neuromuscular disease research project, as well as others, have recently linked protein domain specific (located in the M-motif) dominant variants in this gene to yet another phenotype - mild myopathy, associated with a persistent, posturally pronounced, high-frequency tremor (Shashi et al. 2019). Interestingly, a natural animal model of a calf, carrying a pathogenic variant in the same protein region, had been described previously, presenting with congenital muscle weakness and tremor (Wiedemar et al. 2015).

1.5. Myogenic tremor

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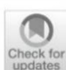

Sarcomeric myopathies associated with tremor: new insights and perspectives

Janis Stavusis[1,2] · Janelle Geist[2] · Aikaterini Kontrogianni-Konstantopoulos[2]

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Abstract
Myopathies are a large and heterogeneous group of disorders associated with mutations in structural and regulatory genes responsible for proper muscle assembly, organization and function. Despite the molecular diversity of inherited myopathies, they have historically been classified by the phenotypic traits observed in affected patients. It is therefore common for myopathies originating from mutations in different genes to be grouped together due to similar physical manifestations, and conversely myopathies resulting from mutations in the same gene to be considered separately due to disparate symptoms. Herein, we focus on an early onset myopathy linked to inherited or de novo mutations in sarcomeric genes that is characterized by muscle weakness, hypotonia and tremor, and further highlight that it may constitute a new form of myopathy, with tremor as its defining feature. Based on recent reports, we also discuss the possible myogenic origin of the tremor that may start at the level of the sarcomere due to structural and/or contractile alterations occurring as a result of the identified mutations. It is our hope that establishment of this form of myopathy accompanied by myogenic tremor as a new disease entity will have important diagnostic and therapeutic implications.

Keywords Sarcomeric genes · Congenital myopathy · Muscle weakness · Hypotonia · Tremor

Introduction

Myopathies constitute a large and heterogeneous group of neuromuscular disorders, associated with structural defects and/or contractile dysfunction of muscle fibers (Schorling et al. 2017). Although they may manifest as variable phenotypes of different severities affecting all body musculature (i.e. generalized myopathies) or limited muscle groups (i.e. distal or proximal myopathies), muscle weakness and/or wasting have been the hallmarks of myopathies (Schorling et al. 2017).

Janis Stavusis and Janelle Geist have contributed equally in the preparation of the review article.

✉ Aikaterini Kontrogianni-Konstantopoulos
akontrogianni@som.umaryland.edu

1 Latvian Biomedical Research and Study Centre, Ratsupites 1, Riga LV-1067, Latvia

2 Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene Street, Baltimore, MD 21201, USA

Prior to the genetic era, myopathies were mainly classified by phenotypic traits and/or histological findings of muscle biopsies (Schorling et al. 2017). However, during the last two decades there has been a major shift towards a genetically based classification, highlighting the congenital nature of myopathies and emphasizing the association of selective features with certain forms (Schorling et al. 2017). A tremor phenotype, defined as rhythmic, involuntary, oscillatory movement of a body part, may stem from a wide range of pathologies, most originating from abnormalities in central and/or peripheral/sensory circuits (Morales-Briceño et al. 2018). Herein, we focus on tremor as a distinctive phenotypic characteristic accompanying an early-onset myopathy originating from mutations in genes encoding sarcomeric proteins, likely due to structural and/or contractile alterations instead of neurological etiologies. Notably, such mutations are found in both thick and thin filament proteins, exhibit dominant or recessive inheritance, and affect both genders similarly. In particular, mutations in the thick filament proteins myosin heavy and light chains, and slow skeletal myosin binding protein-C (sMyBP-C) (Fig. 1 and Table 1), and the thin filament proteins troponin,

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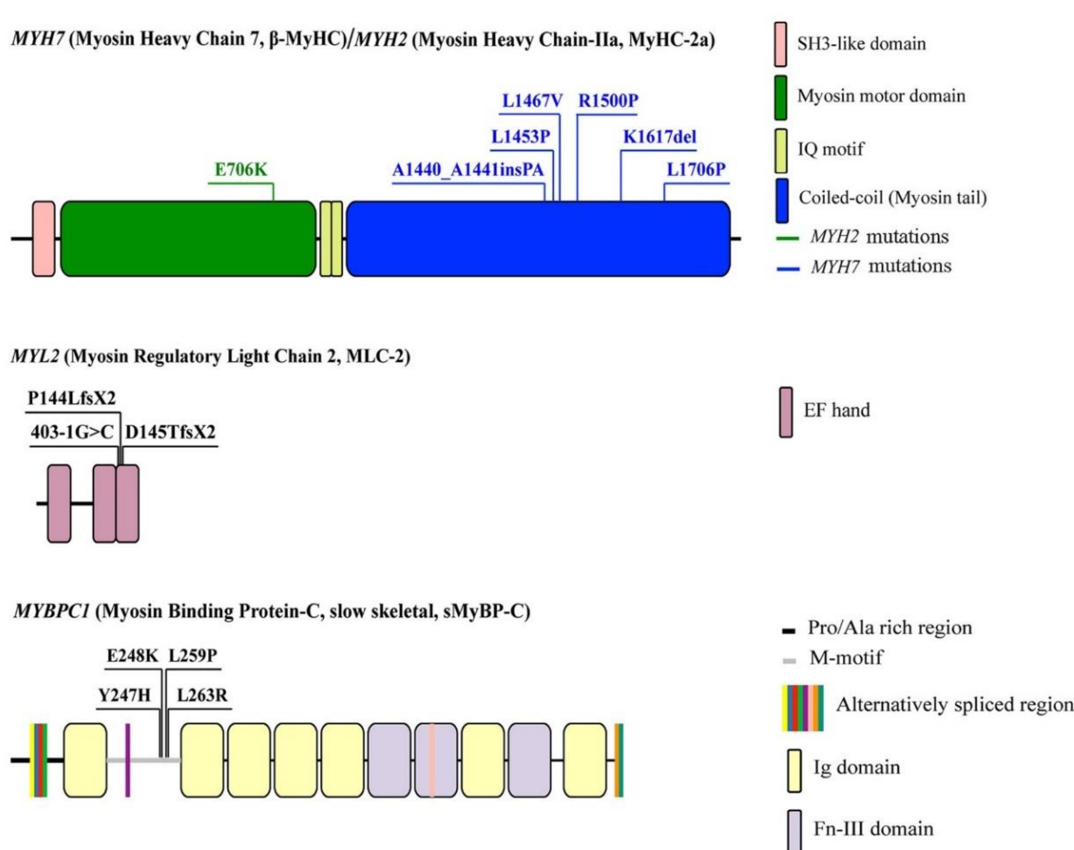

Fig. 1 Schematic of thick filament associated proteins showing the location of mutations co-segregating with myopathy characterized by tremor

tropomyosin and nebulin (Fig. 2 and Table 1) have been shown to lead to the development of myopathy with tremor.

Given that tremor pathogenesis has been traditionally associated with neurological defects (Morales-Briceño et al. 2018), its presence in myopathic patients and spontaneous or preclinical animal models in the absence of apparent neuropathy is intriguing and perplexing. The following is an overview of myopathic cases due to purely sarcomeric mutations, reporting tremor as a prominent feature, which we argue should be grouped as a novel, singular myopathic disease entity.

Thick filament associated mutations

Myosin heavy chain 7 (*MYH7*)

Campus syndrome-c.4320_4321insCCCGCC p.A1440_A1441insPA: One of the first cases of tremor causatively linked to a sarcomeric mutation was described as Campus syndrome in Pietrain pigs (Wissel et al. 1997). Affected pigs showed tremor in their extremities when standing and walking (Wissel et al. 1997), but not at rest (Richter et al. 1995; Murgiano et al. 2012). Further studies on piglets bred from the original swine father, Campus, determined that the syndrome was inherited in an autosomal dominant fashion and Campus was a gonadal mosaic founder (Murgiano et al. 2012). Onset occurred between 2 and 9 weeks of age and was progressive, with most pigs dying of heart attack before maturity (Murgiano et al. 2012). Additional phenotypic characteristics of the syndrome included muscle weakness throughout the body, postural instability, muscle fiber degeneration and interstitial fibrosis (Murgiano et al. 2012). Treatment with drugs targeting myoclonus, defined as involuntary muscle twitching, proved to be ineffective (Richter et al. 1995). No brain or peripheral nerve alterations were observed and motor nerve conduction velocity appeared to be unaltered (Richter et al. 1995; Murgiano et al. 2012),

Table 1 List of mutations in sarcomeric genes encoding thick and thin filament associated proteins linked to myopathy with tremor

Mutation	Organism	Inheritance	Number of patients with tremor
Thick filament associated proteins			
MYH7 (myosin heavy chain 7)			
A1440_A1441insPA	Pig	De novo/Inherited	Not applicable
L1453P	Human	Inherited	6
L1467V	Human	De novo	1
R1500P	Human	De novo	1
K1617del	Human	De novo/Inherited	5
L1706P	Human	De novo	1
MYH2 (myosin heavy chain-IIa)			
E706K	Human	Inherited	2
MYL2 (myosin regulatory light chain 2)			
403-1G>C	Human	Inherited	11
P144LfsX2/D145TfsX2	Human	Inherited	2
MYBPC1 (myosin binding protein-C, slow skeletal)			
Y247H	Human	Inherited	4
E248K	Human	Inherited	4
L259P	Human	De novo	1
L263R	Human	De novo/Inherited	3
L295R	Calf	De novo	Not applicable
Thin filament associated proteins			
TNNT1 (Slow skeletal troponin T)			
S108X	Human	Inherited	1
E180X	Human	Inherited	>70
L203X	Human	Inherited	9
NEB (nebulin)			
S8042X	Dog	Inherited	Not applicable
TPM3 (tropomyosin α-3 chain)			
ΔE224	Human	De novo	1

whereas semi-tendinous muscle electromyography (EMG) revealed a high amplitude tremor pattern of 14–15 Hz (Wissel et al. 1997).

Linkage mapping pinpointed a six-base pair insertion c.4320_4321insCCCGCC located in exon 30 of the *MYH7* gene, which segregated perfectly with the Campus syndrome phenotype in all pigs heterozygous for the mutant allele (Murgiano et al. 2012) (Fig. 1 and Table 1). The mutation causes an in-frame insertion of two amino acids, proline and alanine, in a highly conserved region of the protein.

MYH7 encodes slow skeletal myosin heavy chain 7, also known as β-myosin heavy chain, which is predominantly expressed in fetal skeletal muscle and in type 1 skeletal muscle fibers post-birth (Wang et al. 2018). In the heart, *MYH7* is switched on in response to physical stress. Interestingly, some carrier pigs reportedly died after stress from sudden heart attack (Murgiano et al. 2012).

Using Marcoil software that examines the existence and location of potential coiled-coil domains in proteins, it was predicted that the insertion of the proline and alanine residues disrupted the conserved coiled-coil region in the COOH-terminus of β-myosin heavy chain, likely affecting the assembly and stabilization of thick myosin filaments and thus contractility and force production (Murgiano et al. 2012).

It is noteworthy that in the Murgiano study (Murgiano et al. 2012), of the ten pigs affected with Campus syndrome, six were homozygous and one was heterozygous for the p.R615C mutation present in the *RYR1* gene encoding the Ca^{2+} release channel ryanodine receptor, that results in malignant hyperthermia syndrome (Fujii et al. 1991). This disease is associated with a stress susceptibility phenotype that is common in Pietrain pigs. Notably, the authors excluded any possible association between the p.R615C mutation and the development of Campus syndrome by the affected pigs, and further suggested that the presence of the *MYH7* mutation (*p.A1440_A1441insPA*) underlies the observed myopathic phenotype.

Laing distal myopathies-p.R1500P, p.K1617del, and p.L1706P: In 2006, Lamont et al. described eight cases, both

TNNT1 (Slow skeletal troponin T, sTnT)

E180X
S108X
L203X
Coiled coil

NEB (Nebulin)

S8042X

Glutamic rich region | Simple repeat | Super repeat (=7 simple repeats) | Serine rich region | SH3 domain

TPM3 (Tropomyosin alpha-3 chain)

ΔE224
Coiled coil

Fig. 2 Schematic depiction of thin filament associated proteins showing the location of mutations linked to myopathy accompanied by tremor

sporadic and familial, with different mutations in the *MYH7* gene, classifying them as distal myopathy-1 (MPD1), also known as Laing distal myopathy (Lamont et al. 2006) (Fig. 1 and Table 1). Out of these eight cases, one patient carried the p.R1500P mutation, five contained the p.K1617del mutation, and one carried the p.L1706P mutation, with tremor reported as a common and defining symptom. Although disease onset varied considerably among cases, patients with tremor exhibited early onset between 1 and 5 years of age. Early symptoms included distal foot muscle weakness followed by finger extensor weakness, often in tandem with tremor of the hands related to both posture and action (Lamont et al. 2006). Mild involvement of the face was noted, with weakness of orbicularis oculi and orbicularis oris, along with weakness of neck flexion. In some individuals the disease progressed to involve the truncal and proximal leg muscles, though the deterioration in strength was very gradual (over the course of many years), and none of the affected individuals were confined to a wheelchair (Lamont et al. 2006). Of note, the study by Lamont et al. mentioned tremor in five out of eight patients with the p.K1617del mutation (Lamont et al. 2006), while a later study by Fiorillo et al. described three (additional) patients carrying the same mutation, without mentioning the presence of tremor (Fiorillo et al. 2016). However, it is unknown if these patients did not develop tremor, or if it was simply not reported.

Examination of serum creatine kinase (CK) levels, an indicator of muscle damage, revealed great variability among patients, as some maintained normal levels while others showed an eightfold increase above the normal upper limit (Lamont et al. 2006). Muscle biopsy findings were also variable, revealing generalized myopathic changes, including predominance of type 1 or type 2 fibers among different families, excessive fiber size variation, small/atrophic type 1 fibers, necrotic and regenerating fibers, central nucleation, autophagic vacuoles in both fiber types, occasional "moth-eaten", "ring" or "whorled" fibers, and a minor degree of fiber splitting. In some samples, intermediate fibers could also be seen, suggesting progressive fiber type switching (Lamont et al. 2006). Interestingly, all three mutations are located in the coiled coil region of the light meromyosin tail domain of the myosin heavy chain β isoform, similar to the Campus mutations described above.

c.4358T p.L1453P: An additional case of Laing distal myopathy that co-segregates with a mutation in *MYH7* and is characterized by tremor was reported by Lefter and colleagues in an Irish family (Lefter et al. 2015). Specifically, six affected individuals were heterozygous for the c.4358T>C p.L1453P mutation located in exon 32, which is in the same COOH-terminal coiled coil light meromyosin tail region as the Campus and Laing mutations described above (Fig. 1 and Table 1). Mutations in this exon are often

associated with cardiomyopathy (Wang et al. 2018), which was observed in one of the carriers, while two other patients presented with respiratory muscle impairment. The disease manifested in early childhood, with patients exhibiting various degrees of proximal upper and lower limb weakness. Notably, two out of the six patients exhibited postural tremor, and three others were severely disabled by middle age. Muscle biopsy samples showed fiber size variation, increased amounts of central nucleation, and rimmed vacuoles (Lefter et al. 2015).

Multiminicore disease (MmD)-c.4399C>G p.L1467V: Two cases of multiminicore disease (MmD) were reported by Cullup et al. associated with two different heterozygous missense mutations in *MYH7*, c.4399C>G p.L1467V and c.4763G>C p.R1588P, which reside in exons 32 and 34, respectively (Cullup et al. 2012) (Fig. 1 and Table 1).

Of the two mutations, only c.4399C>G p.L1467V was associated with marked action tremor in late adulthood. The patient showed both dilated cardiomyopathy and skeletal muscle deficits with prominent distal weakness primarily in the lower limbs that developed throughout adulthood, and non-progressive proximal weakness, which affected mostly the upper limbs. Mild facial weakness was also noted. In addition to tremor, the patient developed cardiorespiratory failure, muscle wasting, contractures, scapular winging and spinal rigidity during late adulthood (Cullup et al. 2012). Muscle biopsies revealed an increase in the presence of central nucleation, fiber size variability, type 1 fiber predominance, "ring" fibers, numerous core structures with focal loss of enzyme activity, and modest sarcomeric disorganization (Cullup et al. 2012).

Similar to the aforementioned Campus and Laing associated *MYH7* mutations, the MmD mutations are located in the tail domain of β-myosin heavy chain, possibly disturbing the proper conformation of the coiled-coil region of the myosin heavy chain tail and/or impairing its binding interactions.

Myosin heavy chain-IIa (*MYH2*)

c.G2116>A p.E706K: In 2000, a Swedish family was identified with a progressive autosomal dominant myopathy manifesting as mild proximal muscle weakness with multiple joint contractures and opthalmoplegia (Martinsson et al. 2000). Using linkage analysis, radiation hybrid mapping and single-strand conformation polymorphism analysis, the disease locus was identified as a missense mutation, p.E706K, in exon 17 of myosin heavy chain II-a. Importantly, the E706 residue located within the SH1 helix of the MyHC motor head is 100% conserved in all known myosin classes (Cope et al. 1996; Tajsharghi et al. 2004).

In six patients of the multi-generation Swedish carrier family (Martinsson et al. 2000), type 2A fibers were small and infrequent, while other fiber types appeared normal. Moreover, rimmed vacuoles were frequent with inclusions of 15–20 nm tubulofilaments (Martinsson et al. 2000). Although no tremor was described in the original report that identified the p.E706K mutation (Martinsson et al. 2000), a 2004 study by Tajsharghi and colleagues noted that two patients in the family exhibited hand tremor in addition to muscle weakness and contractures (Tajsharghi et al. 2004). While MyHC mutations may exert dominant negative effects on muscle function by altered myosin-actin interactions, defects in myosin ATPase activity, or interference with filament assembly and sarcomeric structure, the exact effect of the p.E706K mutation has yet to be elucidated (Martinsson et al. 2000).

Myosin regulatory light chain 2 (*MYL2*)

c.403-1G>C, c.431delC p.P144LfsX2, and c.432delT p.D145TfsX2: Eleven affected infants in eight Dutch families and two affected infants in an Italian family with similar clinical and biopsy findings were reported by Weterman et al. in 2013 exhibiting a cardioskeletal myopathy with onset and death during early infancy (four to six months of age) (Weterman et al. 2013). At birth, all patients were vigorous with no obvious signs of cardiac or skeletal muscle pathology with the exception of generalized, high amplitude tremor. The tremor was present while awake but absent during sleep and slowly abated over a period of weeks. Within weeks after birth, rapid and progressive generalized muscle weakness was noted in patients, including presentation with tented mouth or global facial muscle involvement, including facial palsy and ptosis. All patients developed cardiomyopathy, mainly dilated, which led to heart failure and death during infancy. Autopsies performed on deceased patients and a sural nerve biopsy showed no indication of central nervous system (CNS) involvement, which was therefore excluded as the primary causation of the observed tremor phenotype (Weterman et al. 2013).

Genetic investigation revealed a homozygous missense mutation (c.403-1G>C) in the *MYL2* gene located on chromosome 12 in all of the Dutch patients (Weterman et al. 2013) (Fig. 1 and Table 1). The mutation affects the last acceptor splice site in the gene, leading to usage of a cryptic splice site upstream from the last exon (exon 7) that results in frameshift and replacement of the last 32 codons by 20 new codons. Notably, the Italian patients that exhibited similar pathology and phenotypic features were found to be compound heterozygous for two different mutations in *MYL2* (c.431delC p.P144LfsX2 and c.432delT p.D145TfsX2) within the same exon (exon 7) that also leads to a frameshift, premature termination, and truncation of 20 amino acids (Weterman et al. 2013) (Fig. 1 and Table 1). Thus, the mutations identified in both the Dutch and Italian patients appear

to alter the COOH-terminus of myosin regulatory light chain 2 in a similar fashion.

The main histological findings observed in skeletal muscle biopsy samples from all affected patients included fiber-type disproportion with type 1 fiber hypotrophy, myofibrillar lysis, sarcomeric disorganization and loss of sarcomere register. Notably, the mutant protein showed diffuse and weak expression (Weterman et al. 2013).

Myosin regulatory light chain 2, encoded by *MYL2*, binds to myosin heavy chain 7 (encoded by *MYH7)* and has both structural and regulatory roles in muscle contraction (Wang et al. 2018). In both the Dutch and Italian families, the identified mutations affect one of the EF-hand domains, suggested to serve as Ca^{2+} sensor and modulator of Ca^{2+} signaling, that undergoes conformational change upon Ca^{2+} binding allowing for interactions with downstream targets (Wimberly et al. 1995; Grey et al. 2005). Thus, it is likely that the two mutations may impact thick filament assembly and maintenance and/or actomyosin contractility.

Myosin binding protein-C, slow skeletal (*MYBPC1*)

c.739T>C p.Y247H, c.742G>A p.E248K, c.776T>C p.L259P, and c.788T>G p.L263R/p.L295R: Our team recently described a myopathy associated with novel mutations in the *MYBPC1* gene which encodes Myosin Binding Protein-C slow (sMyBP-C) (Ackermann and Kontrogianni-Konstantopoulos 2011, 2013). Previously, mutations in *MYBPC1* were solely associated with severe and lethal forms of distal arthrogryposis (DA) (Geist and Kontrogianni-Konstantopoulos 2016). The encoded protein belongs to a family of sarcomeric accessory proteins consisting of three distinct isoforms that contribute to the stabilization of thick filaments and the regulation of cross-bridge cycling (Martyn 2004; de Tombe 2006; Ackermann and Kontrogianni-Konstantopoulos 2013; Ackermann et al. 2013). *MYBPC1* is located on chromosome 12 and is heavily spliced giving rise to at least fourteen slow variants co-expressed in slow- and fast-twitch skeletal muscles in different ratios (Weber et al. 1993; Ackermann and Kontrogianni-Konstantopoulos 2013; Ackermann et al. 2015). Interestingly, the four reported mutations reside in constitutively expressed exons within the NH_2-terminal M-motif of the protein that contributes to the dynamic binding of sMyBP-C with actin and myosin S2 and the modulation of actomyosin cross bridges in the force generating cycle (Ackermann et al. 2013) (Fig. 1 and Table 1).

The c.739T>C p.Y247H and c.742G>A, p.E248K mutations were identified in several members of two independent families from Germany and Latvia, respectively, with an autosomal dominant pattern of inheritance (Stavusis et al. 2019). The carriers display early-onset, predominantly axial muscle weakness, hypotonia, contractures in the knees and elbows, skeletal deformities (scoliosis), spinal rigidity and thoracic asymmetry as well as lumbar hyperlordosis. Tremor of high frequency and irregular amplitude was also reported, primarily in the hands, which was exacerbated by posture; notably, the tremor was obvious since birth (Stavusis et al. 2019). Patients with the p.Y247H mutation also presented with tongue tremor. EMG results revealed a myopathic pattern without spontaneous activity and a high frequency postural tremor (10 Hz) in the arms and hands. Clinical symptoms progressed slowly until adolescence and then reached a plateau phase, with all patients displaying moderate levels of disabilities and ambulation difficulties. Histopathological examination of muscle biopsies revealed generalized myopathic changes, including an increase of type 1 fibers (Stavusis et al. 2019).

In vitro biochemical studies showed significantly increased binding of both mutant proteins to heavy meromyosin (Stavusis et al. 2019). This was further corroborated by computational modeling indicating that both the p.Y247H and p.E248K mutations introduce additional positive charges in a stable 3-helix bundle of the M-motif, therefore increasing the number of favorable electrostatic interactions with the predicted (negatively charged) binding site on myosin (Stavusis et al. 2019). However, it is noteworthy that the charge of the histidine sidechains can be greatly affected by even small changes in pH, therefore making it more difficult to predict the exact effect of the p.Y247H mutation in vitro (Li and Hong 2011). The purported increase in electrostatic binding between mutant sMyBP-C proteins and myosin may result in the formation of abnormal and deregulated cross-bridges, which in addition to causing a deficit in force production underlying muscle weakness, may act as the primary pacemaker of the observed tremor.

Similar to the p.Y247H and p.E248K mutations, the c.776T>C p.L259P and c.788T>G p.L263R mutations result in a myopathic phenotype that includes severe congenital hypotonia, muscle weakness and tremor at rest, posture, and on intention in extremities (Shashi et al. 2019). The c.776T>C p.L259P mutation was identified in a Caucasian individual in the US as a de novo mutation, whereas the c.788T>G p.L263R mutation was identified in a Caucasian individual in the US as a de novo mutation and in a father/daughter pair in a Korean family as an autosomal dominantly inherited trait (Shashi et al. 2019). Patients with either mutation exhibit dysmorphic features, including downslanting palpebral fissures and hypotonic facies. Interestingly though, the p.L259P and p.L263R carriers do not develop (obvious) contractures or skeletal deformities, but show facial and tongue tremor. The father/daughter Korean family with the p.L263R mutation also exhibited a waddling gait and EMG revealed myopathic motor unit action potential (MUAP) but normal resting potential (Shashi et al. 2019).

Surface plasmon resonance kinetics analysis demonstrated that the p.L263R mutation results in significantly decreased binding to myosin, whereas the p.L259P mutation modestly enhances myosin binding (Shashi et al. 2019). Computational modeling suggested that contrary to the hydrophobic and neutral leucine, the hydrophilic and charged arginine does not pack well into the core of the 3-helix bundle likely destabilizing the M-motif of sMyBP-C (Shashi et al. 2019). As such, R263 binds strongly to E248 (which is not the case for the original L263) possibly interfering with (i.e. diminishing) the ability of E248 to contribute to myosin binding. On the other hand, computational modeling and circular dichroism indicated that the p.L259P mutation affects the structure of the M-motif by reducing its helicity, potentially rendering the mutant protein relatively unstable (Shashi et al. 2019). Consistent with this notion, mutant sMyBP-C carrying the p.L259P mutation is more susceptible to trypsin cleavage compared to wild-type protein (Shashi et al. 2019). Thus, it is possible that although the p.L259P mutation affects helix-defining hydrogen bonds, the substitution is chemically subtle enough to moderately affect myosin binding, and instead result in shorter half-life of the protein in vivo.

Interestingly, the p.L263R mutation corresponds to a previously reported mutation, p.L295R, in a 2-week old calf that exhibited tremor since birth, standing difficulty, and reduced spinal reflexes (Wiedemar et al. 2015) (Fig. 1 and Table 1). Normal musculoskeletal development was noted including absence of atrophy, but the animal displayed ataxia and hypermetria in addition to tremor while standing. Consciousness was normal and cranial nerve examination did not reveal any deficits, but no EMG, nerve or muscle biopsy data were collected. Similar to the identified human patients with the *MYBPC1* mutations, the functional effects of the amino acid change were predicted to be intolerable and damaging for normal muscle structure/function by in silico predictors (Wiedemar et al. 2015; Shashi et al. 2019).

Thin filament associated mutations

Slow skeletal troponin T *(TNNT1)*

Nemaline myopathy-c.G579T p.E180X: In humans, six genes have been associated with the development of nemaline myopathies (NM) (Ottenheijm et al. 2011), establishing it as one of the most common non-dystrophic congenital myopathies (Johnston et al. 2000; Ottenheijm et al. 2011). Amish nemaline myopathy (ANM), common among the Old Order Amish of Lancaster County, PA, is inherited in an autosomal recessive fashion as a distinct form of nemaline myopathy caused by mutations in *TNNT1*, which encodes slow skeletal troponin T (TnT) (Johnston et al. 2000).

Newborn babies with ANM develop tremor within a few days after birth, followed by progressive muscle weakness, rigidity and contractures (Johnston et al. 2000). Some patients also reportedly displayed abnormal tremor in utero. Although tremor subsides over the first 2 to 3 months of life, proximal contractures and muscle atrophy worsen with age to the point that affected children develop severe pectus carinatum deformity with chest wall rigidity and die of respiratory insufficiency, usually by 2-years of age (Johnston et al. 2000; Jin et al. 2003). Muscle biopsies from four ANM patients obtained within the first year of their life displayed prominent type 1 fiber disproportion in quadriceps muscle, Z-band streaming, central refractile rods, myofibrillar disruption, and myofiber degeneration (Johnston et al. 2000).

Examination of more than seventy affected children indicated that the described pathology associates with the presence of a homozygous nonsense mutation in exon 11 of *TNNT1*, c.G579T, that results in a stop codon at amino acid 180, p.E180X, which is present in all known isoforms of *TNNT1* (Fig. 2 and Table 1). Troponin T (tropomyosin binding, TnT) links the troponin complex to tropomyosin and determines Ca^{2+} responsiveness during muscle contraction (Jin et al. 2003; Galińska-Rakoczy et al. 2008). Upon depolarization of the muscle cell, Ca^{2+} released into the cytoplasm binds to troponin C (TnC, Ca^{2+} sensor) resulting in a series of conformational changes that release the constraint exerted by troponin I (TnI, inhibitory) on tropomyosin (Tm), so that the latter no longer inhibits actomyosin binding allowing contraction to occur.

The new stop codon introduced as a result of the p.E180X mutation leads to truncation of the 83 most COOH-terminal amino acids of TnT, which support binding to TnI and TnC and provide a second interaction site for Tm (Johnston et al. 2000). This truncation results in complete loss of the protein, either by accelerated nonsense-mediated decay or decreased stability of the mutant protein fragment (Jin et al. 2003). A selective loss of slow thin filaments was noted in immunohistochemical staining of quadriceps muscle from a 7-week old ANM patient, indicating that TnT may not only be essential for Ca^{2+} regulation of contraction, but also for muscle development and maintenance (Jin et al. 2003). Importantly, in *C. elegans*, truncating or null recessive mutations in the mup-2 gene encoding TnT, result in abnormal body wall muscle twitching and hyper-contraction, suggestive of the tremor phenotype seen in ANM patients (Myers et al. 1996).

c.323C>G p.S108X: In 2015, Marra et al. reported a case of NM with tremor in a non-Amish patient also caused by a mutation in the *TNNT1* gene (Marra et al. 2015). The proband was born to consanguineous parents and presented with neonatal tremor and torticollis. Low muscle tone and lack of head control was noted at 4 months of age. By two and a half years, the patient was referred to a hospital due to hypotonia and developmental delay. Full examination

revealed generalized muscle weakness (proximal more than distal) with tendon reflexes absent in all limbs, high arched palate, pectus carinatum, thoracic kyphoscoliosis and bilateral hip and knee contractures. Dysarthria and facial weakness with tenting of the upper lip and axial weakness with prominent head lag was also reported. Soon after the examination, the patient developed chronic respiratory insufficiency and dysphagia (Marra et al. 2015).

Next generation sequencing (NGS) revealed a homozygous nonsense variant c.323C>G p.S108X in the *TNNT1* gene (Fig. 2 and Table 1). The mutation leads to the generation of a truncated protein that lacks the last 155 amino acid residues (Marra et al. 2015). The missing region contains binding sites for TnI, TnC and Tm, and is therefore crucial for the correct assembly of the Tn complex as well for the regulation of Tm via the Tn complex.

Histological evaluation of muscle biopsies revealed fiber size variation due to the presence of both atrophic and mildly hypertrophic fibers, as well as eosinophilic granular intracytoplasmic inclusions in both type 1 and 2 fibers (Marra et al. 2015). Contrary to previous observations of type 1 fiber hypotrophy in *TNNT1* NM cases (Johnston et al. 2000), type 2 fiber hypotrophy was noted in this patient (Marra et al. 2015). Additional findings included mild increases in central nucleation, a few necrotic and regenerating fibers, some infiltration of chronic inflammatory cells in foci of myonecrosis, and marked endomysial fibrosis.

c.574_577delinsTAGTGCTGT p.L203X: Additional patients of non-Amish origin with NM caused by a mutation in *TNNT1* associated with tremor were described in 2016 by Abdulhaq et al. The authors used the term congenital nemaline body myopathy (NEM) when describing seven families and a total of nine children of Palestinian origin with this disorder, arguing that use of NEM rather than ANM would be more appropriate in the future (Abdulhaq et al. 2016).

Most patients presented with transient tremor of the limbs and occasional mouth tremor during the neonatal period. Motor developmental delay and poor head movement associated with a stiff neck was noted at three to four months of age. Beyond four months, all patients developed progressive spinal rigidity with kyphosis, scoliosis, and limb contractures. All patients had severely delayed motor milestones and were wheelchair bound. Additionally, facial weakness and dolichocephaly was observed. The majority of the affected individuals developed respiratory insufficiency leading to death within the first decade of their lives, while living subjects required assisted mechanical ventilation (Abdulhaq et al. 2016).

In all cases, a previously undescribed homozygous rearrangement in *TNNT1* was found. The mutation was reported as an insertion/deletion, c.574_577delinsTAGTGCTGT, that resulted in an in-frame stop codon and the generation of a truncated protein lacking the last 76 amino acids (Fig. 2 and Table 1), which encompass the previously described TnI and TnC binding sites (Abdulhaq et al. 2016). The authors described a shared short haplotype segment around this mutation, leading them to hypothesize that this is an ancient founder mutation originating from a common ancestor, despite no apparent relation between the currently identified families.

Standard laboratory tests indicated normal CK levels and no cardiac involvement. Evaluation of muscle biopsies indicated generalized myopathic changes, with nemaline rods detected in most samples, yet in low numbers (Abdulhaq et al. 2016). Small groups of atrophic myofibers, abundant whorled-like myofibers, focally increased endomysial fibrosis and type 2 fiber predominance with small type 1 fibers were the most common features observed in patient samples (Abdulhaq et al. 2016).

Nebulin *(NEB)*

Nemaline myopathy-c.52734272 C>A p.S8042X: Recently, tremor-associated mutations were linked with a novel form of NM in American BullDogs (ABD). A five-month old male ABD presented with non-progressive muscle weakness, exercise intolerance, and tremor beginning at 2 months of age (Evans et al. 2016). Atrophy of limb muscles and hypertrophy of triceps muscles were noted. EMG showed spontaneous electrical activity within proximal appendicular muscles of the thoracic limbs and cervical paraspinal musculature that consisted mainly of fibrillation potentials. There was also a mild decrease in latency of the tibial and ulnar nerves as shown by motor nerve conduction velocity tests (Evans et al. 2016).

Whole exome sequencing was performed on the dam, two affected siblings (one of which was the proband), and one unaffected sibling leading to the identification of a nonsense mutation c.52734272 C>A, p.S8042X in exon 169 of the *NEB* gene (Fig. 2 and Table 1) (Evans et al. 2016), encoding the giant, thin filament protein, nebulin (Kontrogianni-Konstantopoulos et al. 2009). The disease characteristics were not seen in either parent of the affected dog, indicating that they were most likely heterozygous for the mutation, and inheritance was autosomal recessive. Characteristic to NM, rod-like structures were observed in both slow and fast twitch fibers. The absence of NH_2-terminal epitopes of nebulin was confirmed in whole tissue gel electrophoresis and western blotting, suggesting nonsense mediated decay (Evans et al. 2016).

Tropomyosin α-3 chain *(TPM3)*

c.673-675delGAA p.ΔE224: Contrary to most of the mutations discussed herein which lead to muscle weakness, a p.ΔE224 mutation present in *TPM3* encoding tropomyosin

α-3 chain (slow α-tropomyosin) (Fig. 2 and Table 1), results in a hypercontractile phenotype with congenital muscle stiffness (Donkervoort et al. 2015).

The p.ΔE224 mutation was described as a de novo mutation in *TPM3* in a 4-year old male who displayed decreased fetal movement, stiffness at birth with arthrogryposis multiplex congenita (AMC), contractures, kyphoscoliosis and fine tremor (Donkervoort et al. 2015). EMG was suggestive of generalized myopathy, and severe reduction in the response amplitude of the right peroneal nerve. The c.673-675delGAA p.ΔE224 mutation was determined via whole exome sequencing in heterozygous form. Gomori trichrome stain of a muscle biopsy showed myofibrillar irregularities with evidence of mitochondrial accumulation.

In vitro motility assays indicated that the p.ΔE224 mutation resulted in increased Ca^{2+} sensitivity for both the fraction motile and sliding speed, indicating gain of function (Donkervoort et al. 2015). In agreement with these findings, permeabilized fibers isolated from muscle biopsies exhibited enhanced Ca^{2+} sensitivity of force generation compared to controls. Cross bridge kinetic studies further showed reduced cycling in the presence of the p.ΔE224 mutation, suggesting that it likely destabilizes the "off" state of the actin-tropomyosin complex, shifting the equilibrium towards the "on" state that favors actin-myosin binding, and increases Ca^{2+} sensitivity, therefore leading to the observed hypercontractile phenotype (Donkervoort et al. 2015).

Conclusions and perspectives

Recently, it has become increasingly clear that tremor is a consistent and possibly defining feature of myopathies originating from mutations in sarcomeric genes in the absence of apparent neuropathy. The purpose of our review was to gather and present all the published reports of myopathic cases that describe tremor as a prominent phenotypic trait. Interestingly, with the exception of *MyHC-IIa* and *TPM3* that exhibit low amounts in brain, yet significantly greater levels in muscle, the remaining affected genes are solely expressed in muscle (Uhlen et al. 2015). We therefore propose that the observed tremor may be of myogenic origin, starting at the level of the sarcomere due to structural alterations that impact myofilament assembly, stability and/or sliding resulting in oscillatory actomyosin movement during the crossbridge cycle. This oscillatory movement may be picked up by interspersed muscle spindles, which serve as mechanoreceptors to provide proprioceptive sensory information to the brain, and undergo centrally looped propagation and enhancement resulting in the generation of the observed tremor (Stavusis et al. 2019).

In several patients, the tremor subsides with age, but is still apparent with posture and/or physical exertion. It is therefore possible that CNS plasticity may allow for the development of adaptive or compensatory mechanisms by fine-tuning and adjusting motor unit control and outcome (Edgerton et al. 2002). Moreover, a certain degree of intra- and inter-familial heterogeneity is observed with regards to the severity of the tremor phenotype. Although the cause of this variability is unknown, yet not uncommon in myopathies, it is likely that genetic (i.e. penetrance and expressivity of the mutant allele) and idiosyncratic factors (i.e. unique to the individual) may contribute to it (Mah and Joseph 2016).

Taken together, we propose to establish this form of myopathy accompanied by tremor, of likely myogenic origin, as a new disease entity with the ultimate goal to improve diagnosis and develop more appropriate and effective therapies. As we are just in the beginning of understanding the molecular and cellular alterations that occur in myopathies with tremor due to mutations in sarcomeric genes (in the absence of neuropathy), generation of the respective preclinical models followed by detailed phenotypic, behavioral, morphological and functional assessment is required. It is our hope that in the immediate future, an emphasis will be given by the muscle scientific community in dissecting the complexity and intricacies of this new form of sarcomeric myopathy with tremor.

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Compliance with ethical standards

Conflict of interest The authors have no conflict to declare.

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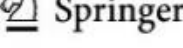

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Internet resources

Human Protein Atlas available from www.proteinatlas.org

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2. MATERIALS AND METHODS

2.1. Subjects

In all cases from Latvia, complete medical history has been collected and all patients have been examined, and their muscle defects evaluated by a neurologist and a clinical geneticist, experienced in neuromuscular disorders.

Data collection was performed in accordance with the permission issued by the Central Medical Ethics Committee of Latvia. All participants or their parents/ legal guardians (in case of children under the age of consent in the respective country) have signed an informed consent form.

During the years 2009-2021, approximately 130 index patients from Latvia, exhibiting NMD disease phenotype, have been enrolled in our research and diagnostic projects. In addition, 59 index patients from Lithuania, exhibiting LGMD disease phenotype, have been enrolled, as a part of scientific collaboration with the Vilnius University Hospital, Santariskiu Clinics. 19 index patients from the Latvian patient group were selected and enrolled in the MYO-SEQ project, based in University of Newcastle, United Kingdom, with additional family members added to our project for segregation analysis, when needed. Out of these, the following groups have been involved in this work.

[*CAV3 gene sequence variations: National Genome Database and clinics*]

This patient group consists of 81 index patients with clinical symptoms of Limb-Girdle Muscular Dystrophy (LGMD) and an autosomal dominant or sporadic inheritance pattern, 40 of them from Latvia and 41 from Lithuania. Additional samples from patient family members were included to perform segregation analysis, following pathogenic variant identification. All patients from Latvia were recruited at the Neurology department, Children's University Hospital, and the Out-Patients Clinic for Neurological Diseases, The Pauls Stradins Clinical University Hospital (Riga, Latvia), and all patients from Lithuania - from the Center for Medical Genetics, of the Vilnius University Hospital, Santariskiu Clinics (Vilnius, Lithuania). Both Latvian and Lithuanian patients presented with predominant pelvic and shoulder girdle muscle weakness as well as elevated creatine kinase (CK) values.

97 individuals with a clinical diagnosis of cardiomyopathy were selected from the Genome Database of Latvian Population. Out of these 49 had a diagnosis of dilated cardiomyopathy (DCM), 16 a diagnosis of hypertrophic cardiomyopathy (HCM) and 32 patients had unspecified and other cardiomyopathies.

The control group consisted of a total of 100 randomly selected healthy, unrelated individuals from the Genome database of Latvian population, representing the general population, age and sex matched to the patient group.

[*Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies*]

This patient group consists of 60 index patients with clinical symptoms of Limb-Girdle Muscular Dystrophy (LGMD), 26 of them from Latvia and 34 from Lithuania. Additional samples from patient family members were included to perform segregation analysis, following pathogenic variant identification. These patients are from the same group as described previously. Same as previously, patients who presented with predominant pelvic and shoulder girdle muscle weakness as well as elevated CK values were chosen. An additional criterion for patients from Latvia was electromyography data demonstrating myopathy or myogenic impairment.

The control group consisted of a total of 394 randomly selected healthy, unrelated individuals from the Genome database of Latvian population, representing the general population, age and sex matched to the patient group. In addition, 186 samples from Latvia and 175 samples from Lithuania representing ethnic groups (ancestry from the last three generations can be traced archaeologically and ethno-linguistically to distinct regions of Latvia and Lithuania) were used.

[*Collagen VI-related limb-girdle syndrome caused by frequent mutation in COL6A3 gene with conflicting reports of pathogenicity*]

The family of probands 1 and 2 was recruited within the framework of our neuromuscular disease research project, targeting all unrelated individuals with predominant pelvic and shoulder girdle muscle weakness and elevated creatine kinase (CK) values.

The family of proband 3 was recruited during a regular visit to Medical Genetics Clinic, CHUL-CHUQ in Quebec City, Canada.

Subjects included from the previous publications by Panadés-de Oliveira *et al.* were retrospectively recruited from the database of the Neuromuscular Disorders Unit of Hospital Universitario 12 de Octubre in Madrid (Panadés-de Oliveira et al. 2019). These patients were re-evaluated and their clinical information as well as MRI data from three patients were added.

Clinical information about proband 7 was collected from the publication by Hunter *et al.*, where they recruited children with clinically diagnosed myopathies that have consented for

participation in research study, according to the Western Institutional Review Board approved protocol (Hunter et al. 2015).

[*Novel mutations in MYBPC1 are associated with myogenic tremor and mild myopathy*]

Initially, from family 1 (Latvia), four individuals were included in the project, with the goal of performing exome sequencing - three affected and one unaffected, from two generations. To perform segregation analysis in the extended family, five healthy and one affected individual were additionally included. The patients represent three generations. In addition, 90 randomly selected healthy, unrelated individuals from the Genome database of Latvian population, representing the general population were used in this project.

Regarding family 2 from Germany, initially only one affected individual was included to perform panel sequencing. In order to perform segregation analysis in the extended family, three healthy and three affected individuals were added. The patients represent four generations. Genetic studies in individuals from family 2 were performed with informed consent, in accord with the Human Genetic Examination Act (Genetic Diagnosis Act-GenDG).

2.2. Direct sequencing

Most of the direct sequencing was performed using standard polymerase chain reaction (PCR) enzyme mix (Solis BioDyne, Tartu, Estonia), except a 1.7 kb fragment amplification of the *FKRP* gene, performed with Long PCR Enzyme Mix (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing reactions were carried out using standard conditions as suggested by the manufacturer. An automatic genetic analyzer (ABI PRISM 3130xl, Applied Biosystems, Foster City, CA, USA) was used in all cases, in combination with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

The data acquired were analyzed using the CLC Genomics Workbench software (QIAGEN, Venlo, Netherlands). The possible pathogenicity of identified sequence variations was determined using the *in-silico* prediction programs: Polymorphism Phenotyping-2 (PolyPhen-2), SIFT, PROVEAN and Combined Annotation Dependent Depletion (CADD). For SNPs located in the coding region, conservation levels were assessed based on data from the UCSC database (Genome Browser, Human Dec. 2013 (GRCh38/hg38) Assembly).

To increase the diagnostic value of testing, segregation analysis was performed and the whole family screened where possible.

[*CAV3 gene sequence variations: National Genome Database and clinics*]

Sequencing of both coding exons of the *CAV3* gene as well as the exon–intron boundaries was performed in all project sample groups.

[*Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies*]

The genomic regions of pathogenic variants c.550delA and c.191dupA in genes *CAPN3* and *ANO5*, respectively, as well as a 1.7kb fragment of the *FKRP* gene, containing exon 4 (including exon-intron boundaries) and the entire protein coding region of the gene were sequenced in the Latvian and Lithuanian project groups. In addition, all variants identified by microarray genotyping were verified by direct sequencing for respective patients and their family members, when available.

[*Collagen VI-related limb-girdle syndrome caused by frequent mutation in COL6A3 gene with conflicting reports of pathogenicity*]

[*Novel mutations in MYBPC1 are associated with myogenic tremor and mild myopathy*]

Next generation sequencing variant validation in *COL6A3* and *MYBPC1* genes as well as segregation analysis were performed using nested PCR.

2.3. Microarray genotyping

At the time the brand new and highly parallel genome-wide VeraCode GoldenGate system (Illumina, San Diego, CA, USA) was used as the platform for variant screening. The panel of LGMD-2 associated variants, to be tested, was selected manually, using open-access databases and publications. The variant selection was based on their pathogenicity, location within genes, allele frequency and population specificity. In addition, the variants selected had to comply with the systems internal criteria - single-nucleotide diallelic variants only, in order to ensure allele specific hybridization, extension, and ligation, furthermore there had to be a distance of more than 60 base pairs between any two selected variants to prevent overlapping oligonucleotides during hybridization. This led to a list of 96 variants to be tested.

All LGMD patient and general population samples were genotyped for the selected variants, using the VeraCode GoldenGate system on a BeadXpress reader (Illumina, San Diego, CA, USA). Genotyping was performed using the standard conditions. To ensure quality control and to evaluate the intra-subject concordance rate, each sample was run in duplicate in one genotyping run.

2.4. Next generation sequencing

[*Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies*]

Ion Torrent’s AmpliSeq (Thermo Fisher Scientific, Waltham, MA, USA) targeted gene enrichment and sequencing technology was performed on four patient samples, according to the manufacturer's protocol. The commercially available AmpliSeq Inherited Disease Panel (Thermo Fisher Scientific, Waltham, MA, USA) that covers 325 genes associated with the most common inherited diseases, including LGMD and other muscle dystrophies, was used for this approach.

The panel consists of three primer pools, used for PCR amplification, followed by treatment with the FuPa reagent (Thermo Fisher Scientific, Waltham, MA, USA) to partially digest primer sequences and phosphorylate the amplicons, which were then ligated to multiplexing barcodes (Thermo Fisher Scientific, Waltham, MA, USA). The created library was purified using AMPure XP magnetic beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA) and amplified using the PCR assay provided, followed by another purification.

The sequencing of the created libraries was done using the Ion Torrent PGM device (Thermo Fisher Scientific, Waltham, MA, USA).

[*Collagen VI-related limb-girdle syndrome caused by frequent mutation in COL6A3 gene with conflicting reports of pathogenicity*]

In collaboration with the MYO-SEQ project, whole exome sequencing was performed for proband of family 1 at the Broad Institute’s Genomics Platform, using Illumina exome capture, 38 Mb baited target, and the Broad’s in-solution hybrid selection process.

The sequencing pipeline consisted of sample plating, library preparation (2-plexing of samples per hybridization), hybrid capture, sequencing (76 bp paired reads), sample identification QC check, and data storage. Hybrid selection libraries cover >80% of targets at 20x and have a mean target coverage of >80x.

Exome sequencing data was processed through a pipeline based on Picard, using base quality score recalibration and local realignment at known indels. BWA aligner was used for mapping reads to the human genome build 37 (hg19). Single Nucleotide Polymorphism (SNPs) and insertions/deletions (indels) were jointly called across all samples using the Genome Analysis Toolkit (GATK) HaplotypeCaller package (version 3.1). Default filters were applied to SNP and indel calls using the GATK Variant Quality Score Recalibration (VQSR) approach. Lastly, variants were annotated using Variant Effect Predictor (VEP).

[*Novel mutations in MYBPC1 are associated with myogenic tremor and mild myopathy*]

Exome sequencing for 4 members of family 1 from Latvia was performed using the Illumina's HiSeq 2500 platform (Illumina, San Diego, CA) and the Nextera Rapid Capture Expanded Exome kit (Illumina, San Diego, CA). Three affected and one unaffected member samples were sequenced to improve the possibility of finding pathogenic variants, by using variant segregation analysis.

Raw sequencing reads were aligned to the human genome build 37 (hg19) and subsequently processed according to Genome Analysis Toolkit's (GATK) best practice guidelines using BWA-MEM, Picard-tools, and GATK. Variants were called by GATK Haplotype Caller and then annotated using Annovar. A stepwise approach of filtering was used on the obtained data, leaving only rare variants.

Pathogenicity of remaining variants was evaluated using several *in-silico* prognostic tools, including PolyPhen2, Mutation Taster, SIFT, and CADD.

2.5. Recombinant protein generation and purification

In order to be used for the overlay assays, human skeletal muscle complementary DNA was used to amplify the NH2-terminal region of sMyBP-C that contained the Pro/Ala–rich motif, Ig domain C1, and the M-motif (amino acids 1–284, XP_006719468.1). The PCR fragment was subcloned into the pGex4T-1 (Amersham Pharmacia, Piscataway, NJ) vector at EcoRI/XhoI sites to generate a GST-fusion protein. The pathogenic variants Y247H and E248K were subsequently introduced into the resulting plasmid. Wild-type and mutant constructs were verified by sequence analysis. Recombinant polypeptides were expressed by induction with 1mM isopropyl-β-thioglycopyranoside (IPTG) overnight at 22°C and purified by affinity chromatography on glutathione-agarose columns, according to the manufacturer's instructions (MilliporeSigma, Bedford, MA).

2.6. Overlay Assay and Immunoblotting

Heavy meromyosin (HMM) and actin purified from skeletal muscle were used (Cytoskeleton Inc., Denver, CO, USA). Equivalent amounts (3μg) of purified actin and HMM were separated by 4% to 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a nitrocellulose membrane. Equivalent loading and transfer were confirmed by staining the nitrocellulose membrane with Ponceau red. Blots were incubated in buffer A (50mM KCl, 20mM MOPS, 4mM MgCl2, 0.1mM EGTA, 2mM DTT, 3% BSA, 10mM NaN3, 0.5% Tween-20, and 0.5% Nonidet P-40) for 8 hours at room temperature, followed by incubation with buffer A containing 0.5μg/ml of the indicated

glutathione S-transferase (GST)-fusion proteins overnight at 4°C. Blots were then washed extensively with buffer A, followed by PBS, blocked in 5% milk, and subsequently probed with antibodies to GST (1:10,000; Novagen, Billerica, MA). Immunoreactive bands were detected with the Tropix Chemiluminescence kit (Applied Biosystems). Quantification of immunoreactive bands was performed using densitometric analysis and ImageJ software (NIH, Bethesda, MD).

2.7. Protein modeling

[*Collagen VI-related limb-girdle syndrome caused by frequent mutation in COL6A3 gene with conflicting reports of pathogenicity*]

The C1/11th VWA domain of the COL6A3 protein (residues 2393–2597; accession number NP_004360.2) was modeled using Phyre2. Molecular dynamics were performed on this model using the YASARA suite. After 100 ns equilibration, the desired mutation (Lys2483Glu) was created using the "swap" command, and this mutated structure was allowed to equilibrate for an additional 100 ns.

[*Novel mutations in MYBPC1 are associated with myogenic tremor and mild myopathy*]

Models of the M-motif of sMyBP-C were generated using Phyre2, RaptorX, and iTASSER, based on the human cardiac version of this domain (PBD: 2LH4; 80% identical to sMyBP-C). The model was then allowed to equilibrate using the computer program YASARA for ~90ns. Individual mutations were introduced to the model by the "swap" command in YASARA, and the resulting models were allowed to equilibrate for ~100ns. Docking of wild-type and mutant sMyBP-C to myosin was performed using equilibrated sMyBP-C models and the S2 fragment of myosin (PDB: 2FXO) with the HADDOCK program. The best models, as judged by low z-scores and low root mean square deviation, were then further refined for ~30ns.

3. RESULTS

In the eleven-year period of this diagnostic and research initiative, 32 index patients from Latvia have received molecular diagnosis, with the success rate increasing considerably over the last few years. From the 59 index patients provided by our Lithuanian collaborators, 19 individuals have received molecular diagnosis as part of our projects.

12 patients received molecular diagnosis in the framework of our project that utilized whole-gene Sanger sequencing and massive parallel genotyping, with the majority of cases associated with pathogenic variants in the *CAPN3* gene (see below). 12 patients had molecular diagnosis established or a potential disease-associated candidate variant found through the use of collaborational next-generation sequencing, in the form of the MYO-SEQ project. The acquired results showed dystrophies associated with variants in the *DMD* gene in female patients, and with variants in the *CAPN3* gene as the most common ones in this group of random undiagnosed rare neuromuscular disease patients. One patient and his extended family received their molecular diagnosis through an international multi-institute collaboration that resulted in the establishing of a new myogenic tremor phenotype (see below). The most recent, however, is our success in establishing diagnosis for 7 out of 11 patients in the first batch of rare inherited disease patients (including rare neuromuscular diseases), using local sequencing hardware and data analysis capabilities that we have honed over the years of collaboration with other specialists in the field. Each of these cases is unique, with no repeating disease associated genes.

In all cases the confirmed diagnoses have resulted in genetic counseling and evaluation of the associated genetic risks for the extended family as well as the probands.

The results of this work are presented here as original publications. The author's contribution to them is as follows.

Review paper (presented in literature overview)

Stavusis J, Geist J, Kontrogianni-Konstantopoulos A. Sarcomeric myopathies associated with tremor: new insights and perspectives. J Muscle Res Cell Motil. 2020 Dec;41(4):285-295. doi: 10.1007/s10974-019-09559-1.

Contribution: Wrote part of the manuscript and prepared visual and part of the tabular materials.

Original manuscript I (presented in results)

Stavusis J, Inashkina I, Jankevics E, Radovica I, Micule I, Strautmanis J, Naudina MS, Utkus A, Burnyte B, Lace B. *CAV3* gene sequence variations: National Genome Database and clinics. Acta Neurol Scand. 2015 Sep;132(3):185-90. doi: 10.1111/ane.12369.

Contribution: Carried out the experimental work and data analysis, prepared graphical materials and wrote most of the manuscript.

Original manuscript II (presented in results)

Inashkina I, Jankevics E, **Stavusis J**, Vasiljeva I, Viksne K, Micule I, Strautmanis J, Naudina MS, Cimbalistiene L, Kucinskas V, Krumina A, Utkus A, Burnyte B, Matuleviciene A, Lace B. Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies. BMC Musculoskelet Disord. 2016 May 4;17:200. doi: 10.1186/s12891-016-1058-z.

Contribution: Carried out part of the experimental work and data analysis and wrote part of the manuscript.

Original manuscript III (presented in results)

Stavusis J, Micule I, Wright NT, Straub V, Topf A, Panadés-de Oliveira L, Domínguez-González C, Inashkina I, Kidere D, Chrestian N, Lace B. Collagen VI-related limb-girdle syndrome caused by frequent mutation in *COL6A3* gene with conflicting reports of pathogenicity. Neuromuscul Disord. 2020 Jun;30(6):483-491. doi: 10.1016/j.nmd.2020.03.010.

Contribution: Carried out part of the experimental work and data analysis, prepared part of the graphical and tabular materials and wrote part of the manuscript.

Original manuscript IV (presented in results)

Stavusis J, Lace B, Schäfer J, Geist J, Inashkina I, Kidere D, Pajusalu S, Wright NT, Saak A, Weinhold M, Haubenberger D, Jackson S, Kontrogianni-Konstantopoulos A, Bönnemann CG. Novel mutations in *MYBPC1* are associated with myogenic tremor and mild myopathy. Ann Neurol. 2019 Jul;86(1):129-142. doi: 10.1002/ana.25494.

Contribution: Carried out part of the experimental work and data analysis, prepared part of the graphical and tabular materials and wrote part of the manuscript.

3.1. Neuromuscular disease diagnostics in Latvia - starting from basics (CAV3 gene sequence variations: National Genome Database and clinics)

This part of the project focuses on initial LGMD testing in Latvia, using simple Sanger sequencing in the pre-NGS era. The goal of this work was to use whole-gene sequencing on a smaller LGMD associated gene - *CAV3*, mutations in which are relatively frequent, and trying to figure out variant frequencies in the population of Latvia, and, perhaps, solve some of the dominant LGMD cases in our database. In the patient group, two likely pathogenic disease associated variants, c.183C>A, p.S61R and c.216C>G, p.C72W, were found. This led us to the conclusion that in our patient group 1–2% of patients with LGMD phenotype have variants in the *CAV3* gene. Other than a discrepancy with one of the variants found, no major deviations from published data were found in Latvian population. Interestingly, two people carrying variants with possible disease related significance were found in our control population.

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CAV3 gene sequence variations: National Genome Database and clinics

Stavusis J, Inashkina I, Jankevics E, Radovica I, Micule I, Strautmanis J, Naudina MS, Utkus A, Burnyte B, Lace B. *CAV3* gene sequence variations: national Genome Database and clinics.
Acta Neurol Scand 2015: 132: 185–190.
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Introduction – Caveolinopathies are a group of untreatable, degenerative muscle diseases associated with caveolin 3 (*CAV3*) gene mutations. *Objectives* – The goal of this study was to characterize the role of the *CAV3* gene in patients with limb-girdle muscular dystrophy, hyperCKemia, cardiomyopathies, as well as utilization of the National Genome Database in clinical applications. *Materials and methods* – We sequenced the coding region and exon/intron boundaries of *CAV3* gene in 81 neuromuscular disorder patients, a sample group from the National Genome Database, consisting of 97 individuals with cardiomyopathies, and also random selection of 100 persons. Immunohistochemical staining of muscle biopsy was performed to verify findings in one case, as the setup for the project was to use less invasive molecular biology methods. *Results* – We identified three novel sequence variations (c.183C>G, p.S61R; c.220C>A, p.R74S; c.220C>T, p.R74C) and found evidence that one was associated with hypercreatine kinase-emia. Two previously reported mutations in families with limb-girdle muscular dystrophy were found. No mutations were identified in the cohort of patients with cardiomyopathies. *Discussion* – *CAV3* gene encodes muscle-specific protein with dominant negative type of missense mutations in it causing various phenotypes. Our study confirmed *CAV3* gene involvement in neuromuscular disorders, but found no evidence in the group of patients with cardiomyopathies. Persons included in the National Genome Database could be screened for late onset Mendelian diseases.

J. Stavusis[1], I. Inashkina[1], E. Jankevics[1], I. Radovica[1], I. Micule[1], J. Strautmanis[1], M. S. Naudina[1], A. Utkus[2,3], B. Burnyte[2,3], B. Lace[1]

[1]Latvian Biomedical Research and Study Center, Riga, Latvia; [2]Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University, Vilnius, Lithuania; [3]Centre for Medical Genetics, Vilnius University Hospital Santariškių Klinikos, Vilnius, Lithuania

Key words: cardiomyopathy; CAV3; caveolinopathy; hyperCKemia; LGMD1C

J. Stavusis, Ratsupites 1-1, Riga, Latvia, LV-1067
Tel.: +371 67808200
Fax: +371 67442407
e-mail: janis.stavusis@biomed.lu.lv

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Introduction

Caveolinopathies are a group of currently untreatable degenerative muscle diseases that cause muscle fiber degeneration or regeneration, limb proximal muscle weakness and atrophy, hypertrophy of lower limb muscle, and positive Gower sign, in addition to different levels of restriction of human movement. These diseases are genetic in their origin and caused by decrease in the level of the membrane protein caveolin-3 (1). Caveolin-3 is a 151 amino acid containing protein that forms flask-shaped invaginations on the cytoplasmic surface of sarcolemmal membranes (2). It is expressed in all muscle cells, where it regulates sarcolemmal stability and the activity of various signaling pathways, as well as vesicular transportation (3). *In vitro* functional research has shown that caveolin-3 is an important differentiation and survival regulator in myoblast cells (4).

The gene encoding caveolin-3, *CAV3*, is the only known locus associated with caveolinopathies, with mutations identified in 99% of all caveolinopathy cases (5). *CAV3* itself is located on chromosome 3p25 and has two coding exons (6). *CAV3* expression has been detected in all muscle cells, as well as in glial cells and early postnatal peripheral nerves (2). It is a highly evolutionarily conserved gene, suggesting a vital function; therefore, frequent sequence variations at this locus are not expected (2). *CAV3* mutations mainly disturb normal cell signaling

pathways, change muscle cell structural integrity, and lead to apoptosis (7, 8). Most caveolinopathies are transmitted with an autosomal dominant inheritance (2).

The five main phenotypes connected with *CAV3* mutations are limb-girdle muscular dystrophy autosomal dominant type C (LGMD 1C), isolated hyperCKemia, rippling muscle disease (RMD), hypertrophic cardiomyopathy (HCM), and distal myopathy (DM), although a few other rarer phenotypes have been identified over the years (9). It is possible for people from one family with the same mutation to have different phenotypes, overlapping disease phenotype being described in some of the families with caveolinopathies; no certain phenotype–genotype correlations have been observed (10). Patients mainly complain of muscle pain, while muscle weakness is a varying indication that does not correlate with length of disease. Serum creatine kinase (CK) levels increase steadily over time, but do not correlate with the progress of caveolinopathies (2). In two percent of all cases, mutations in *CAV3* gene were found in patients with long QT syndrome suggesting the role of caveolin-3 protein in the cardiac excitability regulation (11). In summary, it was suggested that caveolinopathies account for the 1–2% of unclassified LGMD and other phenotypes, including hypertrophic cardiomyopathy (HCM) (12).

Materials and methods

Subjects

Eighty-one patients with clinical symptoms of limb-girdle muscular dystrophy were recruited. Forty were from Latvia and 41 were from Lithuania, and all 81 probands (family members included only for further segregation analysis in cases when mutations were identified) had clinical symptoms and an autosomal dominant or sporadic inheritance pattern. Patients were recruited at the Neurology department, Children's University Hospital, and the Out-Patients Clinic for Neurological Diseases, The Pauls Stradins Clinical University Hospital (Riga, Latvia) and the Center for Medical Genetics, of the Vilnius University Hospital, Santariskiu Clinics (Vilnius, Lithuania). The mean age at onset of symptoms in Latvian patients was 14 years (range, 1 month to 53 years) and the male:female ratio was 1.4:1. The mean age of Lithuanian patients at presentation was 23 years (range, 4–60 years) and the male:female ratio was 1:2.4.

Ninety-seven individuals with a clinical diagnosis of cardiomyopathy (excluding pregnancy, puerperium, and ischemic cardiomyopathy) were selected from the Latvian Genome Data Base without information about familiar cases. Of these, 44 were female and 53 were male, with a mean age of 66 years (range, 26–92 years). A diagnosis of dilated cardiomyopathy (DCM) (I42-ICD10) was established for 49 patients, HCM (I42.1; I42.2) for 16 patients, and 32 patients had unspecified and other cardiomyopathies (I42.8; I42.9).

A group of 100 unrelated Latvian population controls was selected randomly within the framework of the national 'Latvian Genome Data Base' project. The mean age of the controls was 61 year (range 50–80 years). They represent divergent group of Latvian population, and none of them had established diagnosis of neuromuscular disease.

Data collection was performed in accordance with the regulations issued by the Central Medical Ethics Committee of Latvia. All participants have signed an informed consent.

Sequencing of CAV3

Sequencing of both coding exons of the *CAV3* gene and of the exon–intron boundaries was performed using standard polymerase chain reaction (PCR) and an automatic genetic analyzer (ABI PRISM 3130XL, Applied Biosystems, Foster City, CA, USA). For PCR, primer pair AGG GAC TAA CCC CAC TTC C/AGG AGC GTC ACA GTA GTA AG was used for first exon and primers TCC TGC ACA GAT CAC AGA CC/CTT GCA GTA GCT GCC TCT TG for second exon, with annealing temperatures of 56°C and 58°C, respectively. For sequencing reactions, primers GGC AGG CTG AAG TTA TGT GG/TTT CAG GGA AAC TGT GTC TGC were used for first exon and GTA GGG TCC AGC CAC CAA G/TCA TGG GGT ATG GAG CAG TC for second exon, with annealing temperature of 58°C for both primer pairs. Sequencing reactions were carried out using standard conditions as suggested by the manufacturer. The data acquired were processed using the program CLC Genomics Workbench (QIAGEN, Venlo, Netherlands). The possible impact of identified sequence variations on protein structure and function was determined using the *in silico* prediction programs: Polymorphism Phenotyping-2 (PolyPhen-2), SIFT, and PROVEAN (13). For SNPs located in the coding region, conservation levels were assessed based on data from the

UCSC database (Genome Browser, Human Feb. 2009 (GRCh37/hg19) Assembly). When identifying mutations in patient samples, segregation analysis was performed and the whole family screened where possible.

Immunochemical staining with CAV3 antibodies (BD Biosciences, San Jose, CA, USA) was performed by standard protocol to verify findings in one patient, who underwent muscle biopsy due to the rapid progression of the disease. In the other cases, we chose to maintain the study as less invasive as possible. Paraffin embedding was used for muscle biopsy samples, and samples from unaffected individual were used as controls.

Results

CAV3 exons and intron–exon boundaries were sequenced in samples from eighty-one patients diagnosed with limb-girdle muscular dystrophies. In this group, 17 gene sequence changes were identified, fifteen of which were present in SNP databases. Two amino acid changes, resulting from mutations found, are reported to have a pathogenic role.

The first variation leading to an amino acid change is NM_033337.2:c.183C>G, NP_001225.1: pSer61Arg, a missense mutation identical to rs116840796 (NM_033337.2:c.183C>A, NP_001225.1:pSer61Arg) in amino acid change, but having nucleotide change from C to G instead of C to A. The variation is located in the second exon, in the middle of the scaffolding domain. This is a highly conserved region, responsible for binding to various signaling proteins. Using *in silico* prediction tools SIFT and PROVEAN, variation was predicted as damaging and deleterious, respectively (14, 15). However, based on UCSC conservation data, the exact nucleotide position is not very highly conserved. The proband was a 6-year-old boy born to healthy non-consanguineous parents. He was born at term of uneventful pregnancy after C-section which was performed due to abnormal position of the fetus. Psychomotor development was normal in the first years of life, but patient started walking on his toes and would fall often, starting at the age of four. At the same time, increased levels of serum creatine kinase (CK) were detected—1923 U/l (normal values up to 175 U/l). On examination, head circumference, as well as muscle tone and strength, was normal. Patient did have hypertrophy of calves. ECG showed no abnormalities, and muscle biopsy has not been performed for this patient.

The second variation found in this group leading to an amino acid change was NM_033337.2: c.216C>G, NP_001225.1:p.Cys72Trp (rs116840776), a missense mutation located in the second exon, at the end of the scaffolding domain. Variation was predicted as tolerated by SIFT; however, PROVEAN predicted it as deleterious. Based on UCSC database information, the exact amino acid position is not highly conserved. The proband was an 8-year-old girl, who had initial symptoms less than 2 years ago, starting with a complaint about an inability to climb the stairs. Proximal muscle weakness and atrophy were observed on physical examination. Winging scapula was noted, Gower's sign was positive, and CK level was highly elevated at 7663 IU/l. The proband's father and brother had mildly elevated CK levels at 571 and 768 IU/l, respectively, without any clinical symptoms. The proband's paternal grandmother has been diagnosed with progressive muscle weakness and elevated CK (up to 1177 IU/l) and has had walking difficulties since the age of 40; disease is progressive. Physical examination revealed proximal and distal muscle weakness. As proband's muscle biopsy sample was available, CAV3 immunohistochemistry staining was performed to determine protein localization and expression levels (Fig. 1). When compared to a sample from unaffected individual, no notable changes were observed.

Despite this disease pattern, the *CAV3* c.216C>G mutation was identified in samples from the proband's brother and mother. Mother did not present with any disease symptoms or CK elevation.

In the cardiomyopathy group, 14 gene sequence variants were found. Seven of these, including all from the coding regions, overlapped with those found in the population group, and four were shared with LGMD patient samples. The remaining three variants could be found in gene variation databases and are not reported to be associated with caveolinopathy or have incomplete accompanying information. There were no persons identified with mutations in *CAV3* gene known to cause a disease.

One hundred random DNA samples were analyzed from the National Genome Database, among which 12 nucleotide sequence changes were identified. Nine of these could be found in SNP databases, and nucleotide sequence change rs116840801 (c.257T>C, p.L86P) has been previously associated to rippling muscle disease in homozygous form (16). The other three changes (two coding and one non-coding: c.220C>A, p.R74S; c.220C>T, p.R74C; and c.115-45_115-31del, respectively) were novel and could not be found in databases (Fig. 2).

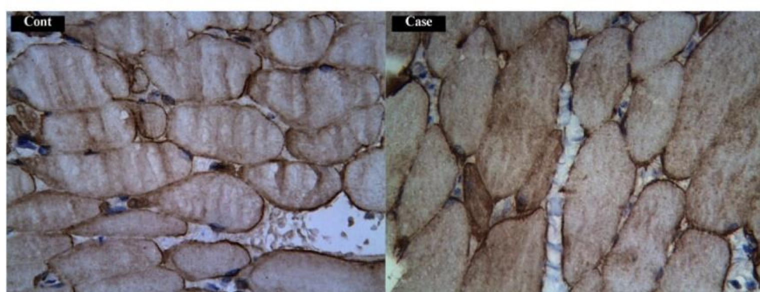

Figure 1. Controls' (Cont) and probands' (Case) muscle biopsy CAV3 protein staining. Original microscope magnification ×400.

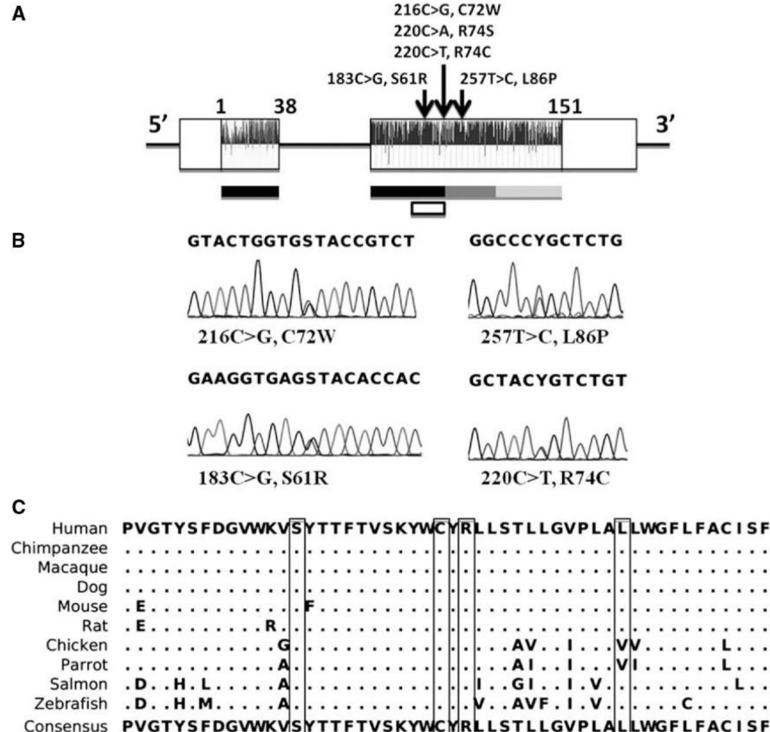

Figure 2. (A) Schematical representation of *CAV3* gene. Different domains are color coded. Black—N-terminal region, dark gray—a central hydrophobic transmembrane domain, light gray—C-terminal region, and white—scaffolding domain. The approximate locations of mutations are marked with arrows. Bars inside coding regions represent the level of conservation in 100 vertebrates (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly). (B) Sequencing results for amino acid changing variations. (C) Protein region conservation in vertebrates, affected amino acids marked with boxes.

The impact of the three SNPs resulting in amino acid changes was determined *in silico* by assigning a grade on the PolyPhen scale from 0.0 to 1.0. *In silico* prediction tools SIFT and PROVEAN predict all three variations to be damaging and deleterious. All variants generated scores at or close to 1.0. According to data found in the UCSC Genome Browser (Human Feb. 2009 (GRCh37/hg19) Assembly), positions 220 and 257 are highly conserved (Fig. 2). A slight elevation (218 IU/l; reference <170 IU/l) in CK level was observed only in a male, at the age of 58 with the genetic change c.220C>T, p.R74C.

Discussion

In the patient group, two potentially harmful sequence variations, c.183C>A, p.S61R and c.216C>G, p.C72W, were found. Both are located in the scaffolding domain of the protein, which is a highly conserved region. The variant c.183C>A, p.S61R has been described by Fulizio et al. in a

heterozygous state in a patient with absent CAV3 protein and dystrophic muscle pathology. It was suggested that mutations in this domain may cause significant consequences on protein and clinical level (6).

An explanation for variations c.216C>G, p.C72W *in silico* prediction could be its position at the very end of the scaffolding domain (aa 55–74), which may indicate decreased functional significance.

This mutation has previously been found in a heterozygous state in a patient with muscular dystrophy. However, it was also found in the patient's siblings, who had no symptoms of muscular dystrophy, suggesting that a single abnormal allele is not sufficient to cause the phenotype. No additional mutations were identified in the proband (17). De Paula et al. also identified this mutation in a heterozygous state in one of 100 normal Brazilian controls and concluded that it is a rare variant that does not cause the disease phenotype when present in only one allele (18).

After investigating the family of our patient, we found that the mutation in proband was inherited from the maternal side, with no symptoms present in the mother, while muscular dystrophy was clearly inherited from the paternal side of the family. Disease shows autosomal dominant type of inheritance in family with variable penetrance. This inconsistency, combined with no observable changes in protein expression and localization in muscle tissue, led us to conclude, consistent with previous reports, that heterozygosity for the c.216C>G, p.C72W (previously C71W) variant does not cause the disease phenotype.

As was expected, 1–2% of patients with LGMD phenotype have mutations in *CAV3* gene. However, this was not the case with cardiomyopathies, where certain controversies still exist about mutations identified.

The first report of *CAV3* mutation in a family with atypical HCM was in a proband, in the absence of CK elevation or any signs of muscle weakness. It was concluded that, in a small percentage of patients with HCM, the disease is caused by mutations in *CAV3* gene (3). In support of this, in another individual with DCM and also presenting with symptoms of LGMD, *CAV3* mutation was described. This mutation can also cause the hyperCKemia phenotype in the heterozygous state, suggesting a high variability of *CAV3* expression and effects on protein interactions (19).

Controversial findings of the small gene *CAV3* lead us to assumption that some of the previously reported sequence variations, confirmed also by cell models, are strongly influenced by the gene–gene interactions *in vivo* and as so could not be considered pathogenic, when present without additional mutations. We believe it is highly unlikely that mutations in the *CAV3* gene commonly cause cardiomyopathies; however, due to the small sample size of HCM and DCM patients in this study, we were unable to draw definite conclusions.

Significant deviation from the expected results was observed in our group of randomly selected individuals from the National Genome Database. According to our sequencing and CK level data, it is plausible that there are two individuals with a caveolinopathy. The person with the sequence variation c.220C>T, p.R74C has slight increase of serum CK. We can hypothesize that the person in question is suffering from an undiagnosed hyperCKemia. In an advanced stage of disease, CK level usually shows tendency to normalize due to the muscle replacement with fibrous or adipose tissues. However, it is impossible to get any further health data about the person to confirm this theory.

Interestingly, we found only one sample with the intronic deletion 115-45_115-29del; however, 61 samples contained another, previously undescribed intronic deletion, 115-45_115-31del. As the latter change was found in all groups, we conclude that it is not likely to be associated with disease phenotype; however, while there are no records of this variation in databases, we have observed relatively high frequency in our samples. As for the other sequence variants found in the control group, their frequencies are comparable and do not differ greatly in our Baltic samples, when compared with data from genome databases.

As previously discussed, a limiting factor of this project was the size of the sample groups, which may not fully represent the Latvian population. The data gathered have approval for use in further research, as well as for genetic testing of suspected caveolinopathy patients. Many neuromuscular disorders remained undiagnosed in our population due to unspecific symptoms of muscle weakness, which sometimes are found also in patient's parents, and therefore perceived as normal familial trait.

The purpose of this project was to determine the role of *CAV3* in various neuromuscular diseases, which has been performed successfully. Data gathered are comparable across our geographic region and approximately 2% of all cases included have been confirmed to have a mutation in the *CAV3* gene. Our study showed that

National Genome Database with 'feedback to the patient' option can be used for screening purposes of Mendelian disorders.

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Conflict of interest and sources of funding statement

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3.2. Neuromuscular disease diagnostics in Latvia - first steps (Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies)

This manuscript focuses on the results of the initial project, trying to tackle LGMD diagnostics in Latvia, using the last massively parallel pre-NGS technology - microarray genotyping. A custom selected panel of the theoretically most common LGMD associated variants was used in conjunction with our LGMD patient and healthy control sample database. As a result of this testing, the most common disease-causing variant in the *CAPN3* gene - the c.550delA was found in eight patients from Lithuania. In addition to this, three variants: c.826C > A, c.404_405insT, and c.204_206delCTC in the *FKRP* gene as well as one variant in the *CLCN1* gene – c.2680C > T were found, each in a single patient sample. In addition, three heterozygous variants - c.5028delG in DYSF gene, c.2288A > G in *CAPN3* gene, and c.135C > T in *FKRP* gene were located in the control samples, with the first two being classified as pathogenic. None of the other selected variants in other genes of choice were found.

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RESEARCH ARTICLE

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Robust genotyping tool for autosomal recessive type of limb-girdle muscular dystrophies

Inna Inashkina[1*], Eriks Jankevics[1], Janis Stavusis[1], Inta Vasiljeva[1], Kristine Viksne[1], Ieva Micule[1], Jurgis Strautmanis[1], Maruta S. Naudina[1], Loreta Cimbalistiene[2,3], Vaidutis Kucinskas[2], Astrida Krumina[1], Algirdas Utkus[2,3], Birute Burnyte[2,3], Ausra Matuleviciene[2,3] and Baiba Lace[1,4,5]

Abstract

Background: Limb-girdle muscular dystrophies are characterized by predominant involvement of the shoulder and pelvic girdle and trunk muscle groups. Currently, there are 31 genes implicated in the different forms of limb-girdle muscular dystrophies, which exhibit similar phenotypes and clinical overlap; therefore, advanced molecular techniques are required to achieve differential diagnosis.

Methods: We investigated 26 patients from Latvia and 34 patients from Lithuania with clinical symptoms of limb-girdle muscular dystrophies, along with 565 healthy unrelated controls from general and ethnic populations using our developed test kit based on the Illumina VeraCode GoldenGate genotyping platform, Ion AmpliSeq Inherited Disease Panel and direct sequencing of mutations in calpain 3 (*CAPN3*), anoctamin 5 (*ANO5*) and fukutin related protein (*FKRP*) genes.

Results: Analysis revealed a homozygous *CAPN3* c.550delA mutation in eight patients and three heterozygous variants in controls: dysferlin (*DYSF*) c.5028delG, *CAPN3* c.2288A > G, and *FKRP* c.135C > T. Additionally, three mutations within *FKRP* gene were found: homozygous c.826C > A, and two compound – c.826C > A/c.404_405insT and c.826C > A/c.204_206delCTC mutations, and one mutation within *CLCN1* gene – c.2680C > T p.Arg894Ter. *ANO5* c.191dupA was not present.

Conclusions: Genetic diagnosis was possible in 12 of 60 patients (20 %). The allele frequency of *CAPN3* gene mutation c.550delA in Latvia is 0.0016 and in Lithuania - 0.0029. The allele frequencies of *CAPN3* gene mutation c.2288A > G and *DYSF* gene mutation c.4872delG are 0.003.

Keywords: Limb-girdle muscular dystrophies, Illumina VeraCode GoldenGate, Calpain 3 c.550delA, Fukutin related protein

Background

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of diseases that predominantly affect pelvic and shoulder girdle muscle groups. LGMD is a rare disorder, and the different forms of LGMD range in prevalence from 1 in 14,500 to 1 in 123,000, depending on the population [1, 2]. The autosomal recessive form of LGMD (LGMD type 2; LGMD-2) is more common, with 23 causal genes and chromosomal loci identified to date [3–7]. The rarer autosomal dominant (LGMD type 1; LGMD-1) form accounts for only 10 % of all cases and has eight causative genes and chromosomal loci currently identified [6, 8, 9]. These genes encode proteins involved in many different aspects of muscle cell biology. Diagnosing of specific types of LGMD is a challenging process due to the many genes involved, including unknown genes within the identified chromosomal loci. Although common mutations have been identified for some causative genes (for example, calpain 3 (*CAPN3*) c.550delA or fukutin related protein (*FKRP*) c.826C > A),

* Correspondence: inna@biomed.lu.lv
[1]Biomedical Research and Study Centre, Ratsupites str. 1, k-1, LV-1067 Riga, Latvia
Full list of author information is available at the end of the article

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a lot of additional mutations were found in different populations. Confirming the diagnosis of LGMD, in addition to subtyping, is usually done using molecular techniques. Directly sequencing the coding regions of the gene is the most common method, but remains often insufficient, due to the size and number of genes involved. Hence, the advancement of LGMD diagnostics was achieved through next generation sequencing of the genome or exome, or through targeted exome capture applications. Furthermore, several commercially available kits include loci for the diagnosis of neuromuscular disorders. The advantage of next generation sequencing lies in the capability of analyzing whole exomes at a reasonable cost without invasive muscle biopsies. Limitations of this method are related to insufficient exon coverage, leading to a potential lack of a representative examination of all exons [10].

The aim of this study was to investigate LGMD type 2 mutation spectrum and allelic frequency in Latvian and Lithuanian populations.

Methods

Patients

We investigated 26 patients from Latvia and 34 patients from Lithuania with clinical symptoms of LGMD. After mutation identification, segregation analysis of the family was performed.

Patients were recruited from the Neurology department of Children's University Hospital, from the outpatient clinic for Neurological diseases at Pauls Stradins Clinical University Hospital (Riga, Latvia), and from the Center for Medical Genetics, Vilnius University Hospital Santariskiu Clinics (Vilnius, Lithuania).

The Latvian patient sample consisted of 26 unrelated individuals with predominant pelvic and shoulder girdle muscle weakness and elevated creatine kinase (CK) values. Patients with electromyography data demonstrating myopathy or myogenic impairment were included in the study. A muscle biopsy was obtained from one patient; however, analysis of this was inconclusive regarding their LGMD subtype. The mean patient age at the onset of symptoms was 14 years (range: 1 month (accidental finding without clinical symptoms until 8 years) to 53 years), and the male:female ratio was 1.4:1.

The Lithuanian patient sample consisted of 34 unrelated patients with predominant pelvic and shoulder girdle muscle weakness and elevated CK values. Mean patient age at presentation was 23 years (range: 4–60 years) and the male:female ratio was 1:2.4.

The data collection was performed with permission from the Central Medical Ethics Committee of Latvia. All participants or their parents (in case of patient's age under 14 in Latvia and under 18 in Lithuania) signed an informed consent forms to participate and publish. Complete patient and family histories were taken at the clinic, and patients were asked about their muscle strength and performance before the onset of symptoms. Patients were interviewed and examined by a neurologist and a clinical geneticist experienced in neuromuscular disorders.

Healthy, unrelated individuals ($n = 204$) randomly selected from the Latvian Genome Database were used as controls representing the general population. When necessary, we supplemented the study with 101 samples, for total number of 394. Additionally, 175 DNA samples from Lithuania and 186 samples from Latvia were used as controls for ethnic groups. They were recruited, if their ancestors from the last three generations could be traced back archaeologically and ethno-linguistically to distinct regions of Latvia and Lithuania [11, 12].

Microarray genotyping

We selected the highly parallel genome-wide VeraCode GoldenGate system (Illumina, San Diego, CA, USA) as the platform for mutation screening [13]. The main resources used for manually selecting mutations, previously published as causative for LGMD-2, were the open access databases [14–17] as well as publications. Mutations were selected based on their pathogenicity, location within exons or splice sites, allele frequency, and the population analyzed. In addition, the mutations had to satisfy the following criteria to comply with the VeraCode GoldenGate system: they had to be single-nucleotide mutations (nonsynonymous single nucleotide polymorphisms (SNPs) or insertions/deletions (indel)); diallelic (rather than triallelic or tetra-allelic) to ensure allele specific hybridization, extension, and ligation; and be >60 base pairs away from any other mutations on the array to prevent overlapping oligonucleotides during hybridization. All LGMD patients and general population controls were genotyped for the selected mutation using a customized VeraCode GoldenGate system on a BeadXpress reader (Illumina). Reactions and quality controls were carried out under the standard conditions. To ensure quality control and to evaluate the intra-subject concordance rate, 48 duplicate samples were processed in one genotyping run, instead of 96 single samples.

The presence of the identified mutations was confirmed by direct sequencing performed using standard PCR conditions. The oligonucleotide sequences are available upon request.

CAPN3 and anoctamin 5 (*ANO5*) mutation detection

The mutations in *CAPN3* and *ANO5* genes (c.550delA and c.191dupA, respectively) in the Latvian and Lithuanian populations were identified by direct sequencing performed using standard polymerase chain reaction (PCR) conditions ($t_{annealing}$ 56 °C) with the oligonucleotides *CAPN3* F1 5'

TCTCAAAAAGCACCCAGTCC 3' and *CAPN3* R1 5' GCCTCTTCCTGTGAGTAGG 3', and *ANO5* F1 5' TGGTATGTTTGCAGCATTTTTG 3'and *ANO5* R1 5' CATACCTCAAATTTTGGATGTTAC 3'.

The frequencies of mutations in the ethnic and general populations were calculated using the Hardy-Weinberg equation under the assumption of the absence of evolutionary forces.

FKRP gene sequencing

A 1.7 kb fragment containing *FKRP* exon 4 and entire coding sequence was amplified using previously published primers [18] and 1.25 Units of Long PCR Enzyme Mix (ThermoScientific, Lithuania). PCR conditions were as follows: 45 cycles, annealing temperature 52 °C, extension temperature 68 °C, and DMSO concentration 4 %.

The exon 4 end exon-intron boundaries were analyzed by direct sequencing performed using published [18] and following oligonucleotides: *FKRP* F1 5'AGAT GCTGTGGTGCTCCTG 3', *FKRP* R1 5'TCCAAGTA GATGCCGAGGTC 3', *FKRP* F2 5'AGCTGCTGGA CTTGACCTTC 3', and *FKRP* F2 5'CCCACGTCCTC CAAGTAG 3'.

An automatic genetic analyzer (ABI PRISM 3130xl, Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used in all cases.

Next generation sequencing using AmpliSeq Inherited Disease Panel

Life Technologies Ion Torrents targeted gene enrichment and sequencing technology AmpliSeq was performed according to a protocol provided by the manufacturer on four patient samples.

The commercially available AmpliSeq Inherited Disease Panel (Life Technologies) that covers 325 gene exons and intron-exon boundaries of most common inherited diseases, including genes of LGMD and other muscle dystrophies, was used for this approach [19]. Standard DNA extraction was followed by PCR amplification using three primer pools included in the panel. The amplicon mixes were combined and treated with FuPa reagent (Life Technologies) to partially digest primer sequences and phosphorylate the amplicons, which were then ligated to multiplexing barcodes (Life Technologies). Library was then purified using Beckman Coulter (Nyon, Switzerland) AMPure XP magnetic beads and amplified using PCR assay provided, followed by another purification. Massive parallel sequencing of library created was done using Ion Torrent PGM device.

Results

A total of 264 DNA samples, 26 from Latvian patients, 34 from Lithuanian patients, and 204 healthy controls, were analyzed by a custom microarray for the selected loci.

We initially selected 209 sequence's variants based on the criteria mentioned above. Of these, 121 were mutations in the *CAPN3* gene, 50 were mutations in dysferlin (*DYSF*), 28 were mutations in sarcoglycan alpha (*SGCA*), sarcoglycan beta (*SGCB*), sarcoglycan delta (*SGCD*), and sarcoglycan gamma (*SGCG*), two were gender controls (amelogenine, X-linked/ amelogenine, Y-linked), and eight were control polymorphisms. This initial number of mutations was reduced considerably due to designs resulting in critical failure scores, as calculated by the Assay Design Tool. To increase the overall performance and sensitivity of the assay, we had to decrease the investigated variants to a final total of 96.

Data cleaning was completed as recommended by the manufacturer resulting in exclusion of 23 samples.

In addition, we did a "paired sample" test in the final quality control step to compare the call frequency values of the duplicate samples, which led to the classification of 13 additional mutations as false positives and decrease the analyzed sample size to 185 (80 %). Additional file 1: Table S1 contains information about quality the parameters. A complete list of the Group 1 mutations, which have the best test performance ($n = 20$) is provided in Additional file 1: Table S2A. The additional mutations with fluctuating results included in the test ($n = 57$) form Group 2 and are provided in Additional file 1: Table S2B. Excluded mutations are provided in Additional file 1: Table S2C. After cleaning the data, we identified four different mutations in our cohort of patients and controls. Our analysis revealed the c.550delA homozygous mutation in *CAPN3* in eight Lithuanian cases (24 %). Controls from the Latvian population did not have *CAPN3* c.550delA, but had two causative mutations: *DYSF* c.4872delG and *CAPN3* c.2288A > C, and one benign SNP *FKRP* c.135C > T (one heterozygous allele each; see Table 1). These three variants were not detected in patients. Re-sequencing of these mutations revealed one false negative case within the data from the Illumina VeraCode GoldenGate assay. Thus, the sensitivity of the test was 89 % (95 % confidence interval (CI): 0.54–0.99), and the specificity was 98 % (95 % CI:0.89–0.99).

Because no controls had the *CAPN3* c.550delA mutation, it was not possible to estimate the frequency of this allele in the populations. For estimating its allele frequency in the Latvian population, an additional 101 individuals were selected from the Latvian Genome Database. One heterozygous carrier was found, and they had no symptoms of neuromuscular disease. Thus, the allele frequency of c.550delA within the Latvian population is estimated to be 0.0016 (0.16 %). However, the situation was different in the Lithuanian samples, in which the c.550delA allele was found in one of 175

Table 1 Mutations identified with LGMD-2 diagnostic test kit

Name	Gene	Mutation	Frequency in Control group	Frequency in the group of patients with NMD	Known mutation frequency
CAPN3_00010 (rs80338800)	*CAPN3*	c.550delA	0 (0.001[a])	0.13	NA
CAPN3_00119	*CAPN3*	c.2288A>C	0.003	0	NA
DYSF_00178	*DYSF*	c.4872delG	0.003	0	NA
rs2287717	*FKRP*	c.135C>T	0.003	0	0.14

[a]frequency estimated for enlarged control group (with additional 101)
NA, data not available
NMD, neuromuscular disorders

samples, making the calculated allele frequency 0.0029 (0.29 %). Using the Hardy-Weinberg equation, the estimated number of persons homozygous for *CAPN3* c.550delA in the Latvian population is 0.3 per 100,000 persons, and in the Lithuanian population, it is 0.8 per 100,000. Other three variants *DYSF* c.4872delG, *CAPN3* c.2288A > C, and *FKRP* c.135C > T had an allele frequency of 0.3 % in Latvian population, and the calculated number of homozygous persons was 1 in 166,000 for each of them.

Data from patients who had been diagnosed with LGMD2A using the GoldenGate VeraCode assay, were summarized. Information about these patients' clinical symptoms is in Table 2. To further ensure our results, we performed mutation analysis in the patients' families, and confirmed mutations in two affected siblings. The segregation analysis was concordant with the clinical picture and the presence of the mutation.

As a recent and population-specific finding, mutation c.191dupA of the *ANO5* gene was also analyzed in patients and ethnic control groups because of its reported frequency in North European populations [20]. Analysis did not reveal the variant in any of the samples, resulting in an allele frequency of zero in Latvia and Lithuania.

Since none of selected mutations were found within Latvian patients' samples, we performed analysis by direct sequencing of entire coding region and 4th exon-intron boundaries of *FKRP* gene for 23 Latvian patients.

Table 2 Description of patients with identified mutations

Gene	Patient ID	Mutation	Symptoms
FKRP		c.826C>A/c.826C>A	Female, disease onset at age 11, shoulder girdle muscle weakness and atrophy, pseudohypertrophy of calves, hyperlordosis, respiratory difficulty, CK level (U/l) 700-1500
FKRP		c.826C>A/c.404_405insT	Male, disease onset at age 5 with rhabdomyolysis, hypertrophic cardiomyopathy, hypertrophy of calves, mild proximal weakness, Gowers' symptom negative, CK level (U/l) 32,000
FKRP		c.826C>A/c.204_206delCTC	Female, age 8, slight proximal muscle weakness, CK level (U/l) 700
CLCN1		c.2680C>T/N	Female, disease onset at age 4, severe proximal muscle weakness, Gowers' symptom positive, myotonia by EMG, CK level (U/l) 13,059
CAPN3	D06	c.550delA/c.550delA	Female, disease onset at age 4, tip toe walking, proximal muscle weakness and atrophy, scoliosis at age 10, ankle and elbow contractures in teen years, significantly reduced physical activity at age 19, still walking after 34 years from onset, CK level (U/l) 4000 at present.
CAPN3	D07	c.550delA/c.550delA	Female, disease onset at pre-school age, walking and gait disorders, proximal muscle weakness and atrophy, CK level (U/l) 9001
CAPN3	D08	c.550delA/c.550delA	Female, disease onset at age 11, tip toe walking, waddling gait, frequent falling and inability to run, scapular winging, hypertrophy of calves, still walking after 16 years from onset, CK level (U/l) 5722
CAPN3	D09	c.550delA/c.550delA	Female, disease onset at age 7 with history of frequent falling and inability to run, short stature, hypertrophy of calves, proximal muscle weakness, scapular winging, walking with assistance at the age 17, CK level (U/l) 4000
CAPN3	D10	c.550delA/c.550delA	Female, disease onset at age 6, muscle weakness and atrophy in the lower extremities with difficulty running, climbing stairs, and frequent falls, waddling gait, scoliosis, dependent on a wheelchair at age 17, CK level (U/l) 18,826
CAPN3	D16	c.550delA/c.550delA	Male, disease onset at age 14, *pes equinovarus* from birth, waddling gait, fatigue in his arms progressively worsened to symmetric weakness in hip girdle, scapular winging, walking with assistance at age 29, dependent on a wheelchair at age 32, CK level (U/l) 6370
CAPN3	D23	c.550delA/c.550delA	Female, disease onset at age 7, proximal muscle weakness, still walking after 34 years from onset, CK level (U/l) 7487
CAPN3	D24	c.550delA/c.550delA	Male, disease onset at age 3, tip toe walking, proximal muscle weakness, hypertrophy of calves, significant loss of physical activity at age 14, CK level (U/l) 854

CK, creatine kinase, *EMG*, electromyography

26 Lithuanian patients, who had not been diagnosed with LGMD2A, were also included in this analysis. Our analysis revealed one c.286C > A homozygous mutation in Lithuanian patient and two compound heterozygous mutations (one in each of Lithuanian and Latvian case) within the coding region of *FKRP* gene. The first is *FKRP* c.826C > A/c.404_405insT mutation, unknown *cis* or *trans* position, another one is *FKRP* c.826C > A/ c.204_206delCTC in *trans* position that is confirmed by segregation analysis, carried out in the patient's parents samples. Information about patients' clinical symptoms is summarized in Table 2.

Additionally, next generation sequencing using AmpliSeq Inherited Disease Panel was performed on four patient samples. These patients had no diagnosis found by other methods described. One heterozygous mutation within chloride voltage-gated channel 1 (*CLCN1*) gene – c. 2680C > T p.894R > X was identified. Patients' clinical features are described in Table 2.

Discussion

This study, using the LGMD-test kit, revealed a homozygous *CAPN3* c.550delA mutation in eight Lithuanian patients and three variants (one heterozygous allele each) in controls: *DYSF* c.5028delG, *CAPN3* c.2288A > G (both were reported as pathogenic), and *FKRP* c.135C > T (benign polymorphism). These three variants were not detected in patients.

The mutation *CAPN3* c.550delA is one of the most frequent among patients suffering from LGMD-2. It has been identified in many countries, including France, Greece, Italy, the Netherlands, Germany, and the United Kingdom, but was relatively infrequent in these populations [21, 22]. Relatively high frequencies (40–70 % of LGMD2A cases) of *CAPN3* c.550delA have been observed and reported in Turkey, Bulgaria, Croatia, the Czech Republic, Poland, and Russia [23–29]. Canki-Klain et al. suggested that the *CAPN3* c.550delA mutation originated in the Eastern Mediterranean, probably spreading widely across Europe, and single haplotype analysis confirms this hypothesis [27].

Our data suggest that *CAPN3* c.550delA is also the most frequent mutation in LGMD patients in Lithuanian populations. The calculated frequency of homozygous persons is 0.8 per 100,000 people, and in Latvia calculated frequency is 0.3 in 100,000. The marked difference observed between allele frequencies in Lithuanian and Latvian populations remains unexplained. We speculate that the disease gradient falls rapidly towards the North and East of Europe.

The *CAPN3* gene mutation c.2288A > G and *DYSF* gene mutation c.5028delG (currently c.4872delG) carrier frequency is 1 in 204 people.

We did not find common mutations in *ANO5*, *SGCA*, *SGCB*, *SGCD*, or *SGCG*. It is possible that these common mutations have distinct founder effects and are sparsely scattered in Northeast Europe.

Additionally, we found three different mutations within *FKRP* gene: c.826C > A, c.404_405insT, and c.204_206delCTC and one mutation in *CLCN1* gene – c.2680C > T.

Currently, the LGMD-2 diagnosis toolkit is not vital for diagnostics; nevertheless, the information about the mutation spectrum we acquired in this study is important for construction a gene panel for LGMD diagnostics and gives insight into the mutation spectrum of this heterogeneous disease.

Conclusions

The LGMD-2 test kit was introduced as a rapid and low cost screening tool for improvement of the healthcare of the neuromuscular disease patients. Genetic diagnosis was achieved in 12 of 60 patients (20 %). The allele frequency of *CAPN3* gene mutation c.550delA in Latvia is 0.0016 and in Lithuania – 0.0029. The allele frequencies of *CAPN3* gene mutation c.2288A > G and *DYSF* gene mutation c.4872delG are 0.003 in Latvia and profiled the known common mutations causing LGMD-2.

Ethics approval and consent to participate

The study was performed with permission issued by the Central Medical Ethics Committee of Latvia (N27 from December 14, 2011). All participants or their parents signed an informed consent form to participate.

Consent for publications

All participants or their parents signed an informed consent form to publish.

Availability of data and materials

Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4662.

Additional file

Additional file 1: Quality parameters and description of all mutations analyzed with Illumina VeraCode GoldenGate assay. (DOCX 69 kb)

Abbreviations

LGMD: limb-girdle muscular dystrophy; CK: creatine kinase; EMG: electromyography; SNP: single nucleotide polymorphism; PCR: polymerase chain reaction; CI: confidence interval; *DYSF*: dysferlin; *FKRP*: fukutin related protein; *ANO5*: anoctamin 5; *CAPN3*: calpain 3; *SGCA*: sarcoglycan alpha; *SGCB*: sarcoglycan beta; *SGCD*: sarcoglycan delta; *SGCG*: sarcoglycan gamma; *CLCN1*: chloride voltage-gated channel 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

II carried out the molecular genetic studies, participated in the mutation selection and drafted the manuscript. EJ participated in the design of the study and helped to draft the manuscript. JS carried out the molecular genetic studies. IV participated in the mutation selection. KV isolated patients' DNA. IM, JS, MSN, LC, VK, BB, AM participated in the recruitment of the patients and their diagnosing as neurologists or clinical geneticists. AK participated in the design of the study. AU participated in the design of the study. BL conceived of the study, participated in its design and coordination, participated in the mutation selection, participated in the recruitment of the patients and their diagnosing and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

Not applicable.

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Author details

[1]Biomedical Research and Study Centre, Ratsupites str. 1, k-1, LV-1067 Riga, Latvia. [2]Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University, Vilnius, Lithuania. [3]Centre for Medical Genetics, Vilnius University Hospital Santariškių Klinikos, Santariškių str. 2, LT-08661 Vilnius, Lithuania. [4]Laval University, Quebec, Canada. [5]Centre hospitalier universitaire de Québec, 2705, boulevard Laurier, Québec, Québec G1V 4G2, Canada.

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3.3. Neuromuscular disease diagnostics in Latvia - dawn of a new age (Collagen VI-related limb-girdle syndrome caused by frequent mutation in COL6A3 gene with conflicting reports of pathogenicity)

The paper looks at one of the many successful results from the collaboration with an international research project (MYO-SEQ) during the initial part of the project. The project was focused on trying to find the genetic cause in cases of undiagnosed rare neuromuscular disorders, using whole-exome sequencing. Here we describe dealing with cases where data on found variations are coming from many sources, with varying accompanying information and many different interpretations. In addition, we try to give our observations and conclusions on a relatively frequent variant in the *COL6A3* gene - c.7447A > G, giving our interpretation of its pathogenicity and connection with the associated disease.

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Collagen VI-related limb-girdle syndrome caused by frequent mutation in *COL6A3* gene with conflicting reports of pathogenicity

Janis Stavusis[a,*], Ieva Micule[a], Nathan T. Wright[b], Volker Straub[c], Ana Topf[c], Luísa Panadés-de Oliveira[d], Cristina Domínguez-González[d], Inna Inashkina[a], Dita Kidere[a], Nicolas Chrestian[e], Baiba Lace[a,f]

[a] *Latvian Biomedical Research and Study Centre, Ratsupites 1, Riga LV-1067, Latvia*
[b] *Department of Chemistry and Biochemistry, James Madison University, Harrisonburg, VA 22807, United States*
[c] *Institute of Genetic Medicine, Newcastle University and Newcastle Hospitals NHS Foundation Trust, Central Parkway, Newcastle upon Tyne, UK*
[d] *Department of Neurology, Hospital Universitario 12 de Octubre, Av. de Córdoba, s/n, 28,041, Madrid, Spain*
[e] *Child neurology department, CHUQ, Laval University, 2325 Rue de l'Université, Quebec City, Canada*
[f] *Medical Genetics department, CHUQ, 2705 Blvd Laurier, Quebec City, Canada*

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Abstract

Recently the scientific community has started to view Bethlem myopathy 1 and Ullrich congenital muscular dystrophy as two extremes of a collagen VI-related myopathy spectrum rather than two separate entities, as both are caused by mutations in one of the collagen VI genes. Here we report three individuals in two families who are homozygous for a *COL6A3* mutation (c.7447A> G; p.Lys2483Glu), and compare their clinical features with seven previously published cases. Individuals carrying homozygous or compound heterozygous c.7447A> G, (p.Lys2483Glu) mutation exhibit mild phenotype without loss of ambulation, similar to the cases described previously as Collagen VI-related limb-girdle syndrome. The phenotype could arise due to an aberrant assembly of Von Willebrand factor A domains. Based on these data, we propose that c.7447A> G, (p.Lys2483Glu) is a common pathogenic mutation.
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Keywords: COL6A3; Collagen VI-related myopathy; LGMD phenotype.

1. Introduction

Bethlem myopathy 1 and the more severe Ullrich congenital muscular dystrophy 1 are both associated with mutations in one of the three collagen VI genes—*COL6A1*, *COL6A2* and *COL6A3*. Since the same genes are involved, these two diseases are now often viewed as the two extremes of a spectrum of collagen VI-related myopathies. Lately, however, there has been also data on involvement of the *COL12A1* gene, with mutations in it being associated with Bethlem myopathy 2 [1–3].

First described in 1976 by Bethlem and Wijngaarden, the Bethlem myopathy patients have a milder and relatively stable myopathy that affects both sexes equally, with predominantly autosomal dominant inheritance, although autosomal recessive cases have been described as well [4]. Onset is usually in early infancy with slow progression [5]. Patients exhibit moderate weakness and atrophy of the trunk and limb muscles, typically involving proximal muscles more than distal, and extensors more than flexors. The hallmark of the Bethlem myopathy, seen in nearly all patients, is the eventual development of flexion contractures of the fingers, wrists, elbows, and ankles [6]. Many affected patients need a wheelchair by their fifties, and some die of respiratory failure [7]. The phenotype shows no overtly aberrant histology, and usually presents without elevated serum creatine phosphokinase levels, though cases of elevated creatine kinase have also been reported [4]. Findings by

* Corresponding author.
E-mail address: janis.stavusis@biomed.lu.lv (J. Stavusis).

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Merlini et al. indicate that mitochondrial dysfunction plays a critical role in the pathogenesis of the disorder [8].

Although precise data on disease frequency is still emerging and incomplete from many regions, there is compelling evidence that collagen VI-related myopathies are among the most common congenital muscular dystrophies worldwide [9,10]. For instance, in the UK, the prevalence of Ullrich congenital muscular dystrophy is 0.13 per 100,000 people and the prevalence of Bethlem myopathy is 0.77 per 100,000, though this could be somewhat underrepresented due to the current state of genetic and clinical testing [11,12]. Another study placed collagen VI-related myopathies as third most common type of congenital muscular dystrophies in the UK [12].

Collagen VI-related myopathies are known to present in both an autosomal dominant and a recessive pattern [12–15]. The genetic heterogeneity observed in patients leads to the hypothesis that collagen VI related myopathies comprise a spectrum of conditions, with variable severity, based on a number of factors [13]. For instance, the same mutation can cause both – a severe (Ullrich) and a mild (Bethlem) myopathy [16]. Interestingly however, it has been shown that haploinsufficiency is not enough to cause the myopathy, suggesting that either previously reported autosomal dominant patients might be carrying an additional unreported mutation or there is some additional regulatory mechanism [13].

Brinas et al. classified COL6A-related muscular dystrophies into three classes, based on the severity of the phenotype [2]. Likewise, Bonnemann proposed the following four phenotypical and functional classifications of collagen IV-related myopathy. First is severe Ullrich congenital muscular dystrophy (CMD), with patients exhibiting early phenotype, not achieving independent ambulation and frequently having severe contractures. Second is typical Ulrich CMD, with patients eventually achieving independent ambulation but then losing it again by the age of 20, loss of ambulation typically occurring between 5 and 15 years of age, frequently having severe contractures. Third is intermediate phenotype, with patient ambulation beyond 20 years of age and more variable contractures. Fourth is Bethlem myopathy, with patients retaining ambulation into adulthood and contractures of variable severity [1]. There are also two more rarely described Col6 phenotypes: a limb-girdle muscular dystrophy (LGMD) like presentation, classified as a subset of the fourth class, and myosclerosis [17].

The gene *COL6A3* itself is located on the reverse strand of chromosome 2q37.3. It codes one of the three alpha chains (alpha-3) of type VI collagen. The alpha chains form heterotrimeric monomers, the basic components of most of collagen VI, and are then secreted into the extracellular matrix where they form collagen VI microfibrils [18]. The collagen VI is a beaded filament collagen that is expressed in most connective tissues.

2. Objective

Here we report three novel individuals in two families, affected by mutation c.7447A> G, (p.Lys2483Glu) in a homozygous state in the *COL6A3* gene. We then compare their clinical features with previously published cases, to discuss possible pathogenicity of the reported mutation.

3. Subjects

The first family, which includes probands 1 and 2 (Fig. 1), was recruited within the framework of a neuromuscular disease research project in Latvia, targeting all unrelated individuals with predominant pelvic and shoulder girdle muscle weakness and elevated creatine kinase (CK) values. Collaboration with the Myo-Seq consortium was established to perform the clinical exome analysis. Data collection was performed with permission from the Central Medical Ethics Committee of Latvia. All participants or their parents signed the informed consent forms. Patients were interviewed and examined by a neurologist and a clinical geneticist experienced in neuromuscular disorders.

The second family of proband 3 (Fig. 1) was assessed during a regular visit to Medical Genetics Clinic, CHUL-CHUQ in Quebec City, Canada. The retrospective study involved information about clinical symptoms and muscle MRI.

Subjects included from previous publications

Panadés-de Oliveira et al. [16] added their clinical information and muscle MRI images of three patients with homozygous mutation c.7447A>G, (p.Lys2483Glu) in the *COL6A3* gene. Their study subjects were recruited retrospectively from the database of the Neuromuscular Disorders Unit of Hospital Universitario 12 de Octubre in Madrid.

Clinical information about proband 7, harboring the same homozygous mutation, was collected from the publication by Hunter et al. [15], where they recruited children with clinically diagnosed myopathies that have consented for participation in research study, according to the Western Institutional Review Board approved protocol.

Concerning materials and methods used with these patients, please refer to relevant publications [15,16].

Muscle MRI was performed for proband 1 and his unaffected father, as well as for proband 3, and patients of Panadés-de Oliveira.

4. Materials and methods

Whole exome sequencing was performed from the DNA samples of family 1 at the Broad Institute's Genomics Platform, using Illumina exome capture, 38 Mb baited target, and the Broad's in-solution hybrid selection process. Exome-sequencing pipeline included sample plating, library

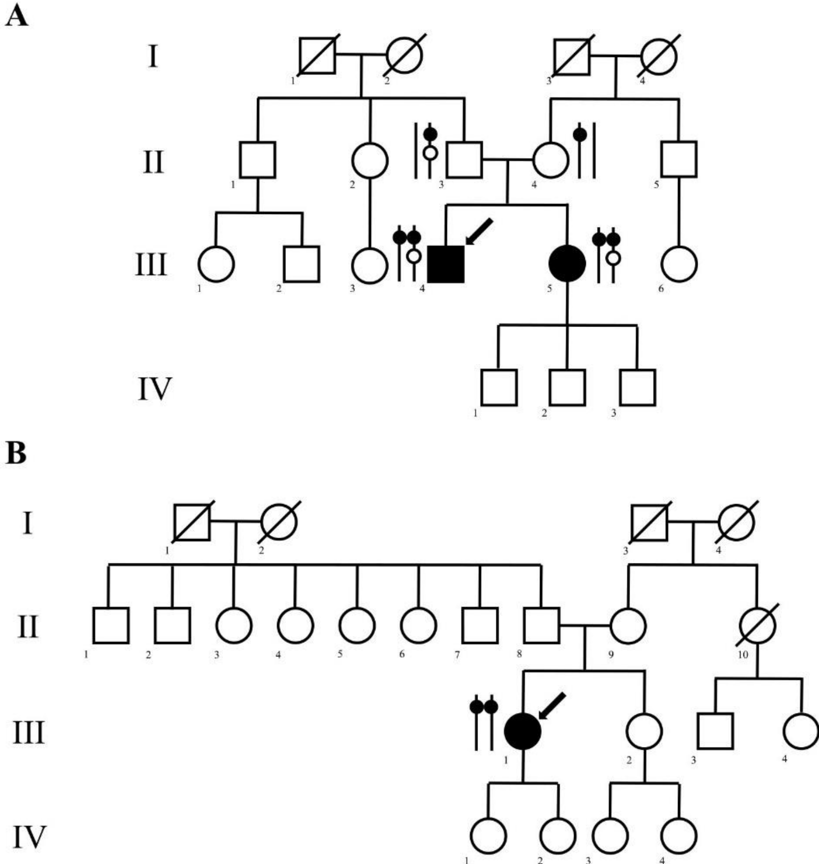

Fig. 1. Family pedigrees. **(A)** Pedigree of the first family indicating proband 1 (III-4) and proband 2 (III-5). **(B)** Pedigree of the second family, indicating proband 3 (III-1). For both pedigrees, black and whilte symbols indicate affected and unaffected individuals respectively, while index patients are denoted with arrows. Gene allele schematics denote patients for whom genetic testing has been done, with filled beads marking the c.7447A>G mutation and empty beads marking the c.8074delT mutation.

preparation (2-plexing of samples per hybridization), hybrid capture, sequencing (76 bp paired reads), sample identification QC check, and data storage. Hybrid selection libraries cover >80% of targets at 20× and have a mean target coverage of >80×. The exome sequencing data was de-multiplexed and each sample's sequence data were aggregated into a single Picard BAM file. Exome sequencing data was processed through a pipeline based on Picard, using the base quality score recalibration and local realignment at known indels. BWA aligner was used for mapping reads to the human genome build 37 (hg19). Single Nucleotide Polymorphism (SNPs) and insertions/deletions (indels) were jointly called across all samples using Genome Analysis Toolkit (GATK) Haplotype Caller package version 3.1. Default filters were applied to SNP and indel calls using the GATK Variant Quality Score Recalibration (VQSR) approach. Lastly, variants were annotated using Variant Effect Predictor (VEP).

Data from a neuromuscular gene panel sequencing was obtained from family 2 by a private company, using the approach where genomic DNA obtained from the submitted sample is enriched for targeted regions, using a hybridization-based protocol, and sequenced using Illumina technology. Unless otherwise indicated, all targeted regions were sequenced with ≥50× coverage depth. Reads were aligned to a reference sequence (GRCh37), and sequence changes were identified and interpreted in the context of a single clinically relevant transcript. All clinically significant observations were confirmed by Sanger sequencing.

The C1/11th VWA domain (residues 2393–2597; accession number NP_004360.2) was modeled with Phyre2 [19]. Molecular dynamics were performed on this model using the YASARA suite, as previously described [20]. After 100 ns equilibration, the Lys2483Glu mutation was created using the "swap" command, and this mutated structure was allowed to equilibrate for an additional 100 ns.

5. Results

5.1. Clinical findings and MRI data

In family 1 the proband was a 35-year-old male of Latvian ancestry, complaining since childhood about muscle weakness and lack of endurance. At age 30, the patient presented with muscle weakness and significant muscle atrophy, mostly in the hamstrings. This patient also had symmetrical proximal muscle weakness in lower extremities 4/5 and milder symmetrical proximal weakness in upper extremities, as well as mild axial weakness. Mild flexion contractures were noted in elbows and carpal joints, as well as medium ankle contractures. His creatine phosphokinase levels were slightly elevated (435 U/L), and an EMG revealed fibrillations and myopathic motor unit patterns. The muscle biopsy showed severe dystrophic changes with fibrosis, fatty replacement, and focal spots of hypertrophy. Pulmonary functional testing performed, revealed no deviation from norm. The sister of proband (proband 2) had similar symptoms; she also had proximal muscle weakness, limiting actions like climbing stairs and running, with mild ankle contractures. However, her disease progression was slower.

Muscle MRI of the proband, T1, STIR cor, PD, PD spair ax, T2 sag showed muscle atrophy with fatty infiltration from the periphery to center, with predominantly severe involvement of adductors, vasti and hamstring muscles, compared to gracilis and iliopsoas muscles (Fig. 2(B)). Muscle MRI of the patient's father was normal (Fig. 2(A)).

Proband 3 from family 2 was a 45-year-old woman, complaining since childhood about predominantly proximal muscle weakness in lower extremities. Patient's ancestry is French Canadian and Irish. Her main concern was leg weakness that had not progressed throughout her life. Muscle strength in upper extremities was symmetrical 4/5 in both proximal and distal muscle groups. Axial weakness was registered as 4/5. Muscle strength in lower extremities was reduced symmetrically 4/5, similarly in both proximal and distal muscle groups, with the exception of muscle quadriceps, which retained strength 5/5. Mild flexion contractures of ankles were noticed. EMG revealed axonal polyneuropathy in lower extremities. Her patellar reflexes were 2/4 and ankle reflexes 1/4. Oxygen saturation in venous and capillary blood was normal. Pulmonary X-ray was without pathology. There were no complaints concerning shortness of breath, cough or other respiratory problems, therefore pulmonary function tests were not performed. The proposed plan of investigation, including mutation analysis for other family members and pulmonary function tests, was refused by the patient, who we believe additionally suffers from anxiety and memory problems.

CK level was 429 U/l, with muscle biopsy showing fiber size variation, mild fibrosis and centralization of the nucleus. Muscle MRI showed diffuse involvement of adductors, hamstrings and vasti muscles (Fig. 2(F)).

Clinical information concerning all probands was collected in Table 1.

5.2. Genetic data

In proband 1, exome sequencing revealed two different mutations in the *COL6A3* gene, both of them downstream from the critical TH domain. The first is a rare homozygous substitution c.7447A>G, (p.Lys2483Glu) (here and further in text based on RefSeq NM_004369, transcript ID: ENST00000295550.8), that was first reported by Brinas et al. in 2010 in a compound heterozygous person, and can be found in the dbSNP database under reference number rs139260335 [2]. The mutation is located in exon 36, in the C1/11th VWA domain (VWA 11), located in a nonhelical region of the protein.

In silico predictory algorithms SIFT and PolyPhen mark it as deleterious and probably damaging, with CADD score of 22.5. The variant can be found in the GnomAD dataset with the frequency of 6.01e−04 in the global population, and is most prevalent in the non-Finnish European and Latino populations, with frequencies of 0.001 and 0.0007 respectively. In this dataset there exists one homozygous individual of non-Finnish European population, this bearing in mind, that GnomAD database curators made all efforts to exclude individuals with severe childhood diseases [21–24]. The affected site is phylogenetically completely conserved in mammals, with the surrounding area being conserved as well, albeit at a lower level.

The associated entry in ClinVar database is marked with conflicting interpretations of pathogenicity, which is not surprising, considering the phenotypic variability observed in association with mutations in COL6A genes, described previously. There are several unpublished reports in LOVD database associated with the c.7447A>G, (p.Lys2483Glu) mutation.

The second mutation is a heterozygous deletion c.8074delT, (p.Tyr2692MetfsTer15) leading to a frameshift and premature termination of the protein. This mutation has not been reported and cannot be found in any of the genetic databases available. It is located in exon 38, affecting the C2/VWA 12 domain, located in the same nonhelical region of the protein, as the other mutation. In addition, due to the premature termination, the truncated protein is missing two glycosylation sites and three disulfide bonds as well as a fibronectin type-III and a BPTI/Kunitz inhibitor domains downstream of the deletion.

The father of the patient is a carrier of both mutations c.[7447A>G; 8074delT], in a heterozygous state, and the mother is a carrier of c.7447A>G in a heterozygous state, both of them being healthy with no phenotypic expression, the proband's sister has the same genotype as the proband.

Proband 3 demonstrated the same homozygous substitution c.7447A>G, (p.Lys2483Glu) in *COL6A3*.

A model of the C1/11th VWA domain suggests p.Lys2483 is solvent exposed. Additionally, simulations of the p.Lys2483Glu mutation do not significantly perturb the structure. Instead, this mutation is likely to alter Col6a3-target binding (Fig. 3), seeing how C1 and C2 domains are binding

Table 1
Clinical information of known cases affected by the c.7447A>G mutation.

Author	Genotype	Gender, age	EMG	CK U/L	Muscle MRI	Muscle biopsy	Weakness	Contractures	Skin involvement	Scoliosis	Respiratory insuffiency
Homozygous carriers											
Panadés-de Oliveira et al. 2019 [16]	c.[7447A>G]; [7447A>G]	M, 42	ND	4000	COL6 disease type	Dystrophy, rimmed vacuoles	Proximal LL	Interphalangeal, ankles	No	No	No
	c.[7447A>G]; [7447A>G]	M, 48	ND	1000	COL6 disease type	ND	No	Interphalangeal, ankles	No	No	No
	c.[7447A>G]; [7447A>G] and COL6A1 c.2435–2A>G	F, 19	ND	830	COL6 disease type	ND	Distal LL	Interphalangeal, ankles	No	No	No
Hunter et al. 2015 [15]	c.[7447A>G]; [7447A>G]	M, 17	Normal peripheral motor and sensory nerve conductions with abnormal spontaneous activity, chronic motor neuropathy	1000	ND	Fiber size variation, fiber type changes suggestive of myopathy	Proximal LL, distal LL	Ankles	No	Yes	Normal FVC and FEV1
Present study, Proband 1	c.[7447A>G]; [7447A>G; c.8074delT]	M, 30	Fibrillations and generalised myogenic impairement	534	COL6 disease type	Severe atrophy of muscle tissue, compensating by focal spots of hypertrophy, suspecting muscular dystrophy	Proximal LL	Elbows, ankles	No	No	Normal FVC and FEV1
Present study, Proband 2	c.[7447A>G]; [7447A>G; c.8074delT]	F, 42	ND	ND	ND	ND	Proximal LL	Ankles	No	ND	ND
Present study, Proband 3	c.[7447A>G]; [7447A>G]	F, 44	Axonal polyneuropathy in lower extremities, which could be caused as a result of chronic neurogenic impairment and/or chronic myogenic impairment with neurogenic recruitment	429	Diffuse and severe fatty infiltration	Fiber size variation, mild fibrosis and centralization of nucleus	Distal LL, proximal LL	Ankles	No	Yes	No
Compound heterozygous carriers											
Brinas et al., 2010 [2]	c.[7447A>G]; [9384_9386del]	M, 12	ND	ND	ND	ND	ND	ND	ND	No	FVC% 78
Hunter et al. 2015 [15]	c.[5480delG]; [7447A>G]	M, 12	Fibrillations	700	ND	Fiber size variation. Dystrophic changes, normal Collagen 6.	Proximal	Ankles	No	No	Normal FVC and FEV1
Panadés-de Oliveira et al. 2019 [16]	c.[8540_8540 delA]; [7447A>G]	F, 27	ND	800	COL6 disease type	Dystrophy	Cervical, proximal	Elbows, ankles	No	No	Reduced maximal expiratory pressure

LL – lower limbs.
CMAP – compound motor action potential.
COL6 disease type MRI – Typical muscle MRI findings of Bethlem myopathy in T1-weighted sequence showing concentric involvement of vastus lateralis and central high signal in rectus femoris (c), as well as peripheral involvement of medial gastrocnemius muscle.

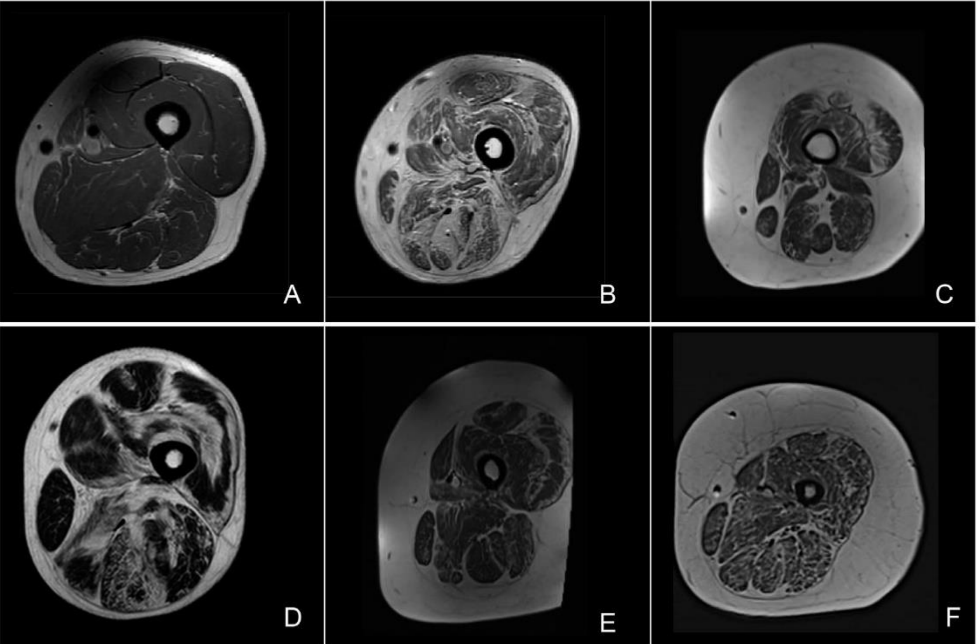

Fig. 2. Muscle MRI of the patients with c.7447A>G mutation.
All T1 weighted axial sequence of thigh.
(A) Father of proband 1 COL6A3 c.[7447A>G; 8074delT].
MRI – normal.
(B) Proband 1 COL6A3 c.[7447A>G; 8074delT]; [7447A>G].
MRI – diffuse and severe fatty infiltration from periphery to center pattern of mm. vastus lateralis and mm. vastus medialis, hamstrings, adductors, but relatively sparing mm. gracilis.
(C) Patient 15. COL6A3 c.[7447A>G]; [7447A>G].
MRI – fatty replacement mainly involving mm. vastus lateralis and mm. vastus medialis with beginning of hamstrings and mm. adductor magnus involvement. Mm. gracilis and mm. sartorius are relatively preserved.
(D) Patient 11. COL6A3 c.[7447A>G]; [7447A>G].
MRI – diffuse and severe fatty infiltration from periphery to center pattern of mm. vastus lateralis, mm. vastus medialis, hamstrings and adductors, but sparing mm. gracilis and mm. sartorius.
(E) Patient 15. COL6A3 c.[7447A>G]; [7447A>G].
MRI – mild fatty infiltration mainly involving mm. vastus lateralis and starting in hamstrings, sparing mm sartorius and gracilis.
(F) Proband 3 COL6A3 c.[7447A>G]; [7447A>G].
MRI – diffuse and severe fatty infiltration, involving all muscles but affecting more severely mm. vasti, adductors and harmstrings.

same domains on other proteins, thus being responsible for monomer assembly [17].

6. Discussion

The alpha-3 chain is a modular protein comprised of 10 Von Willebrand factor A (VWA) domains arranged in tandem at the N-terminus, followed by collagen triple helix (TH) domain, then two more VWA domains, a Fibronectin type-III (FN3-like) domain and a Kunitz domain. The N-terminal VWA domains make Col6A3 significantly larger than its counterparts the alpha-1 and −2 chains (3177 residues), and play a major role in cell adhesion by binding other extracellular matrix proteins [25]. In turn, this cell adhesion event drives the organizing of matrix components and muscle development.

Previous research has shown that homozygous truncating mutations before or within the triple helix (TH) domain is associated with the most severe collagen VI-related myopathies, while the moderate phenotypes are associated with heterozygous mutations resulting in skipping off part of TH domains or affecting the glycine residue of the Gly-X-Y domain [2]. Out of these, mutations affecting the conserved Gly-X-Y motif in the triple helix (TH) domain are the most commonly identified mutations in the collagen VI myopathies.

When analyzing genotype/phenotype correlations of 194 individuals with glycine substitutions in the TH domain of either of the *COL6A* genes, Butterfield et al. noted that in all three genes 89% of the mutations were clustered in the N-terminal region before the 17th Gly-X-Y triplet (TH17) [26]. In *COL6A3* this region contains cysteine residues that form disulfide bonds that stabilize tetramers.

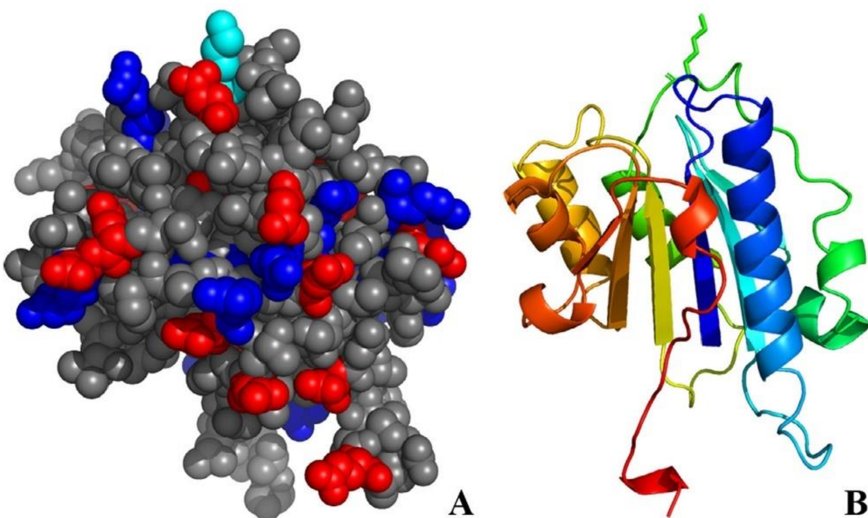

Fig. 3. Modeling of the Col6a3 affected domain.
(A) The charge distribution of our Col6a3 c.2400–2600 model. Blue is + charged residues, red is – charged residues. Cyan, at top, is K2483. **(B)** Model of the c.2400–2600 domain of Col6a3. The green amino acid at the top of the molecule is K2483. It is noteworthy that the C-terminus, in red, likely does not point down but instead snakes its way up the side of the domain so that the C2 domain is oriented somewhere above this C1 domain.

While patients with mutations inside the region of Gly-X-Y triplets 10–15 tend to have a more severe phenotype than those with mutations outside this region, identical glycine substitutions have been associated with both severe and mild phenotypes [26]. The recessive mutations tend to occur in the C-terminal end of the TH domain.

In 2010 Brinas et al. described a compound heterozygous patient carrying c.7447A>G, (p.Lys2483Glu) and c.9384_9386 del (p.Trp3128Cys + p.Tyr3129del) mutations with reduced secretion and intracellular retention of COL6A3 in patients' fibroblasts, presenting with mild Bethlem myopathy phenotype [2].

Since then, there has been an accumulation of information concerning c.7447A>G, (p.Lys2483Glu) carriers in compound heterozygous and homozygous states. To the best of our knowledge, there was until now a total of at least 18 such carriers reported, though the number might vary due to a possible overlap of reports [2,12,15,16,27,28]. In cases where a detailed clinical description is available, all of them are presenting with similar disease course. To summarize the phenotype and the natural history of the disease for the seven cases in the current study, onset of the disease is early, with delay in the development of gross motor skills, like Bethlem myopathy. Course of the disease seems to be slowly progressive, with most of the patients having muscle weakness that is restricting only some strenuous activities, usually becoming noticeable in late adolescence or adult age. Contractures are mild and often only ankle joints are involved [12,15,16]. Nallamilli et al. reported c.7447A>G mutation in a heterozygous state in one of the patients with LGMD phenotype [27].

Fichna et al. reported compound heterozygote mutations p.Arg2142Ter*; p.Lys2483Glu in the *COL6A3* gene in a patient with limb-girdle weakness and preserved ambulation, with first symptoms presenting at the age of 14, CK level was elevated seven times [28].

The associated entry of c.7447A>G in ClinVar database has this mutation marked as a variant with conflicting interpretation of pathogenicity. It seems the most probable reason for this could be the fact that a male from European (non-Finnish) population, homozygous for the mutation, has been registered in the GnomAD database. GnomAD curators have made all efforts to remove all individuals known to be affected by severe pediatric disease. Our hypothesis being that the homozygous person included in the database, did not present severe symptoms at childhood, similar to the rest of our patients [24].

There have been several other genes described as having similar features, namely mutations in them being associated with more than one phenotype, for example, mutations in *POMGNT1, FKTN, POMT2, FKRP* and several others can cause both - a severe, even early lethal phenotype, but also a mild dystrophy [29–32]. Some of the mutations have been observed to exhibit tissue specificity, with symptoms related to only one organ, like isolated cardiomyopathy due to mutations in the *TTN* gene [33], or the *FKTN* gene [34]. Particularly interesting are the dominant mutations of the *ANO5* gene, causing gnathodiaphyseal dysplasia [35], whereas the recessive mutations cause LGMD symptoms. These data highlight the expanding range of variability, observed in phenotypic expression, that is moving beyond the gene-to-disease system and more towards the affected domain-to-

disease. Such a shift is making it more difficult for clinicians, trying to pinpoint the genetic cause of a disease, based on phenotypic traits, but also for classification matters, muddying the gene-to-phenotype association. This peculiarity arises from defects in different functional regions of the protein affecting completely different aspects of the protein function, and therefore manifesting as very distinctive phenotypic traits, often resembling defects in other genes.

Regarding EMG examinations in patients carrying the c.7447A>G, (p.Lys2483Glu) mutation in the *COL6A3* gene, there are no specific findings—patients can have chronic motor neuropathy or fibrillations and positive sharp waves. The muscle biopsy, if not stained by ICH for the COL6 proteins, shows great variability, depending on the stage of disease, with the most common finding being fiber size variations, suggestive of myopathy and later muscular dystrophy. Creatine phosphokinase level is usually elevated from two to four times the normal. Currently muscle MRI has served as the most specific medical examination criteria, with pathognomonic fatty degeneration from peripheral to center of the rectus femoris and vastus lateralis with the typical diamond shape or tigroid pattern, despite its usefulness, muscle MRI is still not routinely used [1]. Our reported patients have MRI findings typical of the COL6 disease (Fig. 2).

Segregation analysis of family 1 showed that the disease is inherited in an autosomal recessive pattern, involving the c.7447A>G, (p.Lys2483Glu) mutation in the COL6A3 gene. We can always speculate that there could be another deep intronic mutation in COL6A genes, responsible for the Bethlem myopathy phenotype, classified by the characteristic pattern seen in muscle MRI. There is accumulating evidence that this rare variant c.7447A>G in a homozygous state can lead to a mild Bethlem myopathy and/or LGMD phenotype without respiratory involvement, however, for the patients with other mutations in a compound heterozygous state, the phenotype severity entirely depends on the second mutation. Looking at the available data, it can be seen that the patient described by Brinas et al., a compound heterozygous carrier for the c.[7447A>G]; [9384_9386 del] variants in the COL6A3 gene, had pulmonary involvement characterized by FVC borderline reduction to 78% at the age of 12. In this case no information is available regarding muscle weakness or contractures [2]. The second person with compound heterozygous genotype c.[5480delG]; [7447A>G] had grade 3/5 muscle weakness of hip abductors and abdominal muscles at the age of 12 [15]. In comparison proband 3, described in this paper, carrier of the c.7447A>G variant in a homozygous state, had muscle weakness of 4/5 in proximal muscle groups at the age of 44.

VWA domains in general give the protein the ability to bind other proteins, while the specific C1 and C2 domains (VWA 11 and VWA 12), affected in family 1, have been shown to be necessary for the assembly of monomers, which suggests that VWA domains interact with specific regions of other VWA domains, with C1 being sufficient for assembly. However lack of or mutations in C2 being associated with disease [17]. Furthermore it was reported already in 1998 by Pan et al. that missense mutation in a VWA domain can be associated with Bethlem myopathy [36]. Therefore, it is possible to hypothesize that a change in one of them might affect the binding potential and therefore modulate the phenotype, when present together with other mutations in the gene.

SAXS work by Beecher et al. suggest that collagen VI-a1, a2, and a3 form a heterotrimer, and this is held together by the VWA domains in the C-terminus of each molecule. It is thus tempting to speculate that p.Lys2438Glu mutation, which substitutes a basic residue for an acidic one, ablates a stabilizing electrostatic interaction and perturbs this heterotrimer. Such a molecular mechanism of action is broadly in line with the functional consequence of this mutation; weakened or misformed collagen VI fibers would affect skeletal muscle structural stability and regeneration.

Based on this, it might be possible to speculate that with premature termination of the protein produced from one of the chains and one of the VWA domains being affected on the other, the overall functions of the protein might be affected enough to lead to the myopathic phenotype observed in proband 1. It has been shown previously that mutations causing premature termination codons have a profound destabilizing effect on the corresponding transcripts, leading to degradation of transcripts harboring these mutations at the post-transcriptional level [2].

The phenotype-genotype correlation observed in family 1 allows us to conclude that the c.8074delT mutation, despite being seemingly more damaging, in a heterozygous form cannot cause symptoms on its own, confirming data described previously.

We would like to classify c.7447A>G, (p.Lys2483Glu) as a common pathogenic mutation, based on the reduced COL6 secretion and intracellular retention in a patient described by Brinas et al., association with phenotype described by Hunter et al. as well as Sframeli et al. and the typical muscle MRI associated with COL6 related myopathy, seen in our reported proband from family 1, as well as the patient reported by Oliveira et al. Homozygous carriers of c.7447A>*G*, (p.Lys2483Glu) mutation exhibit mild phenotype, described previously by Bonnemann as Collagen VI-related limb-girdle syndrome [1,12,15,16] .

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3.4. Cases that are unique (Novel mutations in MYBPC1 are associated with myogenic tremor and mild myopathy)

Here we describe one of the project highlights, where searching for LGMDs, through collaboration with other specialized labs, by using the means of NGS, leads to the discovery of a previously undescribed and very rare disease phenotype, associated with a domain-specific variation in the *MYBPC1* gene. Variations in this gene previously were associated with a completely different phenotype. We describe this phenotype and provide initial thoughts on the possible molecular mechanism that is causing this unique phenotype - a mild myopathy accompanied by a myogenic tremor, now termed myotrem.

Novel Mutations in *MYBPC1* Are Associated With Myogenic Tremor and Mild Myopathy

Janis Stavusis, MSc,[1*] Baiba Lace, MD, PhD,[1,2*] Jochen Schäfer, MD,[3*] Janelle Geist, BSc,[4] Inna Inashkina, PhD,[1] Dita Kidere, MSc,[1] Sander Pajusalu, MD, PhD,[5,6] Nathan T. Wright, PhD,[7] Annika Saak, MD,[3] Manja Weinhold, MD,[3] Dietrich Haubenberger, MD,[8] Sandra Jackson, PhD,[3*] Aikaterini Kontrogianni-Konstantopoulos, PhD,[4*] and Carsten G. Bönnemann, MD[9*]

Objective: To define a distinct, dominantly inherited, mild skeletal myopathy associated with prominent and consistent tremor in two unrelated, three-generation families.

Methods: Clinical evaluations as well as exome and panel sequencing analyses were performed in affected and non-affected members of two families to identify genetic variants segregating with the phenotype. Histological assessment of a muscle biopsy specimen was performed in 1 patient, and quantitative tremor analysis was carried out in 2 patients. Molecular modeling studies and biochemical assays were performed for both mutations.

Results: Two novel missense mutations in *MYBPC1* (p.E248K in family 1 and p.Y247H in family 2) were identified and shown to segregate perfectly with the myopathy/tremor phenotype in the respective families. *MYBPC1* encodes slow myosin binding protein-C (sMyBP-C), a modular sarcomeric protein playing structural and regulatory roles through its dynamic interaction with actin and myosin filaments. The Y247H and E248K mutations are located in the NH_2-terminal M-motif of sMyBP-C. Both mutations result in markedly increased binding of the NH_2 terminus to myosin, possibly interfering with normal cross-bridge cycling as the first muscle-based step in tremor genesis. The clinical tremor features observed in all mutation carriers, together with the tremor physiology studies performed in family 2, suggest amplification by an additional central loop modulating the clinical tremor phenomenology.

Interpretation: Here, we link two novel missense mutations in *MYBPC1* with a dominant, mild skeletal myopathy invariably associated with a distinctive tremor. The molecular, genetic, and clinical studies are consistent with a unique sarcomeric origin of the tremor, which we classify as "myogenic tremor."

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Address correspondence to Dr Carsten G. Bönnemann, Neuromuscular and Neurogenetic Disorders of Childhood Section, Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, NIH, Porter Neuroscience Research Center Building 35, Room 2A-116. MSC 35 Convent Drive, Bethesda, MD 20892. E-mail: carsten.bonnemann@nih.gov; or Dr Aikaterini Kontrogianni-Konstantopoulos, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 North Greene Street, Baltimore, MD 21201. E-mail: akontrogianni@som.umaryland.edu

*These authors contributed equally to the work.

From the [1]Latvian Biomedical Research and Study Centre, Riga, Latvia; [2]Centre Hospitalier Universitaire de Québec, Ville de Québec, QC, Canada; [3]Department of Neurology–Uniklinikum CG Carus, Dresden, Germany; [4]Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD; [5]Department of Clinical Genetics, United Laboratories, Tartu University Hospital, Tartu, Estonia; [6]Department of Clinical Genetics, Institute of Clinical Medicine, Tartu University, Tartu, Estonia; [7]Department of Chemistry and Biochemistry, James Madison University, Harrisonburg, VA; [8]Clinical Trials Unit, Office of the Clinical Director, NINDS Intramural Research Program, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD; and [9]Neuromuscular and Neurogenetic Disorders of Childhood Section, Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD

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Mutations in genes that encode sarcomere-associated proteins underlie an increasingly diverse and growing number of skeletal and cardiac myopathies. Skeletal sarcomeric myopathies encompass histological manifestations ranging from rods and hyaline bodies to various fiber-type disproportion patterns.[1] Remarkably, different mutations in the same sarcomeric protein can lead to strikingly distinct clinical manifestations, ranging from congenital to late onset, and resulting in either muscle weakness or stiffness. These physical symptoms are derived from the molecular repercussions of specific mutations, such as changes in calcium sensitivity or target binding. In addition to mutations causing weakness in conjunction with congenital myopathy, stiffness-inducing mutations have also been described in *TPM2*, *TPM3*, and *ACTA1*.[2] In some cases, patients may present with multiple congenital joint contractures (arthrogryposis multiplex) as a result of prenatal restriction of joint movements.[3] This diversity in disease manifestation underscores the central role of the sarcomere in muscle function and disease. It also highlights that different mutations in sarcomeric proteins may have highly variable pathophysiological consequences, indicating the importance of elucidating their individual molecular pathogenesis.

Myosin binding protein-C (MyBP-C) comprises a family of sarcomeric accessory proteins and includes three isoforms: cardiac (c), fast skeletal (f), and slow skeletal (s).[4] The major roles attributed to the MyBP-C family are stabilization of thick filaments and regulation of cross-bridge cycling.[5–7] Slow myosin binding protein-C (sMyBP-C) is encoded by *MYBPC1*, located on chromosome 12 in humans.[8] Alternatively spliced transcripts of the gene give rise to a subfamily of at least 14 variants regulated by phosphorylation, which are co-expressed in different combinations and amounts in both slow- and fast-twitch muscles.[6,9–12]

sMyBP-C is a modular protein composed of immunoglobulin (Ig) and fibronectin-III (Fn-III) repeats, numbered C1 to C10. The NH_2-terminal Ig, C1, is flanked by unique sequences, the Pro/Ala–rich motif and a MyBP-C specific motif, termed M-motif (Fig 1A).[6,13] The NH_2 terminus of sMyBP-C, including the Pro/Ala–rich motif, C1, and the M-motif, supports binding to actin and myosin subfragment-2 (S2) in a variant-specific manner,[14] whereas the COOH-terminal Ig, C10, binds directly to light meromyosin (LMM), and this interaction is further enhanced by the presence of Ig C8 and Fn-III C9.[15,16] In addition to binding LMM, the COOH terminus of sMyBP-C supports binding to titin, obscurin, four and a half LIM domains 1 (FHL1) protein, and creatine kinase.[16–19]

Earlier reports have linked mutations in *MYBPC1* to development of severe and lethal forms of distal arthrogryposis (DA).[20–22] Here, we report on a distinct clinical phenotype in two independent, three-generation families, manifesting as mild myopathy invariably associated with a persistent, posturally pronounced, high-frequency tremor in all affected individuals that segregates with novel, dominant, missense mutations in *MYBPC1* in each family. We provide pathophysiological insights into this tremor by showing that altered myofilament binding of mutant sMyBP-C appears to be the basis of what we refer to as myogenic tremor. A comprehensive literature search revealed that unexplained tremor associated with myopathy is an overlooked feature of diseases involving mutations in genes encoding sarcomeric proteins.

Materials and Methods

Patients and Clinical Evaluation

Examination of family 1 from Latvia was performed under a protocol approved by the Central Medical Ethics Committee of Latvia, and all subjects and/or their parents have given informed consent. The variant, NM_002465.3:c.742G>A p.(E248K), has been submitted to the ClinVar database. Clinical evaluation of patients was performed by a clinical geneticist and a neurologist. Genetic studies in individuals from family 2 from Germany were performed with informed consent, in accord with the Human Genetic Examination Act (Genetic Diagnosis Act-GenDG).

Quantitative Tremor Analysis

Quantitative tremor analysis was performed in subjects III-2 and IV-1 from family 2 using a Dantec Keypoint Workstation (Natus Medical Inc., Pleasanton, CA). Briefly, accelerometers were attached to the dorsum of the hands, and electromyography (EMG) of forearm flexor and extensor muscles was recorded by surface electrodes fixed to the extensor carpi ulnaris and flexor carpi ulnaris muscles, respectively. Power spectra of the accelerometry and EMG data were calculated by fast Fourier transformation of tremor amplitude and frequency time-series recordings.[23] Tremor analysis was performed at rest and during posture with outstretched hands, both unloaded and with a weight of 500g.

Sequencing

Family 1. Exome sequencing of 4 members of family 1 from Latvia (Fig 1B) was performed using the Illumina HiSeq 2500 platform (Illumina, San Diego, CA) and the Nextera Rapid Capture Expanded Exome kit (Illumina) for best variant segregation. Raw sequencing reads were aligned and subsequently processed according to Genome Analysis Toolkit's (GATK) best practice guidelines using BWA-MEM, Picard-tools, and GATK. Variants were called by GATK Haplotype Caller and then annotated using Annovar. A step-wise approach of filtering was used on the obtained data. Only rare (below 1% in all reference databases, including ExAC, 1000 Genomes, ESP6500, and an in-house next-generation sequencing database from Tartu University Hospital) non-silent variations in autosomal coding regions and splice sites were considered for further analysis. Pathogenicity of variants was evaluated using several in-silico prognostic tools,

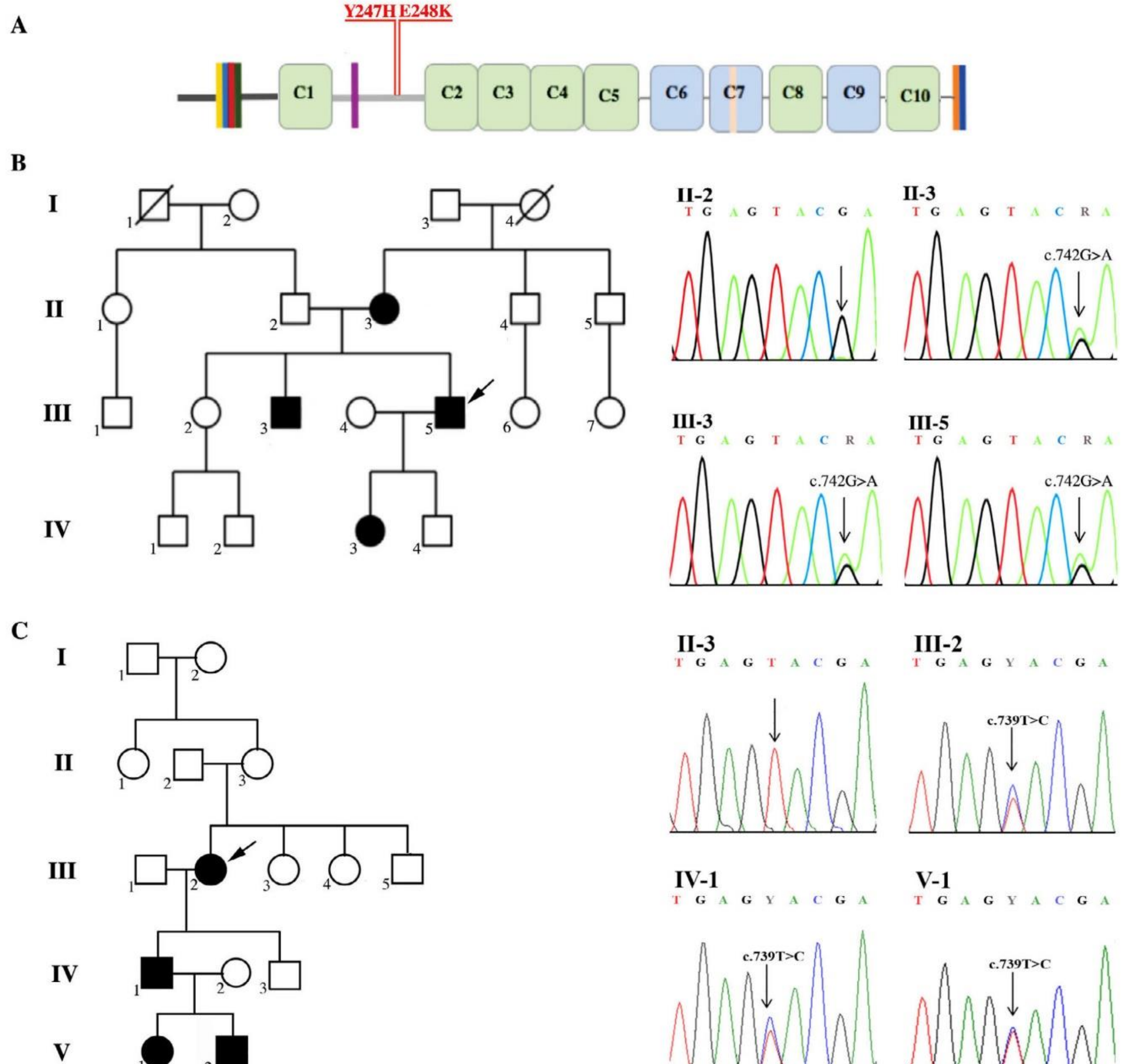

FIGURE 1: Identification of two novel missense mutations in *MYBPC1* underlying the development of skeletal myopathy with tremor. (A) Schematic representation of the structure of sMyBP-C. Dark and light gray horizontal lines correspond to the Pro/Ala–rich region and the M-motif, respectively, whereas vertical colored lines in the NH_2 terminus, domain C7, and the extreme COOH terminus represent alternatively spliced segments. Green and blue rectangles denote immunoglobulin (Ig) and fibronectin-III (Fn-III) domains, respectively. The two novel *MYBPC1* human mutations, Y247H and E248K, and their location in the sMyBP-C M-motif are indicated. (B) Pedigree of family 1 and sequence chromatograms showing the c.742G>A, p.(E248K) mutation in affected individuals II-3, III-3, and III-5 after exome sequencing and the wild-type sequence in the II-2 unaffected individual. (C) Pedigree of family 2 and sequencing chromatograms showing the c.739T>C, p.(Y247H) mutation in affected individuals III-2, IV-1, and V-1 and wild-type sequence at this position in an unaffected individual (II-3). For both pedigrees, black and white symbols indicate affected and unaffected individuals, respectively, whereas index patients are denoted with arrows.

including PolyPhen2, Mutation Taster, SIFT, and Combined Annotation Dependent Depletion (CADD). Notably, during our analysis, we also evaluated close to 100 previously identified myopathic genes as potential candidates, but they were found to be negative.

Variation validation as well as segregation analysis were performed with nested polymerase chain reaction (PCR; initial synthesis primer set: 5'TCTGGGCTTCAAGTACTCAGG3' and 5'CTCACCTGCGCTCTTCTTCT3', and Sanger sequencing primer set: 5'TACCTCGACCTTCGTGGTCT3' and 5'GCTTGAGTCGCTTGAGCAT3') using HOT FIREPol polymerase (Solis Biodyne, Tartu, Estonia). In addition to the previously mentioned 4 members, samples from 6 additional family members were used for mutation

testing and extended segregation analysis. Amplicons were sequenced with an ABI Prism 3130XL (Applied Biosystems, Waltham, MA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing data were analyzed using CLC MainWorkbench (CLCbio, Aarhus, Denmark). Ninety DNA samples from control subjects taken from the Latvian National Genome database were also directly sequenced for presence of the *MYBPC1* E248K mutation.

Family 2. Panel sequencing, using a panel specifically for distal and congenital myopathies, was performed in DNA obtained from the proband III-2 of family 2 from Germany. Confirmation of the observed sequence change in *MYBPC1* was performed by PCR amplification of the affected exon followed by Sanger sequencing in the forward and reverse direction using the BigDye terminator (v1.1) cycle sequencing kit from Applied Biosystems. DNA isolated from clinically affected and unaffected family members was also screened for the sequence change identified in subject III-2 using the same method.

Immunohistochemistry

A biopsy was obtained from the deltoid muscle of the index patient (III-2) from family 2, and histochemical staining (hematoxylin and eosin, cytochrome C oxidase, Gomori, van Gieson, NADH dehydrogenase, ATPase 9.4, PAS, and oil-red O) was performed using standard procedures.

Generation and Purification of Recombinant Wild-Type and Mutant Proteins

For overlay assays, human skeletal muscle complementary DNA was used with sense (5'ATGCCAGAACCCACT3') and antisense (5'TCAATCAAGAATTTTTGC3') oligonucleotides to amplify the NH_2-terminal region of sMyBP-C that contained the Pro/Ala–rich motif, Ig domain C1, and the M-motif (amino acids 1-284, XP_006719468.1). The PCR fragment was subcloned into the pGex4T-1 (Amersham Pharmacia, Piscataway, NJ) vector at *Eco*RI/*Xho*I sites to generate a GST-fusion protein. The Y247H mutation was introduced into the resulting plasmid with the following set of primers: sense (5'GCCAAGCCCAGCGAGCACGAGAAGATCG CCTTC) and antisense (5'GAAGGCGATCTTCTCGTGCTCG CTGGGCTTGGC3'), and the E248K mutation was similarly introduced using the primer set: sense (5'AAGCCCAGCGAGTAC AAGAAGATCGCCTTCCAG3') and antisense (5'CTGGAAGGC GATCTTCTTGTACTCGCTGGGCTT3'). Authenticity of the wild-type and mutant constructs was verified by sequence analysis. Recombinant polypeptides were expressed by induction with 1mM isopropyl-β-thioglycopyranoside (IPTG) overnight at 22°C and purified by affinity chromatography on glutathione-agarose columns, according to the manufacturer's instructions (MilliporeSigma, Bedford, MA).

For circular dichroism experiments, the following set of sense (5'ACTGGAATTCATGCAGGAGGAGGAGCCC) and antisense (5'ACTGCTCGAGTCACTTCTTCTCCTCCCT') primers was used to amplify the M-motif region of wild-type sMyBP-C. The PCR fragment was subcloned into the pET30a+ vector (MilliporeSigma) at *Eco*RI/*Xho*I sites to generate a histidine-tagged fusion protein. The Y247H and E248K mutations were introduced in the wild-type construct using the same primers listed above. Authenticity of the wild-type and mutant constructs was verified by sequence analysis. Recombinant polypeptides were expressed in BL21(DE3) cells (MilliporeSigma) by induction with 1mM IPTG overnight at 22°C and purified by affinity chromatography on Ni-NTA-agarose columns (MilliporeSigma), according to the manufacturer's instructions.

Overlay Assay and Immunoblotting

Heavy meromyosin (HMM) and actin purified from skeletal muscle were purchased from Cytoskeleton (Heavy meromyosin Cat. #MH01-A, Actin Cat. #AKL99-A; Cytoskeleton, Inc., Denver, CO). Equivalent amounts (3μg) of purified actin and HMM were separated by 4% to 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose. Equivalent loading and transfer were confirmed by staining the nitrocellulose membrane with Ponceau red. Blots were incubated in buffer A (50mM KCl, 20mM MOPS, 4mM $MgCl_2$, 0.1mM EGTA, 2mM DTT, 3% BSA, 10mM NaN_3, 0.5% Tween-20, and 0.5% Nonidet P-40) for 8 hours at room temperature, followed by incubation with buffer A containing 0.5μg/ml of the indicated glutathione *S*-transferase (GST)-fusion proteins overnight at 4°C. Blots were washed extensively with buffer A, followed by PBS, blocked in 5% milk, and subsequently probed with antibodies to GST (1:10,000; Novagen, Billerica, MA), as described in a previous work.[24] Immunoreactive bands were detected with the Tropix Chemiluminescence kit (Applied Biosystems). These experiments were repeated at least three independent times, and quantification of immunoreactive bands was performed using densitometric analysis and ImageJ software (NIH, Bethesda, MD). Statistical significance was evaluated with a *t* test, $p < 0.05$.

Modeling of sMyBP-C/Myosin Interaction

Models of the M-motif of sMyB-C were generated using Phyre2, RaptorX, and iTASSER, based on the human cardiac version of this domain (PBD: 2LH4; 80% identical to sMyBP-C).[25–27] The model was then allowed to equilibrate using the computer program YASARA for ~90ns, as previously described.[28,29] Individual mutations were introduced to the model by the "swap" command in YASARA, and the resulting models were allowed to equilibrate for ~100ns.

Docking of wild-type and mutant sMyBP-C to myosin was performed using equilibrated sMyBP-C models and the S2 fragment of myosin (PDB: 2FXO) with the HADDOCK program.[30] The best models, as judged by low z-scores and low root mean square deviation, were then further refined for ~30ns.

Circular Dichroism

Circular dichroism experiments were conducted in a 1-mm pathlength cuvette with wild-type and mutant His-tagged proteins of 15 to 17μM in 20mM phosphate buffer (pH 7.5) and 50mM NaCl. Samples were measured in triplicate at temperatures from 20°C to 90°C in 10°C intervals on a Jasco J-810 spectrophotometer.

Results

Family 1 was ascertained in Latvia by examination of a 30-year-old male (Fig 1B; proband III-5) who presented with muscle weakness and skeletal deformities (Figs 1B and 2A; Supplementary Table 1). The patient had hypotonia and a postural hand tremor since infancy, followed by a delay in gross motor milestone acquisition, with independent walking achieved at 2 years of age. His cognitive functions were normal, and his intellectual development was age appropriate. In early adolescence, symptoms became more noticeable, and the patient developed scoliosis and thoracic asymmetry. Since then, the disease has reached a phase of stability without further deterioration in strength, although the patient reported frequent respiratory infections. Current clinical findings include a high-frequency irregular tremor that is accentuated by posture, most noticeable in the hands (Supplementary Video 1) and, to a lesser extent, in the legs. In addition, the patient presents mild axial muscle weakness, predominantly proximal appendicular weakness, scoliosis with thoracic/sternal deformity, and rigidity of the spine (Fig 2A). Examination of additional family members identified 3 individuals with similar clinical manifestations from three generations, including the index patient's mother, brother, and daughter (Figs 1B and 2A). All 4 affected family members display a similar, predominantly postural, irregular, high-frequency, low-amplitude tremor (Supplementary Video 2).

EMG performed in the proband revealed myopathic changes that were most pronounced in the arms. The family did not consent to a muscle biopsy.

To identify disease-associated genetic variants in this family, whole-exome sequencing analysis was performed on 3 of the affected members and 1 unaffected member, revealing the presence of a single, novel heterozygous variation in the *MYBPC1* gene c.742G>A, p.(E248K) segregating in all 3 affected individuals, but not in the unaffected individual. Directed Sanger sequencing of DNA obtained from additional

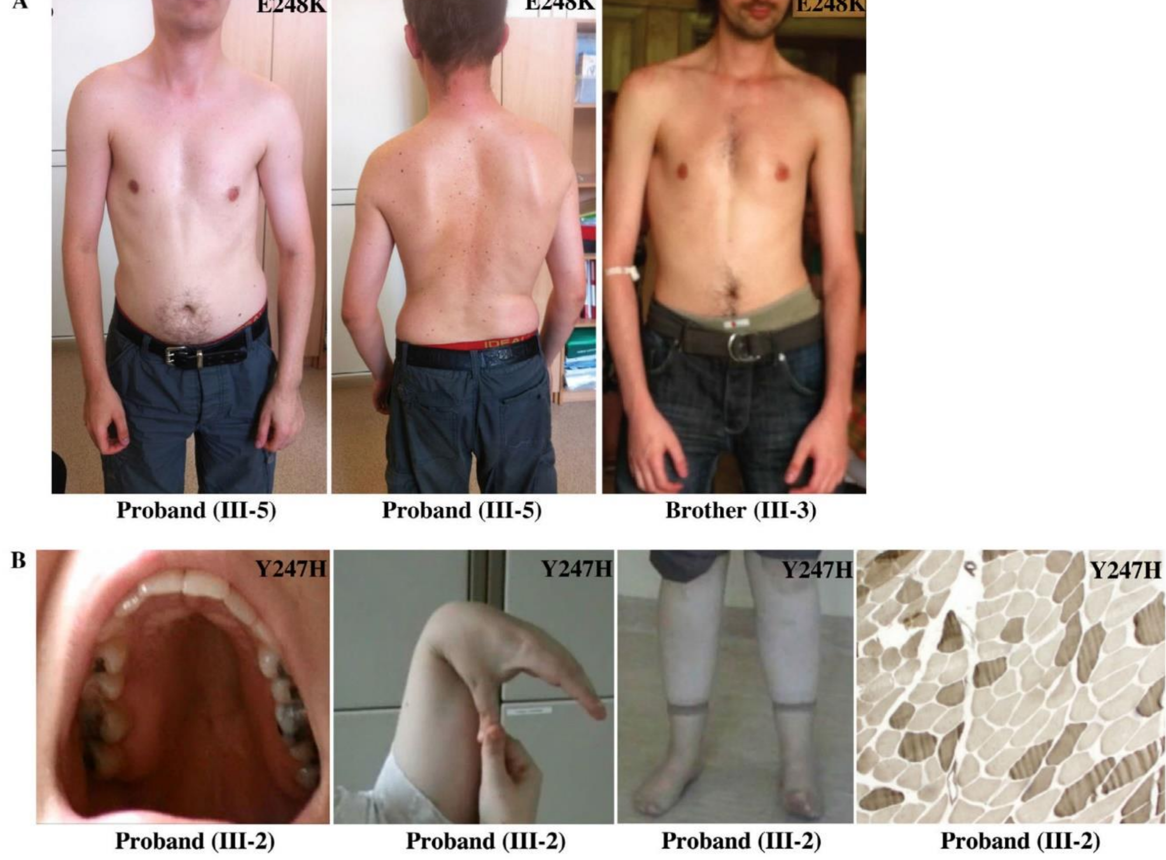

FIGURE 2: Phenotypic traits of individuals carrying the Y247H and E248K MYBPC1 mutations. (A) Photographs of proband (III-5) and his brother (III-3) from family 1, who carry the E248K mutation, indicating the presence of thoracic and sternum deformities, scoliosis, and elbow contractures. (B) Photographs of the index patient (III-2) from family 2, who harbors the Y247H mutation, showing high arched palate, joint hypermobility, contracture of the ankle joint, and histochemical staining (ATPase, pH 9.4) of a biopsy from the deltoid muscle from III-2 showing type 1 fiber predominance (light fibers are type 1, dark fibers are type 2).

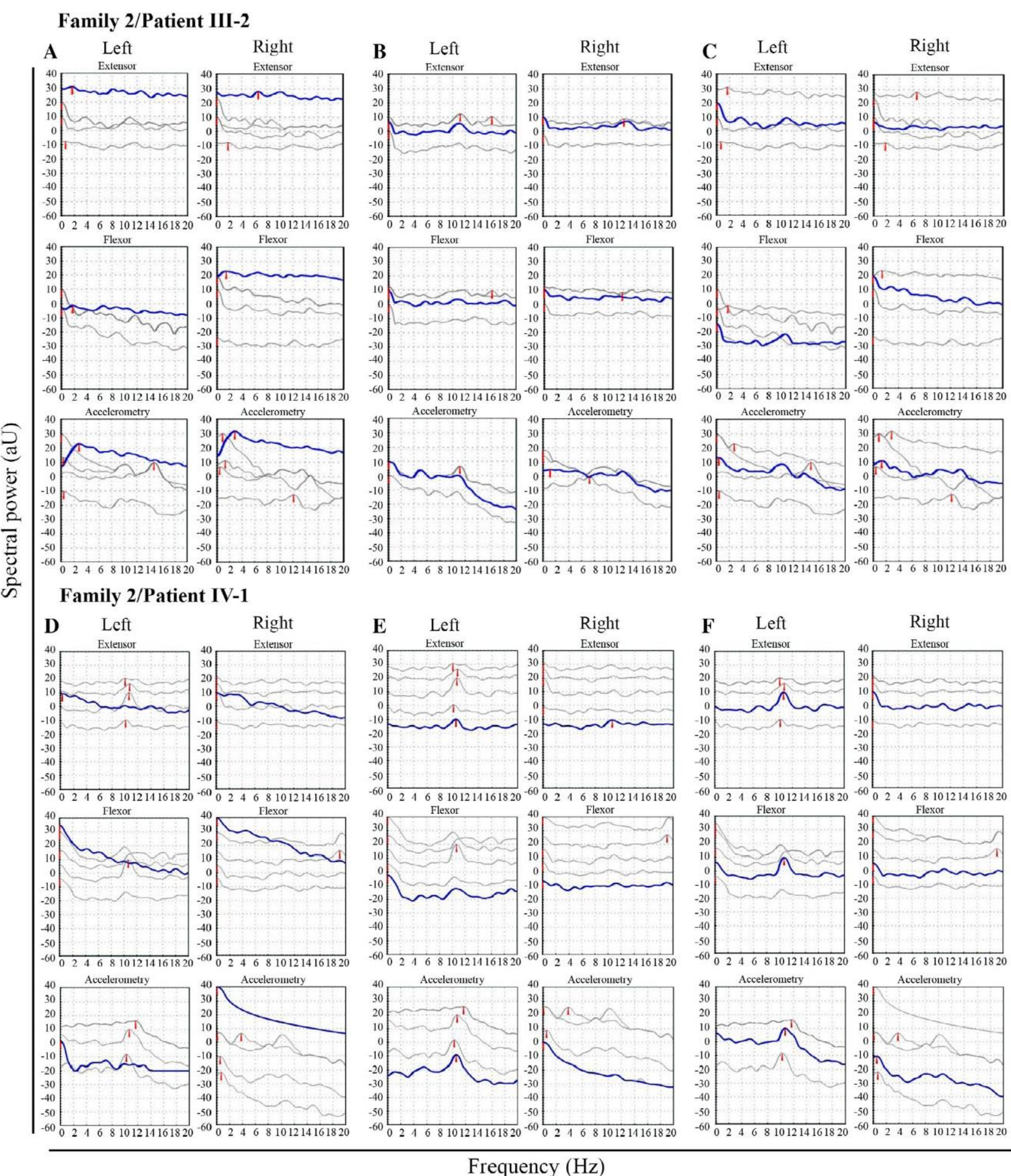

FIGURE 3: Power spectral tremor analysis of individuals III-2 (A–C) and IV-1 (D–F) from family 2 at rest (A,D), with outstretched hands (B,E), and outstretched and loaded hands (C,F). Top panels in (A) to (F) show the electromyography (EMG) in the extensor muscles, the middle panels show the EMG in the flexor muscles, and the lower panels show the accelerometry recordings. At rest, there is no tremor (A,D); with outstretched hands (B,E), and with loaded hands (C,F), there is a tremor peak at 10 to 11Hz in the left hand of both patients, synchronous in the EMG and accelerometry. In the right hand of patient III-2, there is a low-amplitude peak of 13Hz with corresponding EMG peaks, both with unloaded (B) and loaded (C) hands, whereas no clear peak could be identified with loading in the right hand of patient IV-1 (F). Dual-channel EMG shows lack of intermuscular tremor synchronicity in patient IV-1 between the left and right leg (G), but retained synchronicity within the same leg (H). In panels (A) to (F), the multiple thin individual traces correspond to single measurements, whereas the thick line represents the best fit; please note that the red arrowheads were randomly added by the software. In panels (G) and (H), the subdivisions correspond to 100ms.

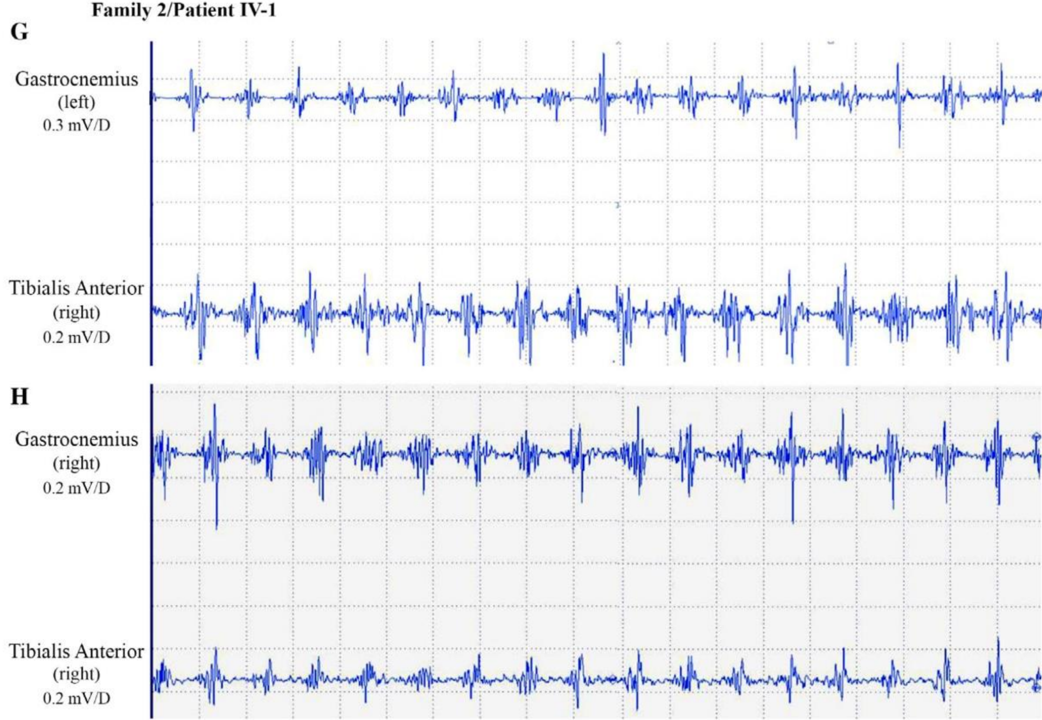

FIGURE 3: (Continued).

unaffected family members as well as of 90 Latvian control samples confirmed that the variant was confined to the 3 symptomatic family members (Fig 1B).

The index patient from family 2, a 50-year-old female (Fig 1C; proband III-2), was first diagnosed at age 46 with a history of slowly progressive, proximal, and axial weakness and myalgia since childhood (Fig 1C; Supplementary Table 1). Moreover, the patient reported myalgia and cramping in the calves at night or following strenuous exercise as well as immediate postural and tongue tremor following muscle exertion and prolonged tongue protrusion. Her symptoms have remained stable since her teenage years until the present, and activities of daily living are only mildly affected with minimal impairment (Supplementary Video 3). On examination, there was mild facial dysmorphia with micrognathia, prominent nasolabial folds, microstomia with pouting lips, and a high-arched palate (Fig 2B; Supplementary Table 1; Supplementary Video 3), in addition to mild facial myopathy with bilateral ptosis (left > right; Supplementary Table 1). Mild, symmetrical weakness (proximal > distal) of the arms and legs, marked axial muscle weakness and atrophy, abdominal muscle weakness, calf pseudohypertrophy, and mild bilateral scapular winging were also noted. The patient had mild axial and appendicular weakness and atrophy (proximal > distal), as well as mild weakness of foot dorsiflexion resulting in stepping gait (Supplementary Table 1). There were no cardiac or ocular symptoms, and hearing was intact.

EMG showed a myopathic pattern without spontaneous activity and a high-frequency postural tremor (10Hz) in the arms and hands. Nerve conduction studies excluded a neuropathy. Histopathological examination of a muscle biopsy obtained from the deltoid muscle revealed mild myopathic changes with NADH and ATPase stains revealing a marked preponderance of type 1 fibers, which, in general, appeared smaller than the few type 2 fibers (Fig 2B).

Examination of additional family members revealed the son (IV-1) and the granddaughter (V-1) of the index patient to be similarly affected, with mild axial and proximal weakness, high- arched palate, and a similar high-frequency, postural hand tremor (Supplementary Table 1; Supplementary Video 3). In all affected adult members, the clinical symptoms progressed slowly until the teen years and then reached a plateau. Patient V-2, a 6-month-old boy, has proximal muscle weakness with poor head control, and the family reported witnessing a hand tremor in this child as well. Power spectral tremor analysis performed in individuals III-2 and IV-1 from family 2 showed no resting tremor (Fig 3A,D). However, with outstretched hands, a narrow frequency peak in the 10 to 11 Hz range was noted by accelerometry in the left hand of both patients with a corresponding EMG peak of flexor and extensor forearm muscles (Fig 3B,E). Recordings with loaded hands showed identical accelerometric peak frequencies compared with the unloaded recording, again with synchronous EMG spectral peaks at 10 Hz (Fig 3C,F). In the

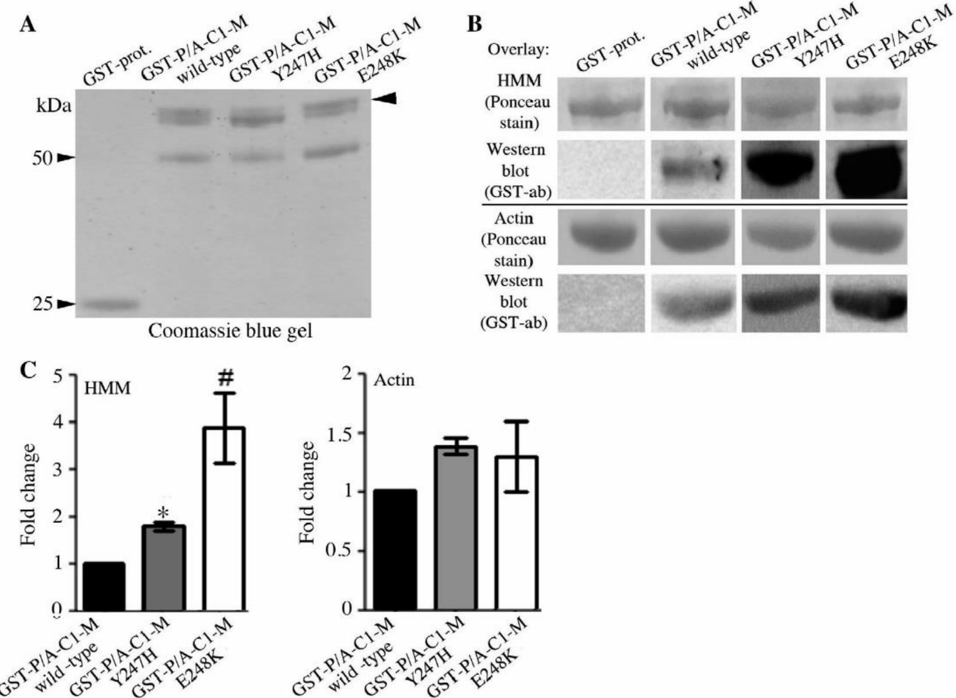

FIGURE 4: Examination of the effects of the Y247H and E248K mutations on the ability of the NH_2 terminus of sMyBP-C to bind actin and myosin in vitro. (A) Coomassie blue–stained gel showing equivalent amounts (1.5µg) of control glutathione *S*-transferase (GST), wild-type GST-P/A-C1-M, or mutant GST-P/A-C1-M proteins used in the overlay assays. Full-length GST-P/A-C1-M recombinant proteins (~58kDa) are denoted with an arrowhead; a degradation product at ~50kDa is present in all three protein preparations. (B) Equivalent amounts (3µg) of purified heavy meromyosin (HMM) or actin were separated by SDS-PAGE, transferred to nitrocellulose membrane, and overlaid with 0.5µg/ml of control GST protein, wild-type GST-P/A-C1-M, or mutant GST-P/A-C1-M carrying the Y247H or E248K mutation. Nitrocellulose membranes (Ponceau stain) and films (western blots probed with GST-ab) were cropped to only include the area of expected signal. (C) Quantification of immunoblots following the overlay assays indicated that both mutant proteins exhibit significantly increased binding to HMM (~2-fold for Y247H and ~3.5-fold for E248K) compared to wild-type protein (n = at least three independent repeats; *t* test; $^{*}p < 0.04$ and $^{\#}p < 0.015$, respectively); control GST protein did not bind to either myosin or actin. GST-ab = glutathione *S*-transferase antibody; SDS-PAGE = sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

right hand of patient IV-1, only a low-amplitude peak (10Hz) was noted with outstretched hands (Fig 3E), but no discernable peak, neither by accelerometry nor in the EMG, could be identified with loaded hands (Fig 3F). This corresponds with the clinical findings (Supplementary Video 3), where the right-sided tremor appeared less prominent and more irregular, thus preventing adequate Fourier analysis. In the right hand of patient III-2, a low-amplitude peak of 13Hz with corresponding EMG peaks of the same frequency was identified, both with unloaded and loaded hands (Fig 3B,C). This constellation of findings essentially excludes the mechanical reflex components of both physiological and enhanced physiological tremor, which are characterized by peripheral limb oscillations with reduction of accelerometric and EMG tremor frequencies after weight loading.[31] Instead, the stability of frequency during loading as observed in our patients would typically be compatible with a centrally mediated component to this tremor. The existence of different tremor frequencies between both arms in patient III-2 (Fig 3B,C) implies multiple tremor pacemakers; this is corroborated by dual-channel EMG recordings in patient IV-1, which showed lack of intermuscular tremor synchronicity between both legs (Fig 3G), but retained synchronicity within the same leg (Fig 3H).

Next-generation sequencing was performed on a multigene panel, revealing a single, novel, heterozygous c.739T>C p.(Tyr247His) sequence change in the *MYBPC1* gene. This sequence change was identified in all affected individuals (III-2, IV-1, V-1, and V-2), but was not found in any of the unaffected individuals tested for the mutation (Fig 1C).

Neither sequence change (p.E248K or p.Y247H) is listed in the NHLBI ESP Exome Variant Server (~13,005 alleles; http://evs.gs.washington.edu/EVS/), the 1000 Genomes server (http://browser.1000genomes.org), or the Exome Aggregation Consortium (ExAC) and Genome Aggregation Database (gnomAD) reference databases, nor were they listed in the

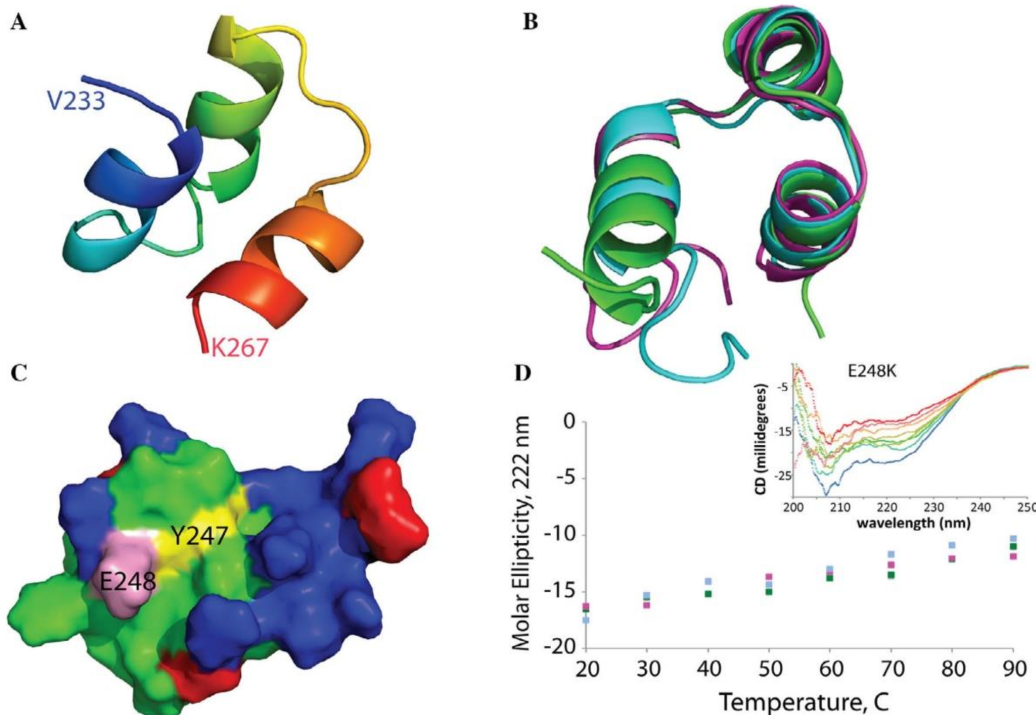

FIGURE 5: Modeling of the sMyBP-C M-motif and effects of the Y247H and E248K mutations. (A) The NH_2-terminal half of the M-motif of sMyBP-C (residues 186–233) is predicted to be partially disordered, whereas the COOH terminus (residues 234–268) folds into a well-ordered three-helix bundle, based on cardiac MyBP-C (80% identical; 92% similar for the M-motif; PDB: 2LHU).[59] (B) Structure prediction programs and molecular dynamics (MD) simulations show this three-helix bundle to be stable, with similar structures for the mutants (wild type in green; Y247H in magenta; E248K in cyan). (C) The M-motif is highly charged, with extensive positive charges (colored in blue) on one side of the structure. Mutations are expected to further increase this concentration of positive charges by replacing a negative charge for a positive charge at the E248 position (pink) or adding a protonatable imidazole ring at the Y247 position (yellow). (D) These MD simulations agree with experimental circular dichroism data, which show the wild-type and mutant domains to be predominantly helical over a wide temperature range (wild type in green, Y247H in magenta, E248K in cyan; inset shows representative CD spectra over a 20°C–90°C temperature range).

ClinVar or HGMD databases of known pathogenic variations preceding our submission of the variants, or in our in-house database of 90 Latvian samples.[32–34] Multiple computational tools, including SIFT (http://sift.jcvi.org), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), Panther-PSEP (http://pantherdb.org/tools/csnpScoreForm.jsp), PaPI (http://papi.unipv.it/help.xhtml), PhDSNP (http://snps.biofold.org/phd-snp/phd-snp.html), SNP&GO (http://snps-and-go.biocomp.unibo.it/snps-and-go), and MutationTaster, supported the deleterious effects of both mutations on the protein, in addition to high phred-scaled CADD scores of 36 for the E248K mutation and 25 for the Y247H mutation.[35–38]

The Y247H and E248K mutations are located next to each other in the unique M-motif of sMyBP-C, which is constitutively expressed in all splice variants. Notably, the M-motif, along with the Pro/Ala–rich motif and Ig C1, support the direct interaction of sMyBP-C with actin and myosin S2 and modulate the formation of actomyosin cross-bridges in the force-generating cycle.[9] To assess the effects of the individual mutations on the ability of the NH_2 terminus of sMyBP-C to directly bind actin or myosin, we performed blot overlay assays. To this end, we generated GST-tagged recombinant proteins containing the NH_2-terminal Pro/Ala–rich motif, Ig C1 and the M-motif (Pro/Ala-C1-M) in the absence and presence of the Y247H or the E248K mutation (Fig 4A), which we tested for their ability to bind actin or HMM immobilized on nitrocellulose membrane. We observed that both mutant proteins exhibited significantly increased binding to HMM (~2-fold for Y247H and ~3.5-fold for E248K) compared to wild-type protein (Fig 4 B,C). Binding to actin appeared slightly increased for both mutations, too; however, this increase in binding did not reach statistical significance (Fig 4 B,C).

We next asked what molecular changes underlie the increased binding of the mutant sMyBP-C proteins to HMM, as compared to wild type. Whereas there is no high-resolution structure of the sMyBP-C M-motif available, the cardiac isoform (80% identical) has been studied extensively.[39] Much of the cardiac M-motif is highly dynamic, but a ~35-residue region (amino acids 233–268) folds into a stable three-helix

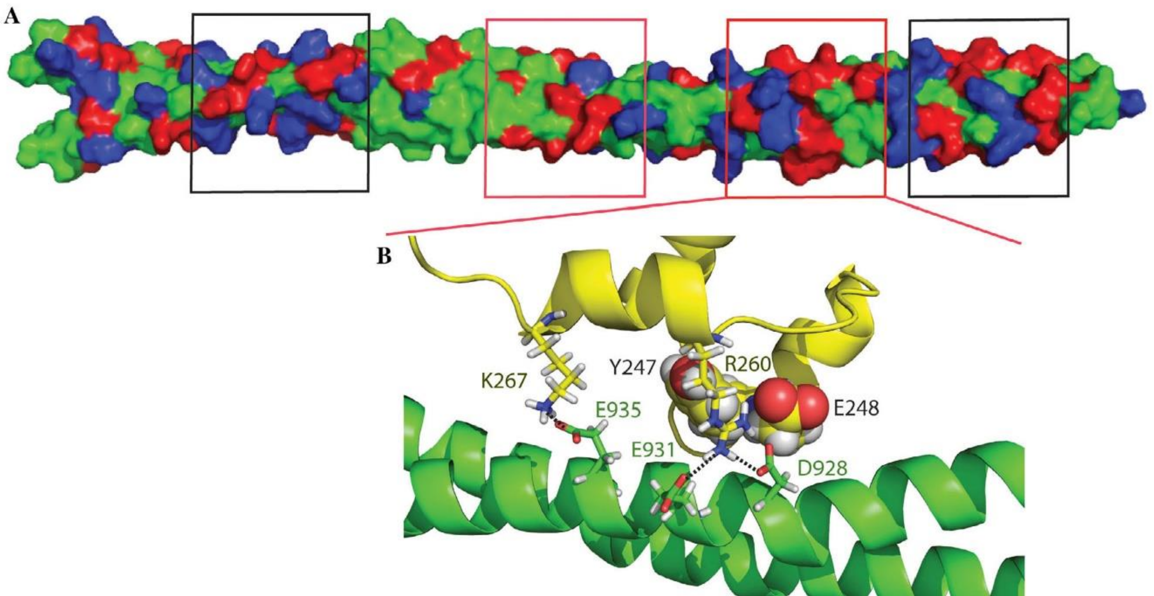

FIGURE 6: Computational modeling supports increased binding to myosin with the Y247H and E248K mutations. (A) Docking and molecular dynamics (MD) simulations predict that the folded M-motif region can bind and form stable interactions with human S2 myosin in two locations, centering around residues 898 and 931 (boxed in red; PDB: 2FXO).[60] (B) One of these potential binding sites, around residue 931, forms multiple charge-charge interactions with the M-motif of MyBP-C. Both the Y247H and E248K mutations (shown as red spheres) are within the binding interface and add extra positive charges to stabilize this complex.

bundle (Fig 5A). Using this structure as a template, we modeled the wild-type and mutant sMyBP-C M-motif and found that neither mutation significantly changed the overall domain fold (Fig 5B). Further analysis of these models showed that one face of the three-helix bundle is enriched in positive charges and both mutation sites expand this basic patch (Fig 5C). Circular dichroism spectra of sMyBP-C confirm that this section of the M-domain is mainly helical, and that the mutations show a similar, predominantly helical structure in agreement with our molecular dynamics (MD) simulations (Fig 5D).

Given the charge distribution and location of the mutations, we reasoned that the M-motif likely binds myosin using this basic side of the three-helix domain (Fig 5C). With this information, we docked this face of wild-type and both mutant models to acidic patches on the myosin S2 domain using HADDOCK. Of the four large acidic regions of S2 (Fig 6A, black and red boxes), productive interactions were successfully modeled for the two middle sites (Fig 5A, red boxes). Further MD analysis on these two sites suggested that the more NH_2-terminal site, centering around myosin residue 898, moves extensively and is not well defined. In contrast, the more COOH-terminal site, surrounding residue 931, produces a stable complex, driven mostly through electrostatic interactions (Fig 6B). Both the Y247H and E248K mutated sites (Fig 6B, spheres) are proximal to this putative binding interface, and both mutant complexes increase the potential number of favorable electrostatic interactions with

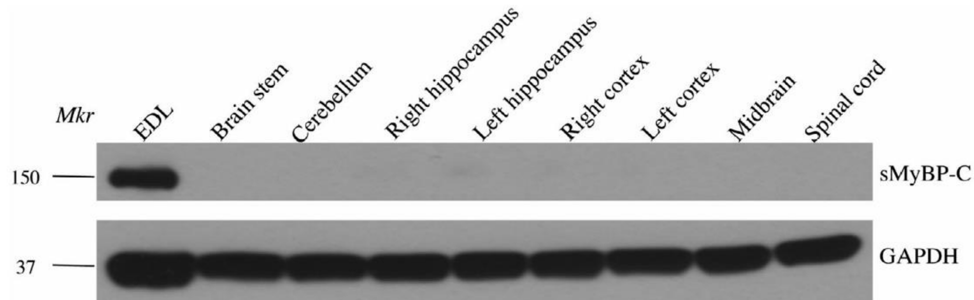

FIGURE 7: Examination of sMyBP-C expression in brain and spinal cord. Western blot analysis of protein lysates (20µg) prepared from mouse *extensor digitorum longus* (EDL) muscle, brain, or spinal cord. sMyBP-C is abundantly expressed in EDL muscle lysates, but is absent from all brain parts tested and the spinal cord. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

myosin. Thus, this model agrees well with our blot overlay data, presents a reasonable binding site on myosin for sMyBP-C, and provides a molecular rationale for why the sMyBP-C Y247H and E248K mutants bind more tightly to myosin. We surmise that this tighter binding to myosin interferes with normal cross-bridge kinetics and serves as the primary generator of the tremor, which, with subsequent central transformation, results in the clinical phenomenology observed in the patients.

Discussion

In this study, we describe the clinical, genetic, and molecular findings in two unrelated, three-generation families, each of which harbors a different novel sequence change in *MYBPC1* resulting in a mild skeletal myopathy with marked, unexplained tremor as the most distinguishing feature. Mild dysmorphic features were noted in some affected members of both families. Based on the familial segregation, absence from the general population, in-silico assessment, and increased target binding conferred by the substituted amino acids, the c.739T>C p.(Y247H) and c.742G>A p.(E248K) mutations can be classified as pathogenic according to American College of Medical Genetics and Genomics guidelines.[40] Notably, a de-novo mutation in *MYBPC1*, c.885T>G p.Leu295Arg, has been identified in a calf with weakness, stiffness, and muscle tremor.[41] The c.885T>G p.Leu295Arg mutation also resides in the M-motif of bovine *MYBPC1* and corresponds to amino acid p.Leu263Arg in exon 11 of the human gene, the same exon affected in our families.

All affected individuals from both families presented with early-onset, mild, predominantly axial muscle weakness, contractures, and tremor of high frequency and irregular amplitude, primarily affecting the hands. Tremor was also noted, but to a lesser extent in the legs of some affected members of family 1 and family 2, and in the tongue of all affected members of family 2. Clinically, the tremor was elicited by muscle usage, such as in assuming postures and/or activity. It is of note that presence of the tremor permitted identification of a newborn child in family 2 as clinically affected even before the development of myopathy. Weakness and tremor progressed slowly during childhood until adolescent years, followed by stability at a moderate level of disabilities, preserving ambulation. An increased susceptibility for prolonged respiratory infections was reported by several affected members of family 1.

The joint contractures of the feet and/or elbows in all affected adults were not present at birth, but developed over the course of the disease and remained mild, and thus differ from the congenital distal contractures characteristic of *MYBPC1* mutations associated with DA in both time of onset and severity. A muscle biopsy in the index patient from family 2 revealed type 1 fiber predominance with some smallness of type 1 fibers. This is a fairly nonspecific histological finding observed in a number of congenital myopathies, including those resulting from mutations in sarcomeric proteins. It is of interest, though, that muscle biopsies from DA1 patients have also shown type 1 fiber atrophy consistent with fiber-type disproportion.[20]

The E248K mutation results in substitution of a negatively charged amino acid (glutamic acid, E) by a positively charged one (lysine, K). This change would be predicted to affect the electrostatic interactions mediated by E248K, which, in turn, could alter the binding affinity of mutant sMyBP-C to myosin and actin. Similarly, the Y247H mutation results in substitution of an amino acid with a hydrophobic side chain (tyrosine, Y) by an amino acid with a potentially protonatable imidazole ring (histidine, H), again possibly altering the binding affinity of mutant sMyBP-C to myosin and actin, although likely to a lesser extent. Consistent with this notion, our in-vitro binding assays indicated that both mutations result in markedly increased binding of the NH_2 terminus of the corresponding mutant, sMyBP-C, to the HMM portion of myosin, with the E248K mutation eliciting a more-pronounced effect. Molecular simulations of sMyBP-C binding to myosin are consistent with the notion of altered binding activity as a result of the mutations. Given that sMyBP-C functions as a regulator of cross-bridge formation, it will now be crucial to understand what exactly the consequences of the enhanced binding of mutant sMyBP-C to myosin would be on cross-bridge formation and cycling and on myosin ATPase activity. This will require further sophisticated biochemical and biophysical investigation along with the generation of the appropriate animal models.

The most distinctive aspect of this new phenotype associated with specific *MYBPC1* mutations is the obligatory tremor in all mutation carriers, which also appeared to be a prominent feature of the spontaneous animal model of the disease.[41] Detailed clinical tremor analysis, including physiological studies, in two patients from family 2 argued against both physiological and enhanced physiological tremor generated by mechanical reflex oscillations, although up to 10% of controls and of individuals with enhanced physiological tremor may show a weight-invariant tremor frequency. The lack of tremor frequency change after weight loading would typically be considered to be compatible with a central pacemaker at the origin of the tremor. However, given that the tremor perfectly segregated with the mutation in the two families so that the mutant sMyBP-C could be inferred as the cause of the tremor, and given that sMyBP-C is not expressed in the central nervous system or in peripheral nerves (Fig 7 and The Human Protein Atlas), it is unlikely that the originator or primary pacemaker of the tremor is located centrally.

Considering the subcellular location of *MYBPC1* and based on our molecular and biochemical results, we postulate

that the tremor-initiating event is located at the level of the sarcomere, which then, through a central loop, leads to synchronization and oscillation of the effector muscles resulting in the clinically visible and recordable tremor characteristics. This is not unlike the hypothesis put forward for tremor generation in Charcot-Marie-Tooth (CMT) disease, where weight-invariant postural tremors may be present.[42] However, even though peripheral, in CMT disease the initial generator does not originate from the muscle itself, but is a result of its denervation, and is hypothesized to lead to an enhancement of the central neurogenic component of physiological tremor. Given that our patients do not have a neuropathy and *MYBPC1* is expressed purely in skeletal muscle, we hypothesize instead that the tremor is sarcomeric and hence myogenic in origin, but, similar to the denervation tremor, is then picked up by stretch receptors, where it undergoes centrally looped propagation and enhancement, thereby rendering it clinically visible. This hypothesis is consistent with the tremor recordings in our patient and establishes "myogenic tremor" as a novel tremor class. We propose that the mutant sMyBP-C results in deregulated cross-bridge cycling, which, in addition to causing a mild deficit in force generation, may take on an oscillatory quality upon contraction, resulting in tremor. This concept of a "myogenic tremor" initiated by deregulation of binding cycles of sarcomeric proteins is corroborated by an animal model with a de-novo *MYH7* mutation, which also showed a progressive high-frequency postural tremor[43] resembling the tremor observed in our patients (Supplementary Table 2). Consistent with this, our group has also observed a similar tremor in a patient with a distinct de-novo *MYH7* mutation (C.G.B. and D.H., unpublished observations). A search of the literature for mutations in additional sarcomeric proteins associated with myopathy and tremor revealed further cases of patients with mutations in genes encoding thick (*MYH2* and *MYL2*) and thin (*TNNT1*, *TPM3*, and *NEB*) filament proteins (Supplementary Table 2),[44–58] suggesting that cross-bridge dysregulation may emerge as a myogenic tremor generator beyond the *MYBPC1* mutations reported here. Additional studies in transgenic animals to further elucidate the physiology of this novel type of tremor and get more insight into tremor-causing mechanisms are in preparation.

In summary, identification of the novel Y247H and E248K mutations in *MYBPC1* define a new autosomal-dominant syndrome of mild skeletal myopathy with a distinctive tremor of likely myogenic origin, which we propose to add as a new tremor entity.

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Author Contributions

B.L., J.S., A.K.K., and C.G.B. contributed to the conception and design of the study. J.S., B.L., J.S., J.G., I.I., D.P., S.P., N.T.W., A.S., M.W., D.H., S.J., A.K.K., and C.G.B. contributed to the acquisition and analysis of data. J.S., B.L., J.S., J.G., N.T.W., S.J., A.K.K., and C.G.B. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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4. DISCUSSION

Diagnostics of rare neuromuscular disorders has always been difficult. Even dedicated laboratories using the NGS approach still have a maximum success rate of 65% (Wu et al. 2018). It must be noted though that the great phenotypic and genetic heterogeneity of these diseases can be attributed for some part of the decreased diagnostic yield. When large laboratories have such struggles, it is even more true for small laboratories, working on rare diseases in an under-diagnosed population, with no prior knowledge of population specific disease frequency data.

This was the situation for Latvia, where the first limited genetic testing for neuromuscular disease patients became available around the year 2000, with tests for Duchenne muscular dystrophy (DMD) and Spinal muscular atrophy (SMA), with testing for Charcot-Marie-Tooth disease type 1A (CMT1A) becoming available a few years later. A few more years passed and governmental funding for genetic testing abroad, even if limited by paperwork and funding, became available. It was around this time, when it was decided that in order to help as many NMD patients as possible to get a molecular diagnosis, geneticists and molecular biologists will collaborate, and use all available scientific resources, in order to guide and develop neuromuscular disease diagnostics, through research. A dedicated project, based on Sanger sequencing and massive parallel genotyping was started in 2011. However, it was realized that the percentage of diagnosed cases is low and the technology is limited, with only 14 patients receiving the molecular diagnosis. It must be noted though, that NMD cases that reached us were ones not diagnosed in a clinical setting, meaning that the most common and easy to diagnose NMDs were often already excluded.

A few minor international collaborations were organized over the years, giving us access to the newest technology and world-class experts, that have resulted in a couple of genetically complicated cases being diagnosed, including the *MYBPC1* case, described in this work. However, the occasions were few and far between, therefore in 2017 a collaboration with the large-scale international research project MYO-SEQ, spearheaded by the Newcastle University in the UK, was established, granting a selected number of patients the possibility to have their exomes sequenced and analyzed. Not many patients could be enrolled in this project, as they had to fit certain age and phenotypical criteria. Nonetheless, the diagnostic yield for this collaboration was very good, with 12 out of 19 enrolled patients having either molecular diagnosis or a potential disease-associated candidate variant established. In cases of potential candidate variants, more work will have to be done in order to properly classify them. Unsurprisingly, the results up to this point seem to suggest calpainopathies as the most common

LGMD form in the population of Latvia, however by a small margin. More unexpected, however, were several cases of pathogenic *DMD* variants in women, as in females only up to 20% of carriers are expected to have mild to moderate muscle weakness (Yiu and Kornberg 2015).

The results of the MYO-SEQ collaboration showed us that NGS is the way to go, and inspired the beginning of the whole genome sequencing project for rare disease patients in Latvia in 2019, with the focus on rare neuromuscular disorders. The first batch of patients has shown good diagnostic yield, with 7 out of 11 patients having received molecular diagnosis and two more having potential disease associated variants identified. With many more batches already on their way, it must be noted, however, that not all of these are NMD patient cases. Overall, NGS data in the rare neuromuscular disease patient group in Latvia has shown that this population is indeed heterogeneous, with most cases being unique.

Caveolinopathies

When genetic testing and research of neuromuscular disorders in Latvia began, there were still relatively few LGMDs known and the world was rather far from reaching the end of the alphabet in their classification. Therefore, when a family presenting with a seemingly classic form of a dominant LGMD came to the attention of geneticists, the choice at the time was either to do nothing or to try to test some of the smaller genes, known to be associated with this type of disease, by Sanger sequencing. Considering that the gene *CAV3* (RefSeq No. NM_033337.3) was associated with cardiomyopathy as well as dominant LGMD, it is rather small and mutations in it are relatively common, it was decided to sequence this gene in the patient population as well as the general population of Latvia, as one with a potentially higher diagnostic value.

Both of the variants (c.183C>A, p.(S61R) and c.216C>G, p.(C72W)) found in this gene in the patient group are located in the scaffolding domain of the protein. It has been suggested that mutations in this domain may cause significant alterations in the function of the protein, with the variant c.183C>A described in a heterozygous state in a case with absent CAV3 protein and dystrophic muscle pathology (Fulizio et al. 2005). This variant has also been rated as likely pathogenic, based on the American College of Medical Genetics and Genomics (ACMG) guidelines (Kopanos et al. 2019). The other variant c.216C>G has been classified as likely benign. Even though some prediction tools classify it as damaging and it has previously been found in a heterozygous state in a patient with muscular dystrophy, it has been found in a number of healthy adult individuals as well (McNally et al. 1998; Kopanos et al. 2019). This suggests that a single abnormal allele of this variant is not sufficient to cause the phenotype.

The decreased functional significance is most probably due to its position at the very end of the scaffolding domain. No homozygous individuals have been reported though.

The result of 1-2% of patients in the LGMD group having mutations in the *CAV3* gene was to be expected, however no variants associated with the disease were found in the cardiomyopathy group. Even though the role of *CAV3* in cardiomyopathy has been shown previously, our findings make us hypothesize that at least in our target population cardiomyopathy is a rare phenotypic trait of *CAV3* mutations, probably strongly influenced by gene–gene interactions (Woodman et al. 2002; Hayashi et al. 2004). This might, however, also be affected by the relatively small size of our cardiomyopathy patient group.

Interestingly, the one person from the control population group, carrying the c.220C>T, p.(R74C) variant also has a slight elevation of serum CK level, and so can be hypothesized to have an undiagnosed hyperCKemia. This could be supported by the fact that in an advanced stage of the disease, CK level usually shows a tendency to normalize due to the muscle replacement with fibrous or adipose tissues and the person in question was 58 years old at the time of sample collection. Unfortunately, we were unable to get hold of this patient to further confirm our theory. This variation has been classified as likely pathogenic by the ACMG guidelines (Kopanos et al. 2019). We also found an apparently benign intronic deletion (c.115-45_115-31del) in a large portion of our samples, which differs by two base pairs from a well-known benign deletion (c.115-45_115-29del), which we found in only one sample. The frequency of other variants found in our control group is comparable to those found in genome databases.

Even though the size of the sample group may have been a limiting factor, this research allowed us not only to set the foundation for local diagnostic testing and research of rare neuromuscular disorder, but also to start filling in gaps in the previously unknown incidence data for these diseases in the general population of Latvia.

Calpainopathies

When Sanger sequencing of whole genes or frequent mutation regions within genes, associated with most common LGMDs, turned out to be apparently lacking in terms of diagnostic potential, it was only natural to try and incorporate the newest technologies. At that point in time massive-parallel genotyping seemed to be the best way to go, so a custom designed genotyping panel was made and used in conjunction with samples from patient and control populations from Latvia and Lithuania. This panel included 96 of the most common pathogenic variants for the most common LGMD types in Europe.

It is known that calpainopathies are one of the most common LGMD types in the world, with some exceptions in certain specific populations (Wicklund 2019). Since 2004 and 2005, when it was described by Canki-Klain *et al.* and Fanin *et al.*, respectively, the c.550delA variation in the *CAPN3* gene (RefSeq No. NM_000070.3) in a homozygous or compound heterozygous state is thought to be one of if not the most common LGMD R1 associated variant in Europe, with the average allele frequency of 0.000422 in non-Finnish Europeans, according to the GnomAD database (Canki-Klain et al. 2004; Fanin et al. 2005; Karczewski et al. 2020). Therefore, it was somewhat surprising that our testing found this variant in a homozygous state in eight patients from Lithuania, making it the most common in this group, but it was not found in any of the patients from Latvia.

We then proceeded to test this variant in the respective general populations, and came up with a similar result - variant frequency of 0.0016 in Latvia and 0.0029 in Lithuania. In his publication Canki-Klain hypothesized that the c.550delA variant has originated in the Eastern Mediterranean and spread across Europe (Canki-Klain et al. 2004). This indeed seems to be the case, as a gradient can be seen in European countries, generally decreasing from center towards east and west and from south to north (Pogoda et al. 2000; Piluso et al. 2005; Georgieva et al. 2005; Milic and Canki-Klain 2005; Balci et al. 2006; Hermanová et al. 2006; Hanisch et al. 2007; Todorova et al. 2007; Stehlíková et al. 2014; Dorobek et al. 2015). Based on our data, this variant seems to be the most common in Lithuania as well, however its frequency drops rapidly in Latvia. The difference in frequencies remains unexplained, as there are no definite cultural or geographical borders between the two countries and both populations do not seem to be that divergent.

The variants c.2288A>G in the *CAPN3* gene and c.4872delG in the *DYSF* gene (RefSeq No. NM_003494.4), both in heterozygous states and both classified as likely pathogenic, according to the ACMG guidelines, were found in two separate control population samples, allowing us to find two disease carriers and setting the frequency of these two variants at 0.00245 in the general population of Latvia (Kopanos et al. 2019). Interestingly, no common variants were found in the *ANO5*, *SGCA*, *SGCB*, *SGCD* and *SGCG* genes - all with high allele frequencies in other European populations. This only serves to consolidate the previous statement of the heterogeneity of the neuromuscular disease patient group in Latvia, which is possibly even higher than in some other European countries.

Even though the initial thought of massive parallel genotyping as a rapid and low-cost screening tool for improvement of the healthcare became obsolete very quickly, this project allowed us to go one step further in neuromuscular disease testing and helped us fill in another blank space in regards to variant frequencies in the Baltics region. The main issues this

technology faced was the low sensitivity (89% in our laboratory setting) and the heterogeneity of both the diseases and our population, leading to decreased diagnostic yield when testing only certain genes and variants.

Collagen 6-related myopathies

Through international collaborations with world-class laboratories was how we first moved rare disease research and diagnostics in Latvia into the field of next-generation sequencing, with our collaboration in the MYO-SEQ project being the most successful in terms of diagnostic yield. However, it soon became apparent that even using NGS approaches, it was not always easy to reach a diagnosis, even when not faced with theoretically pathogenic variants in genes without a known function. There is only a limited number of pathogenic variants for which any kind of functional research has been carried out, and diagnosis should never be based solely on *in-silico* evaluation. Sometimes, however, the problem is the opposite - relatively common variants, reported by many different sources, with conflicting interpretations of pathogenicity, often associated with heterogeneous diseases.

A good example of this, that is still being debated, is the c.7447A>G, p.Lys2483Glu variant in the *COL6A3* gene (RefSeq No. NM_004369.4), found in a compound homozygous state (c.[7447A > G]; [7447A > G; c.8074delT]) in one of the families involved in our project and in a homozygous state in another of our patients. It is currently classified as a variant of uncertain significance, based on ACMG guidelines (Kopanos et al. 2019). All of COL6 genes are associated with diseases now referred to as collagen VI-related myopathies, that range from a mild Bethlem myopathy phenotype to a more severe Ullrich congenital muscular dystrophy phenotype (Briñas et al. 2010; Bönnemann 2011a). Furthermore, there is now compelling evidence that these myopathies are among the most common congenital muscular dystrophies worldwide (Okada et al. 2007; Peat et al. 2008). The COL6A3 protein itself plays a major role in cell adhesion and through it - organization of matrix components and muscle development (O’Leary et al. 2016).

The variant was described in 2010 by Brinas *et al.* in a compound heterozygous state with the variant c.9384_9386del, in a patient presenting with a mild Bethlem myopathy phenotype and having reduced secretion as well as intracellular retention of COL6A3 in his fibroblasts (Briñas et al. 2010). Since then, there have been at least 18 patients reported, carrying this variant in compound heterozygous or homozygous states, though the number might vary due to a possible overlap of reports (Briñas et al. 2010). The general consensus of disease progression, including our patients, is that onset of the disease is early, with delay in the development of gross motor skills. Course of the disease seems to be slowly progressive, with

most of the patients having muscle weakness that is restricting only some strenuous activities, usually becoming noticeable in late adolescence or adult age. Contractures are mild and often only ankle joints are involved (Hunter et al. 2015; Sframeli et al. 2017; Panadés-de Oliveira et al. 2019).

One of the reasons for the variant being classified as variant with uncertain significance is the fact that there exists a male from European (non-Finnish) population, homozygous for the variant, who is registered in the GnomAD database. The reason this is important, is that curators of GnomAD have tried, to the best of their abilities, to remove all individuals known to be affected by severe pediatric diseases. In this instance, it is our hypothesis that the person in question did not present severe symptoms at a younger age, similar to the rest of our patients, and there has been no retrospective curation of the database for such cases (Karczewski et al. 2020).

Such cases, where variants in the same gene are associated with more than one phenotype, are becoming more common, for example, variants in *POMGNT1*, *FKTN*, *POMT2*, *FKRP* and several other genes can cause both - a severe, even early lethal phenotype, but also a mild dystrophy (Brockington et al. 2001b; van Reeuwijk et al. 2005; Godfrey et al. 2007b; Manzini et al. 2012). A great example of such duality is the *ANO5* gene, where the dominant variants are causing gnathodiaphyseal dysplasia, but the recessive ones - LGMD symptoms (Marconi et al. 2013). This expanding range of phenotypic variability is starting to move the community away from the classic gene-to-disease system and more towards the affected domain-to-disease. Such changes are making it more difficult for clinicians, who at first are trying to pinpoint the genetic cause of a disease based on phenotypic traits. Furthermore, it is also affecting the current gene-to-phenotype based classification. This variability arises from emerging data, explicitly showing that defects in different functional regions of the same protein can affect completely different aspects of the protein function, and therefore manifest as very distinct phenotypic traits, often resembling defects in other genes.

When looking at the clinical data from patients carrying the c.7447A>G variation, it must be noted that EMG provides no specific findings and muscle biopsy, if not stained specifically for COL6 proteins, shows great variability, with the most common finding being fiber size variations, suggestive of myopathy and later muscular dystrophy. Creatine phosphokinase level is usually elevated from two to four times the normal (Bönnemann 2011a). It seems muscle MRI is currently the first-line diagnostic tool with the best prognostic value, revealing the pathognomonic pattern with fatty degeneration from periphery to the center of the *rectus femoris* and *vastus lateralis* muscles, with the typical diamond shape or tigroid pattern. However, despite its usefulness, muscle MRI is still not routinely used (Bönnemann 2011a).

Our patients, included in this project, have the MRI findings typical of the COL6 disease, and segregation analysis, where possible, showed that the disease is inherited in an autosomal recessive pattern, involving the c.7447A>G variant. Taking everything in consideration, there is a basis for hypothesizing that the variant c.7447A>G in a homozygous state can lead to a mild Bethlem myopathy and/or LGMD phenotype without respiratory involvement. It must be noted though, that for patients with other variants in a compound heterozygous state, the phenotype severity entirely depends on the second variant. The previously described cases show that a compound heterozygous carrier for the c.7447A>G; 9384_9386del variants presented with pulmonary involvement characterized by FVC borderline reduction to 78% at the age of 12, however no information regarding muscle weakness or contractures was published in this case (Briñas et al. 2010). A second carrier of compound heterozygous variants c.5480delG; 7447A>G had grade 3/5 muscle weakness of hip abductors and abdominal muscles at the age of 12 (Hunter et al. 2015). In comparison, the person in our project, carrying the c.7447A>G variant in a homozygous state, had muscle weakness of 4/5 in proximal muscle groups at the age of 44, and patients carrying the compound variants presented with muscle weakness, mild contractures and muscle atrophy at the age of 30. It is always possible to speculate that the c.7447A>G variant in a homozygous state by itself leads to a milder phenotype, or that there could be another deep intronic variant in COL6A genes, responsible for the Bethlem myopathy phenotype, present in such cases.

The alpha-3 chain is a modular protein comprised of 10 Von Willebrand factor A (VWA) domains, with the N-terminal VWA domains having a major role in cell adhesion by binding other extracellular matrix proteins (O'Leary et al. 2016). VWA domains of COL6A3 protein in general give it the ability to bind other proteins. The C1 and C2 domains (VWA 11 and VWA 12), affected in the family, involved in our project, have been shown to be necessary for the assembly of monomers, with C1 being sufficient for assembly, but lack of or mutations in C2 being associated with disease (Bönnemann 2011b). It has also been reported previously that missense variants in a VWA domain can be associated with Bethlem myopathy (Pan et al. 1998). Therefore, it is possible to hypothesize that a change in one of them might affect the binding potential and therefore modulate the phenotype, when present together with other variants in the gene. It has been shown that collagen VI a1, a2, and a3 form a heterotrimer that is held together by the VWA domains in the C-terminus of each molecule (Beecher et al. 2011). It is thus tempting to speculate that variants like p.Lys2438Glu, which substitutes a basic residue for an acidic one, ablates a stabilizing electrostatic interaction and perturbs this heterotrimer. The resulting weakened or misformed collagen VI fibers might be able to affect structural stability and regeneration of skeletal muscle. This leads to a possible hypothesis that

in the case of our patients that are carrying compound variants, a terminated protein would be synthesized from one of the DNA chains and one of the VWA domains would be affected for the protein synthesized from the other. Both of these changes, taken together, might affect the overall functions of the protein enough to lead to the myopathic phenotype observed. Furthermore, it has been shown previously that variants causing premature termination codons have a profound destabilizing effect on the corresponding transcripts, leading to degradation of transcripts harboring these variants at the post-transcriptional level (Briñas et al. 2010). Interestingly, the phenotype-genotype correlation observed in the family of compound variant carriers, allows us to conclude that the c.8074delT variant, despite being seemingly more damaging, in a heterozygous form cannot cause disease symptoms on its own.

Cases of variants with hard-to-classify effects are becoming more common, as genetic data is being generated at an unprecedented rate. They do present challenges for both clinicians and researchers, hopefully leading to a shift towards even more individual variant functional testing in the future.

Myosin Binding Protein C1-related phenotypes

The most notable case in the history of our project is highlighting a somewhat similar and yet totally different problem, when dealing with neuromuscular disorders. The family affected in this case was first examined by a geneticist more than ten years ago. In this family there are four patients in three generations, all presenting with a peculiar phenotype - mild myopathy, skeletal deformities and tremor.

It was decided to start a collaboration with professor Carsten Bönnemann and the section of neuromuscular and neurogenetic disorders from National Institutes of Health (NIH), Bethesda, USA, led by him. After examining the patients, he came up with a hypothesis that this is a new and undescribed phenotype. Whole exome sequencing was performed for several members of this family at the Tartu University's Institute of Genomics.

After more than a year of data analysis a dominant variant in the *MYBPC1* gene (RefSeq No. NM_002465.4), rated as damaging by predictive algorithms, was proposed to be the cause of the disease. However, at that point pathogenic variants in this gene were described only relatively recently and were associated with severe phenotypes, including distal arthrogryposis and lethal congenital contracture syndrome.

After performing genetic testing in the extended family, we did indeed observe that the variant in question c.742G>A p.(E248K) segregates perfectly with the disease phenotype. In order to start planning some functional testing, we began yet another collaboration - this time with professor Aikaterini Kontrogianni-Konstantopoulo's group from University of Maryland,

USA, that had been working on functional testing of MYBPC1 protein for some time already. Luckily, in a year's time another group came to our attention, this time from Germany, led by doctor Jochen Schäfer, who had found another family, harboring a variant in the neighboring amino acid (c.739T>C p.(Y247H)) and presenting with a similar phenotype, thus providing us with more concrete proof that ours was not a random finding.

We were now able to base our research around the clinical, genetic, and molecular findings in two unrelated, three-generation families, each of which harbors a different, yet neighboring novel sequence change in the *MYBPC1* gene, resulting in a mild skeletal myopathy with marked, unexplained tremor as the most distinguishing feature. We have classified both of these variants as pathogenic, according to ACMG guidelines, based on the familial segregation, absence from the general population, in-silico assessment, and the increased target binding conferred by the substituted amino acids, however they have also been classified as likely pathogenic by other sources (Richards et al. 2015; Kopanos et al. 2019). Only later it came to our attention, that there has been described a natural animal model - a calf with weakness, stiffness, and muscle tremor, carrying a c.885T>G p.(Leu295Arg) variant in the *MYBPC1* gene (Wiedemar et al. 2015). The variant is located in the M-motif of bovine MYBPC1 and corresponds to amino acid p.Leu263Arg in exon 11 of the human gene, the same exon affected in both families, included in our project.

The phenotype of all affected individuals from both families can be summed up as early-onset, mild, predominantly axial muscle weakness, mild contractures, and tremor of high frequency and irregular amplitude, primarily affecting the hands. In some affected individuals the tremor was also present in legs and, interestingly, in all affected members of the second family it was noted in the tongue as well. The tremor was elicited by muscle usage, such as in assuming postures and/or activity. As observed in the second family, tremor is present in newborns even before the development of myopathy. Weakness and tremor progress slowly during childhood, until patients reach adolescent years. This is followed by a plateau period of stability at a moderate level of disabilities. Ambulation is preserved in all patients. Noteworthy, several affected members of the first family reported an increased susceptibility for prolonged respiratory infections, allowing hypotheses about a mild weakness of the diaphragm muscles as well.

The joint contractures in these two families were not present at birth, but developed over the course of the disease and remained mild. Therefore, both time of onset and severity differs from the congenital contractures that are characteristic of distal arthrogryposis and previously associated with variants in *MYBPC1*. Muscle biopsy findings were nonspecific.

Due to the c.742G>A p.(E248K) variant, a negatively charged amino acid is being replaced by a positively charged one. Similarly, to this, the c.739T>C p.(Y247H) variant causes an amino acid with a hydrophobic side chain to be replaced by an amino acid with a potentially protonatable imidazole ring. Both of these changes can be predicted to lead to alteration of the binding affinity of the mutant protein to two of its known major binding partners - myosin and actin, although possibly to a lesser extent in the case of the second variant. Consistent with this prediction, when in-vitro binding assays were performed, the data showed a markedly increased binding of the NH_2- terminus of the corresponding mutant protein to the heavy-meromyosin (HMM) portion of myosin. Furthermore the p.(E248K) mutant protein did indeed elicit a more pronounced binding. Molecular simulations of MYBPC1 and myosin binding uphold the hypothesis of altered binding activity as a result of the mutations, through increased electrostatic interaction. It must be noted, however, that it has been shown that even small changes in pH can affect the charge of the histidine side chains in a major way, therefore making it more difficult to predict the exact effect of the p.(Y247H) mutation (Li and Hong 2011). As MYBPC1 functions as a regulator of cross-bridge formation, the next logical step is to try and figure out the impact of increased binding potential of mutant proteins on cross-bridge formation and cycling. The best way to approach this would be through biochemical and biophysical testing, as well as generation of appropriate animal models that could lead to possible drug or therapy testing in the future.

What makes this new phenotype, associated with domain-specific variants in the *MYBPC1* gene, interesting and unique is the tremor, present in all affected, as well as the spontaneous animal model (Wiedemar et al. 2015). Detailed clinical analysis of tremor in two patients from the second family allows us to exclude with some certainty both physiological and enhanced physiological tremor generated by mechanical reflex oscillations. The peculiarities of tremor - no changes in frequency after weight loading, would normally make us consider a central pacemaker at the origin of the tremor. However, the tremor segregation with pathogenic variants in both families, allows us to propose the mutated protein as the main cause of tremor, and seeing that MYBPC1 is not expressed in the central nervous system or in peripheral nerves (The Human Protein Atlas and our results), it is unlikely that the originator or primary pacemaker of the tremor is located centrally.

Considering the subcellular location and function of MYBPC1 as well as our results, we propose that the initiation of tremor is happening at the sarcomeric level, with the signal then going through a central loop and leading to synchronization and oscillation of the effector muscles. Such a mechanism would not be unlike the hypothesis behind tremor generation in Charcot-Marie-Tooth (CMT) disease, where weight-invariant postural tremors have been

observed (Saifee et al. 2015). The main difference though being that in CMT the initial tremor generator does not originate from the muscle itself, but is rather a result of its denervation, which then leads to an enhancement of the central neurogenic component of physiological tremor. Given that neuropathy has been excluded in our patients and MYBPC1 is expressed only in skeletal muscle, we hypothesize that the tremor in this phenotype is sarcomeric and therefore myogenic in origin, but is then picked up by stretch receptors (possibly muscle spindles) that provide proprioceptive sensory information to the brain, where it undergoes centrally looped propagation and enhancement, thus creating the visible tremor. Tremor testing, performed in our patients, supports such hypotheses. Based on all of the aforementioned, we propose establishing "myogenic tremor" as a novel tremor class. Our proposed disease mechanism is based on deregulation of cross-bridge cycling that leads to mild deficit in force generation and may create an oscillatory movement and signaling upon contraction that results in tremor. An animal model, carrying a pathogenic variant in the *MYH7* gene and presenting with a progressive high-frequency postural tremor, that resembles the tremor in our patients, corroborates the concept of myogenic tremor that is caused by deregulation of sarcomeric protein binding (Murgiano et al. 2012). Consistent with this, a similar tremor has been observed in a patient with a distinct *de-novo MYH7* variant (unpublished observations). Our proposition has been recognized by the team curating the Online Mendelian Inheritance in Man (OMIM) database, who have added it to said database and termed it MYOTREM (Amberger et al. 2019). Right after our publication, Shashi *et al.* described four additional patients from three independent families, all carrying either of the two dominant missense variants - c.788T>G p.(Leu263Arg) and c.776T>C p.(Leu259Pro), located in the M-motif of the *MYBPC1* gene. Interestingly, even though the predicted effect on protein is somewhat different, the observed phenotype is very similar to our cases, and the authors come to the same conclusions (Shashi et al. 2019). Taken together, this means that myogenic tremor, associated with variants in the M-motif of the MYBPC1, has been observed in the two multi-generation families from our project, four additional individuals, described by Shashi *et al.* and the spontaneous natural calf model. Furthermore, mouse models harboring the p.(E248K) and p.(Y247H) variants have been created that fully duplicate the phenotype observed in humans (unpublished data).

During an in-depth research of literature aimed at finding additional cases of sarcomeric proteins associated with myopathy and tremor, we noticed that in all the cases we found, the affected genes are either solely or majorly expressed in the muscle (Uhlen et al. 2015). Therefore, it is reasonable to propose that the tremor described in these cases may be of myogenic origin as well, with underlying molecular mechanisms similar to that described previously, arising from structural alterations that impact myofilament assembly, stability

and/or sliding. In several of these cases, including families in our research, the tremor abates with age, but can still be noticeable with posture and/or physical exertion. This leads us to think that adaptive or compensatory mechanisms may be present, that might be based on central nervous system plasticity and could fine-tune and adjust motor unit control (Edgerton et al. 2002). Noteworthy, a certain degree of heterogeneity can be observed with regards to the severity of the tremor phenotype, both intra and inter-familial. Even though the cause of this is yet unknown, heterogeneity is one of the hallmarks of myopathies, and therefore it is reasonable to consider that genetic and individual-unique factors may be contributing here as well (Mah and Joseph 2016). The plethora of cases where tremor, of likely myogenic origin, has been observed, but dismissed as not being part of the phenotype, only give more importance to our claim that this form of myopathy accompanied by tremor should be considered as a new disease entity.

This project has yet again shown the value of international collaboration and has allowed us to put forward a previously undescribed autosomal dominant syndrome (congenital myopathy with tremor). This claim is still challenged by neurologists, as it goes against the dogma of primary neurological damage being the sole underlying cause of tremor. Furthermore, the case of myogenic tremor does once-again highlight the previously discussed phenomenon of protein-region-specific phenotypes that can be dramatically distinct (as in the examples of lamin A/C, emerin, nesprin 1 and 2, and TMEM43 (LUMA) pathogenic variant association with several different phenotypes) and therefore create difficulties for anyone involved in patient diagnostics and care (Bertrand et al. 2011; Amberger et al. 2019). Based on this information it is reasonable to assume that we will see more and more of a shift towards the protein-region-to-phenotype kind of classification, as new data becomes available, and not only in the field of neuromuscular disorders.

The initial data and findings described and discussed here, regarding the novel *MYBPC1* associated phenotype, have also allowed us to move forward with research of transgenic animal models, initially focusing on detailed phenotypic, behavioral, morphological and functional assessment, in order to elucidate the physiology of this novel type of tremor and gain more insights in the molecular mechanisms underlying it.

Concluding remarks

An added positive benefit of research projects like these, is the fact they help attract more attention to rare neuromuscular disorders from both the general public and specialists nation-wide, thus spreading awareness. It is believed that many neuromuscular disorders remain undiagnosed in our population due to some individuals presenting with unspecified symptoms

of muscle weakness, which sometimes can also be found in parental generation, and therefore perceived as a normal familial trait. This might be especially true for the slow progressing forms. This idea of our population being underdiagnosed partially stems from the neuromuscular disease incidence numbers reported in other European countries, where, assuming our population is not that different, we should see more cases of neuromuscular diseases. Our research has shown the population of Latvia to be quite heterogeneous, without any distinct known founder-effect variants. It seems, however, that calpainopathies are indeed the most common form of rare neuromuscular diseases in Latvia.

This work highlights the evolution of rare neuromuscular disease diagnostics and research in Latvia, spanned over a time period of more than 10 years. It starts from humble beginnings and basic methods that are cheap and easy to interpret, but have low result yield. Then evolves through now-obsolete technologies that seemed promising at the time, slowly gaining knowledge base and filling in blank spaces with regards to variant and disease frequencies in the local population. Now it can be said to have reached a world-class level, where next generation sequencing is the tool used for undiagnosed rare disease cases, with custom built data analysis and development of local databases. In the time of international collaborations as a norm for rare disease research, in order to pool patients and data, we have built lasting and fruitful connections and are utilizing all the options currently available. However, there is still room to grow - moving into the field of functional research of pathogenic variants and gaining the much-needed data, that is paramount for establishing proper diagnosis, and hopefully moving towards effective long-term therapy. Furthermore, we have been able to not only help patients of rare neuromuscular diseases get molecular diagnosis but have also been able to provide new knowledge to the field, and will continue to do so in the future.

5. CONCLUSIONS

- Through the use of our neuromuscular disorder patient database and the Genome database of Latvian population, we have managed to determine the allele frequencies for some of the most common variants, associated with LGMD forms in the population of Latvia, establishing calpainopaties as the most common form.

- Even though frequencies of the majority of tested neuromuscular disease associated variants in the population of Latvia do not differ significantly from the rest of Europe, there are certain variants, frequent in other parts of Europe, that are not common in Latvia. Most notably, LGMD R12 anoctamin5-related which is now thought to be one of the most common LGMD forms in Europe, is not common in Latvia, and the variant c.550delA in the *CAPN3* gene, thought to be the most common LGMD associated variant in Eastern Europe, is common in Lithuania, but not in Latvia.

- Based on available information, variant segregation, molecular testing and modeling, we classify the relatively common variant c.7447A>G in the *COL6A3* gene, currently classified as a variant of unknown significance, as pathogenic, as it is able to cause a mild disease phenotype, when present in a homozygous state. Compound cases show variable severity, based on the second variant in the gene. MRI imaging is proving to be the most specific first-line diagnostic tool, when dealing with collagen 6 associated myopathies.

- We have described a new disease phenotype, associated with M-motif specific variants in the *MYBPC1* gene that presents with a mild myopathy and myogenic tremor (MYOTREM). The initial functional testing suggests a possible disease mechanism, based on sarcomeric contraction dysregulation through altered cycling of actomyosin cross-bridges.

- In line with previous reports, the next generation sequencing approach currently grants the highest diagnostic yield, when dealing with rare neuromuscular disorders, due to the genetic and phenotypic heterogeneity present in this group of diseases. However, it is true only for diseases caused by small genetic variants, as applicability is still limited for diseases like facioscapulohumeral muscular dystrophy, caused by larger changes in the genome.

MAIN THESIS FOR DEFENSE

Thesis I

Variant c.7447A>G in gene *COL6A3* is associated with a mild collagen 6 associated myopathy phenotype in a homozygous state.

Thesis II

Pathogenic variations in the M-motif of the *MYBPC1* gene are associated with a new pathological phenotype, characterized by mild myopathy and myogenic tremor (MYOTREM).

Thesis III

Myogenic tremor does not have a primary neurological component, but is rather arising on the sarcomere level, due to dysregulation of sarcomeric protein binding during muscle contraction, and is then propagated and enhanced through the central nervous system.

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SUPPLEMENTAL MATERIALS

Supplemental table 1. Changes in LGMD nomenclature. Forms recognized as LGMD under the new nomenclature marked in bold.

Previous nomenclature	New nomenclature	Gene	Notes / Reasons for exclusion from LGMDs
Dominant forms			
LGMD 1A	Myofibrillar myopathy	*MYOT*	Distal weakness
LGMD 1B	Emery–Dreifuss muscular dystrophy (EDMD)	*LMNA*	High risk of cardiac arrhythmias; EDMD phenotype
LGMD 1C	Rippling muscle disease	*CAV3*	Main clinical features rippling muscle disease and myalgia
LGMD 1D	**LGMD D1 DNAJB6-related**	***DNAJB6***	
LGMD 1E	Myofibrillar myopathy	*DES*	Primarily false linkage; distal weakness and cardiomyopathy
LGMD 1F	**LGMD D2 TNP03-related**	***TNP03***	
LGMD 1G	**LGMD D3 HNRNPDL-related**	***HNRNPDL***	
LGMD 1H	Not confirmed	-	False linkage
LGMD 1I	**LGMD D4 calpain3-related**	***CAPN***	
Bethlem myopathy dominant	**LGMD D5 collagen 6-related**	**COL6A1, COL6A2, COL6A3**	
Recessive forms			
LGMD 2A	**LGMD R1 calpain3-related**	**CAPN**	
LGMD 2B	**LGMD R2 dysferlin-related**	**DYSF**	
LGMD 2C	**LGMD R5 γ -sarcoglycan-related**	**SGCG**	
LGMD 2D	**LGMD R3 α-sarcoglycan-related**	**SGCA**	
LGMD 2E	**LGMD R4 β-sarcoglycan-related**	**SGCB**	
LGMD 2F	**LGMD R6 δ-sarcoglycan-related**	**SGCD**	
LGMD 2G	**LGMD R7 telethonin-related**	**TCAP**	
LGMD 2H	**LGMD R8 TRIM 32-related**	**TRIM32**	
LGMD 2I	**LGMD R9 FKRP-related**	**FKRP**	
LGMD 2J	**LGMD R10 titin-related**	**TTN**	
LGMD 2K	**LGMD R11 POMT1-related**	**POMT1**	
LGMD 2L	**LGMD R12 anoctamin5-related**	**ANO5**	
LGMD 2M	**LGMD R13 Fukutin-related**	**FKTN**	
LGMD 2N	**LGMD R14 POMT2-related**	**POMT2**	
LGMD 2O	**LGMD R15 POMGnT1-related**	**POMGNT1**	
LGMD 2P	**LGMD R16 α-dystroglycan-related**	**DAG1**	
LGMD 2Q	**LGMD R17 plectin-related**	**PLEC**	
LGMD 2R	Myofibrillar myopathy	DES	Distal weakness
LGMD 2S	**LGMD R18 TRAPPC11-related**	**TRAPPC11**	
LGMD 2T	**LGMD R19 GMPPB-related**	**GMPPB**	
LGMD 2U	**LGMD R20 ISPD-related**	**ISPD**	
LGMD 2V	Pompe disease	GAA	Known disease entity, histological changes
LGMD 2W	PINCH-2 related myopathy	PINCH2	Reported in one family
LGMD 2X	BVES related myopathy	BVES	Reported in one family
LGMD 2Y	TOR1AIP1 related myopathy	TOR1AIP1	Reported in one family
LGMD 2Z	**LGMD R21 POGLUT1-related**	**POGLUT1**	
Bethlem myopathy recessive	**LGMD R22 collagen 6-related**	**COL6A1, COL6A2, COL6A3**	
Laminin α2-related muscular dystrophy	**LGMD R23 laminin α2-related**	**LAMA2**	
POMGNT2-related muscular dystrophy	**LGMD R24 POMGNT2-related**	**POMGNT2**	