



**233rd ENMC International Workshop: Clinical trial readiness for calpainopathies, September 15 – 17, 2017 Naarden, The Netherlands**

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This paper is dedicated to the memory of our friend and colleague, Dr. Hiroyuki Sorimachi, who recently passed away. He discovered calpain 3 and made ground-breaking research contributions to the calpain field. He will be missed by all of us.

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**Highlights:** Thanks to all the information exchanged during the workshop, it was possible:

- to get an accurate picture of the distribution of patients worldwide
- to get an accurate picture of the clinical presentation of patients worldwide
- to propose a series of actions regarding clinical readiness for calpainopathies

## 1. Introduction

Eighteen researchers and clinicians and two representatives of patient organizations (Associazione Italiana Calpaina 3, Italy and Coalition to Cure Calpain3, USA), from 9 countries (France, Germany, Italy, Denmark, Spain, UK, Japan, Brazil and USA), met in Naarden, Netherlands from September 15 to September 17, 2017 to discuss clinical trial readiness for Limb Girdle Muscular dystrophy type 2A (LGMD2A; OMIM 253600), also classically referred to as calpainopathy. LGMD2A is due to mutations in the CAPN3 gene encoding a calcium-dependent cysteine protease named calpain 3 (1). This disease is characterized by slowly progressive muscle weakness affecting selectively the musculature of both girdles. There is no effective treatment for this disease to date. The emergence of novel therapeutic approaches in the field, such as gene therapy, has prompted a much awaited discussion among physicians and researchers about the readiness for clinical trials in calpainopathy.

## 2. An historic perspective of limb-girdle muscular dystrophy

**Michel Fardeau** elaborated on the fascinating saga that led to the identification of the calpain 3 gene, from the very first clinical and pathological descriptions made by Erb in Germany to the molecular elucidation of the disease in 1995. The term itself of limb girdle muscular dystrophy (LGMD), actually coined during World War II and popularized by Walton & Nattrass in 1954, remains hotly debated even nowadays. In the late 70s, Michel Fardeau had the opportunity to revisit Erb's concept of juvenile-onset muscular dystrophy thanks to a genetically isolated population called the '*Petits Blancs des Hauts*' and located in the Reunion Island, an overseas French territory in the Indian Ocean. Collecting clinical data and processing of DNA samples led to a first mapping to chromosome 15. Additional samples

collected notably in the Amish communities of the USA and other countries (Brazil) made possible, via reverse genetics, the identification of the *CAPN3* gene at Genethon while clearly pinpoint the genetic heterogeneity of LGMD. Nowadays, calpainopathy remains the most frequent form of LGMD in the majority of countries with reported prevalence between 10 to 70 per million inhabitants (2-4).

**Andoni Urtizberea** gave an overview of what is currently known of calpainopathy, mainly from a clinical perspective. Thanks to a contribution from Lopez de Munain, (San Sebastian, Spain) and based on his personal experience, he reviewed the classical and non-classical clinical presentations of calpainopathy. Calpainopathy is sometimes capable of mimicking many other neuromuscular disorders such as, for instance, inflammatory myopathies (eosinophilic myositis) or metabolic myopathies. A growing number of benign cases are now also being reported. The recent discovery of an autosomal dominant form of calpainopathy adds to the clinical spectrum of calpainopathies. The existence of several founder mutations was highlighted as well as some degree of clinical heterogeneity even in families belonging to supposedly homogeneous genetic isolates. The fact that the NGS is nowadays substantially changing diagnostic algorithms was also addressed. Better knowledge of these clinical variants also matters in the context of future trials.

### 3. Calpain 3 family and function

**Yasuko Ono** (Japan) presented a review of current knowledge on the calpain family and the regulation and function of calpain 3 (**Figure 1**). Calpain 3 is a member of the calpain family of non-lysosomal calcium-activated cysteine proteases. Although some isoforms were identified in lens and brain, the classical full-length form of *CAPN3* is primarily expressed in skeletal muscle (5). Of note, the expression in the heart is at least 100 times lower than in skeletal muscle with a level that varies among species (6, 7).

In muscle, the enzyme is present as a dormant enzyme probably through interaction with one of its partner, the giant protein titin (8, 9). It was shown that calpain 3 self-activates by autolysis through the removal of an internal peptide to free the catalytic site for substrate accessibility (10, 11). Although autolysis might be involved in the activation process of calpain 3, not all LGMD2A mutant proteins lose their autolytic activity. As one of recently identified mode of calpain 3 functions, intermolecular complementation for restoring protease activity after autolysis provides a new aspect in the effect of pathogenic mutations (12).

The precise function(s) of calpain3 and the mechanism by which it causes LGMD2A are not fully understood although several evidences pinpoint a role in cytoskeleton remodelling (13-15). As an example of research investigating calpain 3 role in muscles, **Ametz Saenz** (Spain) presented a study of the muscle cell protein turnover in myotubes of LGMD2A patients aiming at shedding some light on the pathophysiological mechanisms in LGMD2A and identifying therapeutic targets that may stop muscle degeneration (16). LGMD2A patients' mature myotubes showed deficit in the transition from the transmembrane integrin  $\beta$ 1A isoform to the  $\beta$ 1D isoform which is seen under normal physiological conditions during myoblast fusion and muscle fibre maturation. They observed that Frizzled (FRZB), a protein of the Wnt pathway, regulates integrin  $\beta$ 1D expression and that its silencing increases integrin  $\beta$ 1D expression to levels similar to those in controls, suggesting that silencing FRZB may rescue the normal expression of proteins involved in the fusion process in LGMD2A. This could suggest that the Wnt pathway could represent a therapeutic option for LGMD2A, in addition to other pathways that have been proposed as potential targets (14, 17-20).

#### **4. Clinical presentations and natural history studies in LGMD2A**

**Bruno Eymard** (Paris) presented the French experience on Calpainopathy. Overall, 231 cases of calpainopathy are registered in the French reference centre database with a mean age of onset around 13 years. In the two French genetic laboratories performing molecular diagnosis

of calpainopathy, respectively in Paris (Dr F Leturcq, Cochin hospital) and Marseille (Dr M Krahn, La Timone hospital), 96 index cases with two calpain gene mutations have been identified between 2012 and 2016. The proportion of calpainopathy is around 20% of the all genetically tested LGMDs. Although the disease begins in most of patients during early adolescence, the age at onset is variable from early childhood to middle adulthood. These data are similar to these obtained in smaller series reported in 1996 and in 2016 (21, 22). The disease course is always progressive, with worsening of weakness in both girdles, resulting in functional degradation. In a recently published series including 20 French patients, with age at inclusion ranging from 19 to 65 y (mean age of 42 y), followed at the Institute of Myology (Paris) for a natural history survey, 52% of patients were non ambulant and 14% required a cane or walker aid (22). In the same report, 30 patients from Reunion Island and 35 patients from the Spanish-Basque population were also described: the age at onset and severity were similar in the 3 groups. In this study, as in our previous experience (21), the clinical pattern was homogeneous with both girdle involvement, major involvement of *serratus anterior* with scapular winging, *gluteus maximus* and *medius*, selective posterior compartment of thigh hamstrings and adductors (22). Beside the classical presentation, it is possible to observe variations in the phenotype. For example, in one patient followed at the Institute of Myology, classified as retractile calpainopathy, contractures were prominent, affecting elbows and hips. No heart involvement was found in these patients and respiratory involvement was generally moderate. In nine patients with calpain gene mutations, followed at the Institute of Myology, calpain expression assayed by Western-blot, was normal or minimally reduced. Interestingly, all patients but one were women and age at onset was late from age of 18 years to 47 y, mean 32.1 years. Only 2 out of 9 were non-ambulant, after a long duration, respectively 24 and 32 years.

**Andoni Urtizberea** presented in detail an observational study of clinical manifestations and disease progression where a total of 85 genetically confirmed LGMD2A patients, aged 14-65

years, were recruited in three centres located in metropolitan France (Institut of Myology Paris), in Spain (San Sebastian), and on the Reunion Island (Saint-Pierre). They were followed up every 6 months for 2 years and a subgroup was assessed annually thereafter for two more years. Data collected for all patients included clinical history, blood parameters, muscle strength assessed by manual muscle testing (MMT) and quantitative muscle testing, functional scores, and pulmonary and cardiac functions. In addition, CT scans of the lower limbs were performed in a subgroup of patients (**Figure 2**). This study confirms the clinical description of a slowly progressive disorder with onset in the first or second decade of life with some degree of variability related to gender and mutation type. The null mutations lead to a more severe phenotype while compound heterozygote patients are the least affected. Muscle weakness is remarkably symmetrical and predominant in the axial muscles of the trunk and proximal muscles of the lower limb. There was a high correlation between the weakness at individual muscle level as assessed by MMT and the loss of density in CT scan analysis. All the generated data will help to determine the endpoints for further clinical studies.

**Alicia Alonso** and **Jordi Diaz-Manera** (Spain) reviewed and presented the data on calpainopathy patients in Spain. Spanish patients are followed-up in several different centres. Although there are at this moment five Neuromuscular Disorders Units recognized as reference centres by the Spanish Ministry of Health (three in Barcelona, one in Valencia and one in Sevilla), the genetic study of the *CAPN3* gene is performed in a single public centre located in San Sebastian-Donostia. The Spanish Neuromuscular Disorders registry (NMD-ES) contains at this moment clinical and genetic data of 83 calpainopathy patients. Thirty-six (43%) of these patients are women. The mean age at onset of symptoms in this cohort is 12.4 years old. At last visit, 97% of the patients had muscle weakness involving lower limbs more severely than upper limbs. Scapular winging was found in 64% of the patients. Up to 70% of the patients were non-ambulant while only 10% of the patients were able to walk independently without any help. The most common mutation detected in this cohort is the

p.Arg788Ser-fs14\* (present in 59% of the patients), which is usually known as the “Basque mutation” due to its high prevalence in patients from the Basque country. Other frequent mutations are the p.Gly222Arg (present in 10% of the cohort) and the p.Arg748Gln (present in 6% of the cohort).

**Michela Guglieri** (UK) described a cohort of 56 LGMD2A UK patients, followed up at the John Walton Muscular Dystrophy Research Centre (JWMDRC) in Newcastle, UK. LGMD2A represents 21% of the total LGMD population followed up at the JWMDRC, which is in line with previously reported incidence studies. Patients were equally distributed between males and females (29 vs 27); mean age was 38 years (range 14-83 years). Approximately 50% of the cases reported onset of muscle symptoms between the age of 10 and 20 years with however a wide range (4 to 60 years). Mean time at loss of ambulation was 12 years after disease onset (SD: 11 years). Early onset cases seemed to have a more severe course and earlier loss of ambulation while none of the seven patients with a late disease onset (> 40 years) used a wheelchair (age 50-83 years). Respiratory impairment was observed and correlates with the severity of skeletal muscle involvement; however even when detected, it was usually mild or moderate [Forced Vital Capacity (FVC) between 40 and 80 % of predicted value]. None of the patients showed cardiac involvement. Homozygous or compound heterozygous mutations were confirmed in all patients, except two patients in whom only one mutation was identified (Multiplex ligation-dependent probe amplification studies are ongoing).

**Maggie Walter** discussed a German cohort of 35 calpainopathy patients and reported on age of onset, disease duration, status of ambulation, Creatine Kinase (CK) levels, MRI findings, type of mutation, along with clinical and genetic heterogeneity. Mean onset was usually during adolescence, varying between 2 and 50 years of age; loss of independent ambulation occurred between 15 and 25 years after clinical onset. There was faster progression in patients with juvenile onset, similarly to the UK cohort. Most frequent mutations were c.550delA and



c.1468C>T (p.Arg49Trp). The median age of symptom onset for patients with 2 null alleles (predicting loss of CAPN3 open reading frame and thus absence of calpain protein) was lower than in patients with one or zero null alleles. MRI findings showed that while mutations in genes encoding proteins of the Dystrophin Associated Complex (dystrophin, sarcoglycans) predominantly affect the anterior thigh muscles, proteins with enzyme function (calpain-3, FKRP) predominantly involve the posterior thigh muscles. However, during the course of the disease, the anterior compartment becomes equally affected in calpainopathy. Of note, in contrast to severe MRI changes, some patients are able to walk well into their forties.

**Claudio Semplicini** pointed out that LGMD2A represent the most frequent LGMD in Italy (23, 24), with an estimated prevalence of 1:105,000 in the North-East of the country (3). He reviewed the data of 49 LGMD2A patients (age 8-68 years) from three centres (Padova, Milano and Bosisio Parini) included in a cross-sectional clinical evaluation protocol. Natural history data were collected, different motor function tests were performed [i.e. 6-minutes walking test (6MWT), Performance of Upper Limb (PUL), North-Star Ambulatory Assessment (NSAA), grip strength, timed tests] as well as cardiac and respiratory function evaluations. Psychometric Tests of Individual Neuromuscular Quality of Life (INQoL) and impact of fatigue (Fatigue Severity Scale) were also included. Motor function was correlated with age (young better than old), sex (females better than males), residual protein on Western Blot (presence better than absence) and number of frameshift/null alleles (0 better than 1 and 2). Specific mutations are associated with peculiar phenotypes, such as mutations in exon 11, recurrent in the Venice area, that are associated with a relatively late onset and loss of ambulation, a marked weakness of *biceps brachii* and a normal expression of CAPN3 on WB (autocatalytic defect) (25). NSAA, timed tests and proximal items of PUL seem, in the Italian cohort, the most clinically meaningful tests for future clinical trials. The collection of longitudinal data will help in better defining the disease evolution and clinic-molecular

heterogeneity.

**Mariz Vainzof** (Brazil) highlighted that Brazilian families with LGMD2A included families studied since 1990, when they also contributed to the identification of the CAPN3 gene. Since then, patients have been screened through protein and/or DNA analyses, first by individual gene screening and nowadays using NGS, at the Human Genome and stem cell Research Center, a reference center for neuromuscular diseases in Brazil. LGMD2A was appointed as the more common form in Brazil, corresponding to about 32% of the LGMD cases. Genetic results observed in 63 patients, from 57 unrelated families were presented. A total of 31 different mutations were identified, with six newly described ones that include five non-sense mutations. Consanguinity in these families was very high (72%), with even two families presenting an autosomal dominant-like transmission. Some of the most common mutations in Europe and USA were also the more frequent in Brazil, such as the p.Arg788Serfs\*14 (Basque mutation) and p.Arg110\* mutations. The correlation of type of mutation and the protein was positive, with non-sense mutations showing total absence of the protein while some mutations were compatible with the presence of some calpain 3 protein in the muscle. Interestingly, some specific mutations, such as p.Lys254del and p.Arg490Trp showed the presence of almost normal calpain 3 in the muscle. Finally, using the Brazilian 80 plus bank of controls (26), it was estimated that the frequency of LGMD2A pathogenic mutation described in ClinVar, a public database of the relationship between human variations and phenotypes (<https://www.ncbi.nlm.nih.gov/clinvar/>), represents a proportion of 1/90 heterozygous, and 1/32000 affected LGMD2A in the Brazilian population.

**Vincent Carson** reported the case of the Amish community where a founder effect was reported for calpainopathies. He presented the history of installation of this community in the US, leading to two main settlements in Pennsylvania and Indiana through migration from Germany and Switzerland. The homozygous Amish mutation is present in the population from

Northern Indiana. Of note, this mutation was also identified in two patients in Europe.

**John Vissing** presented the dominantly inherited form of calpainopathy, which was reported as a new form of calpainopathy in 2016 (27). Likely, the mechanism of disease is quite similar to the recessive form, as calpain 3 expression is also severely down-regulated in the dominant form, which could be due to a dominant negative effect of the mutant protein on the wild-type protein from the healthy allele. This means that all single *CAPN3* variants cannot cause a dominant form of LGMD; only those that result in production of an aberrant protein that can polymerize with or otherwise disturb the wild-type protein. Unlike some cases of LGMD2A, where calpain 3 is lost completely, some wild-type protein is always present in the dominant form, albeit in severely reduced levels. Maybe for this reason, the phenotype of dominant calpainopathy is milder than the recessive form (27). The pattern of muscle affection closely resembles that in the recessive form, but in the dominant form, onset of disease is later (34 years vs. 16 in LGMD2A) and the muscle weakness is milder. Also, plasma CK levels are not always elevated, which is almost always the case in LGMD2A. Future clinical investigations should be aimed at identifying new dominant cases, which may in some cases go unnoticed because of the milder nature of the disease. Vissing presented such a new condition related to a c.1715G>C missense mutation in *CAPN3*. As with the 10 dominantly inherited families reported with the same c.643\_663del21 mutation in *CAPN3*, the disease in the three generations of the c.1715G>C mutation family showed loss of calpain3 in muscle, variable muscle affection in generations with muscle affection similar to LGMD2A, and exome sequencing could not identify any other cause of the disease. In looking for dominantly inherited cases of calpainopathy, the clinician should look for; 1) segregation of a new *CAPN3* variant with the disease, 2) calpain 3 loss on Western blot, 3) a phenotype which resembles LGMD2A, just milder, 4) and discard other diagnostic possibilities, by for instance, whole-exome sequencing.

In conclusion, it appears that, in most countries, LGMD2A is usually the most frequent form of LGMD. Most of the patients present a classical clinical phenotype with a significant, selective involvement of the posterior compartment of the thigh. The disease course is usually slow but with an possible correlation between an early onset and a higher severity. Respiratory function may be compromised in a proportion of patients. Cardiac issues are rarely observed and are probably coincidental. Although it seems to be rarely severe in LGMD2A, assessment and monitoring of respiratory function should be part of the standards of care. Unusual presentations of calpainopathy with pseudo-metabolic or prominent joint contractures, benign hyperCKemia or non-conventional mode of transmission (autosomal dominant) have been presented and discussed.

## 5. Diagnosis aspects

Genetic diagnosis for calpainopathies has been available on a routine basis using Sanger sequencing since early after the initial identification of *CAPN3* in LGMD2A in 1995 (1). It is now available mainly using Next Generation Sequencing (NGS), including *CAPN3* analysis within different strategies (in example gene-panel analyses including all known LGMD genes, or all known myopathy genes; Whole-Exome Sequencing; etc.). As for many other genes causing muscular dystrophies, *CAPN3* has a large mutational spectrum, with currently more than 400 different pathogenic sequence variants reported, including well characterized founder mutations (Leiden Muscular Dystrophy Pages [www.dmd.nl](http://www.dmd.nl)) (28, 29).

The molecular diagnosis of calpainopathy is complicated by the fact that, in a number of cases, the protein level is preserved in Western Blot or when a decrease is observed, it may be caused by secondary deficiencies. Within the course of this ENMC workshop, current challenges in the genetic diagnosis have been pointed out. The main discussed issues related to different diagnostic situations encountered in practice are summarized in **Table 1**.

**Bruno Eymard** stated that secondary reduction of calpain expression without calpain gene mutation (secondary calpainopathies) are not rare in other limb girdle muscular dystrophies. In the molecular genetic laboratory of Cochin hospital (Dr F Leturcq), 62% of the calpain deficient LGMD tested between 2012 and 2014, was secondary calpainopathies. In the Institute of Myology cohort, nine patients with a secondary calpainopathy are recorded. In six of these cases, the primary genetic defect was identified: *FKRP*, *ANO5*, *TTN*, in respectively 1, 2 and 3 patients. The clinical phenotype was always clearly different from a primary calpainopathy. In three patients of this group, harbouring recessive titin gene mutations, two features differ clearly from the classical calpainopathies: diffuse contractures and inclusion body pattern at muscle biopsy (30).

A possible correlation between protein expression, type of mutation and disease severity has been reported but collaborative studies are required for a full understanding of the mechanism behind this. In this context, experts highlighted the value of muscle biopsies for diagnosis and research purposes. Despite the introduction of Next Generation Sequencing in the diagnostic algorithm of primary calpainopathy, biopsy analysis can be of tremendous help for a better understanding of the correlation between protein expression and clinical course and for potentially provide patients with prognostic information.

## **6. Outcomes measures/ Biomarkers/ Clinical endpoints/ Clinical trial design**

### 6.1. Outcomes measures: The Institute of Myology experience

**Jean-Yves Hogrel** presented retrospective and prospective natural data in LGMD2A. He first showed data on manual muscle testing (MMT) in 25 patients followed in Paris and Saint-Pierre de la Réunion. As a conclusion only 1 point change in MMT score was observe every 17 years on average. This demonstrates that MMT would not represent a good outcome

measure for LGMD2A, in view of the short duration of most clinical trials. However, a mapping of muscle wasting and a mapping of weakness progression could be used to select certain muscles more prone to change after a given duration of the disease. A multicentre prospective natural history study confirmed this very slow progression using other methods of strength quantification (fixed dynamometry and isokinetic dynamometry) during the follow-up of 85 patients over 4 years (22). The Motor Function Measure (MFM) and CT-scan imaging also attested the slow progression of the disease. The natural history showed that the compound heterozygotes (one null allele, one missense allele) had a less severe phenotype compared to homozygous mutations. Women presented generally with a less severe phenotype compared to men. Focusing on muscle strength, the results confirmed the non-linearity and the poor discrimination power of the MMT. What was more surprising was the variation in correlation between the two quantified methods, which are supposed to measure the same quantity. Of note, this variation depended on the muscle function tested. Correlation between methods was almost perfect for knee extension while it was moderate for knee flexion. This implies that even quantified methods may not be interchangeable depending on the muscle function tested. Finally, most of the functional variables correlated with disease duration. Altogether, the results of these studies underlined the very slow but ineluctable progression of the disease. The choice of neuromuscular measures for future therapeutic trials is thus challenging.

## 6.2. Respiratory evaluation

Presented by **Hélène Prigent**, respiratory involvement has been reported since the initial clinical description of calpainopathy (4). According to the different cohorts, its prevalence has been observed in 21% to up to 60% of the patients (2, 4, 22, 24, 31). Respiratory dysfunction follows a restrictive pattern of respiratory failure with a reduction of lung volumes (4, 32). The severity of the respiratory dysfunction seems to be usually moderate but severe respiratory

failure has been reported in a fraction of patients (22, 31). In the population studied by Richard et al, 11% of the patient had a vital capacity <50% of predicted value (22). Ventilatory support with mechanical ventilation may be required in a minority of patients (22, 31) and respiratory failure has been reported in a few cases as cause of death (4, 31, 33). Little information is available on the progressive alteration of the respiratory dysfunction in LGMD2A; however, respiratory failure seems to correlate more with disease duration and motor dysfunction than to patients' age (31). Although the natural history of respiratory involvement in calpainopathy needs to be further investigated, respiratory dysfunction while usually moderate may be severe in some patients leading to respiratory failure. Therefore, respiratory monitoring should be included in the management of calpainopathy, especially in non-ambulant patients.

### 6.3. Imaging

**Robert-Yves Carlier** is coordinating a collaborative effort on imaging of LGMD2A patients in the framework of the MYO-MRI project (<http://myo-mri.eu/>). This project involves several neuromuscular centers in Europe and USA, and aims at collecting MRI scans of muscular dystrophy patients to describe the imaging pattern and spectrum of muscle involvement in these disorders and to identify clues to help to distinguish the different forms of muscular dystrophies. Muscle imaging through MRI has been increasingly exploited as a method to investigate several genetic and acquired muscle disorders, and to derive information potentially useful for the design of clinical trials (34). So far, full body scans have been collected and analyzed in more than 50 genetically confirmed LGMD2A patients carrying different mutations. On coronal and axial sections scans, the proximal muscles, including paraspinal muscles, are the most affected. Interestingly, it appears that there is a dichotomy of presentations distinguishing a severe and a moderate group with correlation to type of mutation. Minor asymmetry has been observed in some patients. From a global perspective, a

muscular impairment specific to LGMD2A patients is present, but no there is no specific sign in term of muscle texture compared to other MDs.

## **7. Registries and database, patient foundation**

### 7.1 TREAT-NMD experience in patient registry

**Michela Guglieri** presented the TREAT-NMD experience with patient registry. Patient registries are useful tools to evaluate feasibility of trials, recruit patients in clinical trials, establish a link between research community, provide more equality of access to trials and the patients and help developing care standards. The examples of the two TREAT-NMD global patient registries, Duchenne muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMA), were presented. Advantages and disadvantages of national and international registries and of different types of registries were discussed during the meeting. National registries are generally set-up for conditions where there is deemed to be enough patients present in that country to warrant a country specific registry, such as DMD and SMA. However, for ultra-rare diseases international registries are seen to be more appropriate. Patient self-reported registries have the limitation of subjectivity of the data, but have a higher registration rate and are more likely to be kept updated. Health professional reported only registry could potentially allow collection of natural history data but are often time consuming and require engagement of clinicians. The patient self-reported international LGMD2A registry created by Coalition to Cure Calpain 3 (C3) currently has more than 800 patient registered; genetic confirmation is however not required for registration and subscription is mainly limited to the US. National registries for LGMD2A exist in Spain and France, and both are health professional reported only registries.

### 7.2 Coalition to Cure Calpain3



**Jennifer Levy** shared the mission and programs of Coalition to Cure Calpain 3 (C3), a patient foundation for which she is the Scientific Director. C3 is committed to treating and ultimately curing calpainopathy. C3's mission is to fund high potential research and clinical trials while educating the global community about this disease. Their research grant program funds several projects that are related to understanding the calpainopathy disease mechanism and testing potential therapeutics in animal models. C3 also has initiatives to support genetic testing of patients with undiagnosed limb girdle weakness and the generation of new research tools. The foundation organizes calpainopathy-focused meetings and increases awareness through social media campaigns, LGMD-Info.org (curated educational information and resources for the LGMD community), and LGMD Awareness Day (held annually on September 30<sup>th</sup>).

C3 administers an international registry of calpainopathy patients. The registry, which can be accessed at [www.lgmd2a.org](http://www.lgmd2a.org), allows patients to self-report on their contact information, year of diagnosis and genetic testing results, neurologist and clinic information, disease symptoms, family members with calpainopathy, and current medications and adaptive equipment used. Questions are in plain English with no medical knowledge required, and estimated time burden is about 5 minutes. At the time of the ENMC workshop, the registry had 890 submissions. C3 is currently evaluating potential improvements to the registry, including translation of the forms into additional languages and confirmation the registrants' diagnoses.

### 7.3 Associazione Italiana Calpaina 3, Italy

**Bruno Kullmann** presented the AICa3-Italian Association Calpain3, which is the first and only Italian association focused on LGMD2A. The aims of the association can be grouped in 3 categories: 1) Representation: giving voice to patients and their families. 2) Research: stimulate and drive research projects, particularly clinical trial. 3) Information: diffuse

knowledge about the disease, about the on-going research projects, about the centres with expert of the fields.

Preparing the venue to the 233th ENMC Workshop, a survey was conducted between the Italian patients with LGMD2A. All patients were asking to speed up the diagnosis, the development of therapies, the research and the formation of new experts. In addition to the long-term studies with gene- or cell-mediated therapy, there is a huge request for short-term studies aiming to slow down the disease progression and improve quality of life through new treatments or devices able to reduce the disease-related limitations. Patients are asking to be involved in the design and the development of research projects, which could result in a more efficient translation of research into clinical practice. Closer collaboration between patients and researchers would ensure that studies and research projects are tailored to patient needs and will ensure patient engagement. AICa3 is willing to support innovative patient-centred projects, promoted by new centres of excellence in the treatment of neuromuscular disorders.

## **8. Calpain 3 therapies**

### 8.1 Stem cells and C3

**Mariz Vainzof** presented the study done by Dr. Eder Zuconni and Mayana Zatz, from the HGRC, which included the evaluation of the ability of human adipose mesenchymal stem cells (MSC) and human umbilical tissue cord SC to graft into muscle, to express calpain-3, and to improve functional capacity in the C3KO mouse. During 6 months, 4 weekly and 5 monthly injections of  $10^6$  cells were applied in the caudal vein of the mice, and functional evaluation were performed at the same time. No calpain-3 protein was detected through western blot analysis in the muscles of any of the studied animals. However, functional analyses were variable in the studied groups, with some tests suggesting the possibility of a beneficial effect of the injected cells. A more recent discussion about these “negative” data

involves information about the possible paracrine effect of the MSC cells on the regenerative intrinsic process of muscle regeneration. Several groups of this centre are now studying the effect of the secretoma of these cells, and more specifically, the regenerative potential of extracellular vesicles, extracted from different source of cells with a good myogenic potential

## 8.2. AAV gene transfer

**William Lostal** presented the evaluation of AAV-mediated transfer of C3 in a knock-out mouse model of LGMD2A. In an attempt to develop a therapeutic strategy based on gene transfer for LGMD2A, a series of experiments using recombinant Adeno-Associated Viral (rAAV) vectors, the current standard tool for gene transfer in skeletal muscle was performed. After local injections of rAAV vectors expressing *CAPN3* in skeletal muscle of a murine model deficient in calpain 3, an efficient *CAPN3* transgene expression in skeletal muscles with a correct localization at the sarcomere was observed (35). This expression was associated with a restoration of the calpain3 proteolytic activity and rescue of the contractile force deficits. However, when testing a systemic route of administration of these vectors, a cardiac toxicity was detected and confirmed to be related to ectopic expression and unregulated activity of calpain 3 in the heart (36). A second generation of vectors was then designed using new promoters and by introducing a sequence target of a cardiac specific microRNA (miR-208a) in the 3'UTR. These modifications suppress the cardiac toxicity while conserving the therapeutic effect in the skeletal muscles (36). Even if the cardiac toxicity was circumvented by preventing the expression in the cardiac tissue using a miR-regulated vector, it was of importance to investigate the safety of the vectors in a model as close as possible of the human situation. A preclinical study in normal macaca fascicularis has now been conducted to evaluate the biodistribution and safety of AAV9-calpain 3 vectors with a deep analysis of the heart. Upon delivery of  $3 \times 10^{13}$ vg/kg and after one month of expression, the single IV infusion lead to a correct distribution of the vectors in heart but did not lead to observable adverse

effects or detectable toxicity in NHP.

## **9. Trial design and requirement**

**Marie- Laurence Gourlay** addressed the regulatory and methodological requirements for the clinical development of new therapies for rare diseases in the EU. Regulatory requirements for a clinical trial in view of a marketing authorization for a rare disease are similar to those applicable to common diseases since patients suffering from rare diseases require medicinal products that are as safe, as effective and of the same quality as any other commonly used medicinal products. The specificities linked to the clinical development in rare diseases refers to the rarity of patients, of experts, of reference treatment centres, the huge heterogeneity in patient symptoms, age and severity and the lack of sensitive and specific outcome measures and tools. In addition, as far as treatment availability is concerned in rare diseases especially when there is a medical need, early access to treatment through compassionate use and conditional approvals should be envisaged. In the EU, clinical development in rare disease should comply with the Good Clinical Practices and European Medicines Agency (EMA) Guidelines. Some do specifically apply to rare disease as it the case for the Committee for Medicinal Products for Human Use (CHMP) guidelines on the conduct of clinical trials in small populations and the CHMP guideline on clinical investigations of medicinal products in the paediatric population. Some are specifically dedicated to the conduct of clinical trial in specific rare diseases such as the EMA guideline on the clinical investigation of medicinal products for the treatment of Duchenne and Becker muscular dystrophy. Moreover, some others do address the issues specifically linked to the development of Advanced Therapies Medicinal Products such as the CHMP guideline on the follow up of patients administered with gene therapy medicinal products. Any deviations from these accepted rules could be acceptable if adequately justified. Early interactions with patient associations for protocol feasibility and Patient Reported Outcomes assessments and EMA for Scientific Advice or

Protocol Assistance are determining factors to succeed in the conduct of such complex clinical development.

## **10. Conclusion**

The group agreed that patient registries and collection of natural history data represent important steps for trial readiness in LGMD2A. Ideally, a global registry for LGMD2A, collecting data from national registries should be created; the extension of C3 registry to countries outside US would be difficult due to country specific regulatory requirements. A working group should meet to establish the core mandatory items and there was a general agreement that genetic confirmation of the diagnosis should be a mandatory item.

The discussion pointed out that for now; no specific clinical outcomes for LGMD2A have been clearly defined so far, highlighting the need of additional data on the natural course of LGMD2A in preparation of future clinical trials. The example of the Natural History study in dysferlinopathies, another rare LGMD, was presented. The Clinical outcome study in Dysferlinopathies, funded by the Jain Foundation, represents a successful example of international, multi-centre natural history study in a rare LGMD, and has led the identification of possible clinical outcomes which can be used for power calculation for clinical trial designs and potentially for measuring drug effect. However, cost of large, international studies was discussed and identified as a burden to design a natural history study in LGMD2A at this time.

In the last session, and among other therapeutic options, an AAV-mediated gene transfer approach was presented with promising results. Nevertheless, it is clear that more pre-clinical studies are still needed to first reach a better understanding of the function of calpain 3 in skeletal muscles.

The following key deliverables were achieved:

1. Constitution of an integrated network for clinical readiness for LGMD2A, comprising all the participants of this meeting and extended to other investigators from La Reunion island, Eastern countries, the Amish communities because of the prevalence of the patients in these areas.
2. Establishment of a natural history working group and registry working group. These working groups will define which type of outcome measures can be defined for evaluation in clinical trial and how to proceed to establish an adequate registry.
3. Constitution of a group working on biochemical outcomes, including blood and urine biomarkers.

#### **10. Participants – ENMC C3 Workshop Study Group**

Alicia Alonso-Jiménez (Barcelona, Spain); Robert-Yves Carlier (Garches, France); Vincent Carson (Lancaster, PA, USA); Bruno Eymard (Paris, France); Michel Fardeau (Paris, France); Marie-Laurence Gourlay (Evry, France); Michela Guglieri (Newcastle, UK); Jean-Yves Hogrel (Paris, France); Bruno Kullmann (Milan, Italy); Jennifer Levy (USA); William Lostal (Evry, France); Yasuko Ono (Tokyo, Japan); Helene Prigent (Garches, France); Isabelle Richard (Evry, France); Amets Saenz (San Sebastian, Spain); Claudio Semplicini (Padova, Italy); J. Andoni Urtizberea (Hendaye, France); Mariz Vainzof (Sao Paulo, Brazil); John Vissing (Copenhagen, Denmark); Maggie Walter (Munich, Germany)

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**Figure 1: Calpain 3** **A/** Structure of the calpain 3 protein. **B/** Localisation of calpain 3 at the level of the sarcomere. Modified from Ojima K, Kawabata Y, *et al.*, 2010, JCI. **C/** Calpain 3 and its interaction at different subcellular compartment.

**Figure 2:** CT-scan of an LGMD2A patient. **A/** Upper thigh. 1: *rectus femoris*. 2: *vastus lateralis*. 3: *vastus medialis*. 4: *vastus intermedius*. 5: *sartorius*. 6: *adductor longus*. 7: *adductor magnus*. 8: *gracilis*. 9: *semi membranous*. 10: *semi tendinosus*. 11: *biceps femoris*  
**B/** Lower thigh. 1: *rectus femoris*. 2: *vastus lateralis*. 3: *vastus medialis*. 4: *vastus intermedius*. 5: *sartorius*. 6: *gracilis*. 7: *semi membranous*. 8: *semi tendinosus*. 9 and 10: *biceps femoris*. **C/** Upper calf. 1: *tibialis anterior*. 2: *extensor digitorum longus*. 3: *peroneus longus*. 4: *tibialis posterior*. 5: *soleus*. 6: *gastrocnemius lateralis*. 7: *gastrocnemius medialis*.  
**D/** Lower calf. 1: *tibialis anterior*. 2: *extensor digitorum longus*. 3: *peroneus longus*. 4: *tibialis posterior*. 5: *flexor digitorum longus*. 6. *flexor hallucis longus*. 7: *soleus*.

**Table 1:** Issues related to different diagnostic situations in LGMD2A

Diagnostic situation	Encountered challenges	Anticipated solutions and/or consequences
Patient cases with « clear » clinical and/or calpain-3 protein level (deficiency or absence) orientation towards primary calpainopathy	Pathogenicity interpretation of novel, not previously reported sequence variants (in particular for missense, isosemantic and intronic variants)	Systematization and inter-lab coordination for the use of consensual bioinformatics predictive tools  Homogenization and inter-lab coordination for the classification of sequence variants (based on ACMG guidelines [38] [39])  Development and diagnostic implementation of functional tests (for example for possible splicing defects: systematic transcriptional analyses or mini-gene assays)
	Lack of identification of <i>CAPN3</i> pathogenic sequence variants in particular:  - “Missing second mutations, i.e. situations in which only one pathogenic allele is identified in an autosomal recessive segregation assumption  - No pathogenic sequence variants identified in situations with calpain-3 protein deficiency	- Development of systematic genetic screening for « atypical » mutations (deleterious Copy Number Variations, variants in deep-intronic regions or in regulatory element, etc.) using genomic whole- <i>CAPN3</i> locus or transcriptional analyses  - Accurate characterization of possible secondary calpainopathies (implicating other genes including <i>TTN</i> , <i>FKRP</i> , <i>DYSF</i> , etc.)
	Presence of additional pathogenic variants in other genes implicated in myopathies, in situations where a genetic diagnosis of calpainopathy is clearly established (in particular through NGS strategies)	Characterization of possible disease-modifying effects of associated sequence variants
	Characterization of additional pathogenic <i>CAPN3</i> sequence variants implicated in autosomal dominant transmission	Development of systematic genetic screening of <i>CAPN3</i> in autosomal dominant myopathies
	Genetically characterized primary calpainopathies without protein deficiency	Characterization of underlying pathophysiology
Genetically characterized primary calpainopathies without initial « clear » clinical and/or calpain-3 protein level (deficiency or absence) orientation towards primary calpainopathy	Identification of <i>CAPN3</i> pathogenic sequence variants using “broad” first-instance NGS mutational screening for myopathies	Extension of phenotypic spectrum

Figure 1.

Figure 2.

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