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169th ENMC International Workshop Rare Structural Congenital Myopathies 6–8 November 2009, Naarden, The Netherlands

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1. Introduction

This international ENMC workshop assembled 18 clinicians and scientists from Europe, the United States of America, South America, Japan and Australia to discuss “Rare Structural Congenital Myopathies (CM)”. This workshop can be considered a follow-up to an earlier one [1], then and now excluding classical CM on which separate workshops have repeatedly been held at ENMC and respective consortia exist such as on nemaline myopathies, centronuclear myopathies, core myopathies, as well as protein aggregate myopathies. CM can be classified according to CM-specific morphological features, certain epidemiological aspects or on molecular grounds. This workshop addressed those rare CM which, to date, have not been assigned to any known genes by virtue of identifying disease causing mutations. Of the approximately 10 CM discussed at the earlier workshop [1] seven have now been clarified molecularly. The workshop concentrated on the remaining three and other rare CM, i.e., tubular aggregates myopathy, cylindrical spirals myopathy, crystalline body myopathy, as well as fingerprint and Zebra bodies myopathies with the goal to characterise them nosologically and to develop further strategies towards their molecular clarification. Since these CM are very rare, it was crucial to perform archival searches in major large neuromuscular centres across the globe and to obtaining relevant clinical findings from patients and their families in preparation for this workshop. Moreover, the suitability of various investigative techniques and pathways to gene discovery was reviewed to apply them to rare CM within individual working groups that were formed amongst the participants of this consortium.

2. Background and available techniques

Angela Hübner (Dresden, Germany) presented a linkage facility for a screening of unclassified families with CM and other neuromuscular disorders, which is one of the service structures of the German Muscular Dystrophy network MD-NET.

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Within the last two decades the application of molecular genetic strategies has led to a delineation of subgroups of clinically indistinguishable neuromuscular disorders and disclosed marked disease overlap. The expanding number of molecularly defined NMDs requires new strategies to classify overlapping and clinical indistinguishable phenotypes. To date, more than 84 different gene loci have been linked to the different forms of muscular dystrophies (MD), CM, congenital myasthenic syndromes (CMS) and myotonias, and for many of them the responsible genes have been cloned and characterised. This has already led to a partial reclassification of NMD, now guided by genotypes rather than phenotypes. Determining the genotype for an individual patient has important implications for prognosis, management, genetic counselling and the potential for prenatal diagnosis. The knowledge about additional gene loci underlying neuromuscular diseases is rapidly increasing so that it becomes more and more difficult for physicians to meet the justified expectations of patients and relatives for professional counselling. Furthermore, the financial and methodological resources required to achieve a diagnosis have increased in a way that new and more economical diagnostic strategies are warranted.

Good examples for the validity of this approach are the genetically heterogeneous limb girdle muscular dystrophies (LGMD) or the myofibrillar myopathies (MFM) which present with a marked phenotypical overlap and are difficult to differentiate in early stages. The recent mapping and cloning of a number of novel genes involved in neuromuscular function such as the genes for Dok-7 (*DOK7*), cofilin 2 (*CFL2*) and amphiphysin 2 (*BINI*) have substantially widened our understanding of the pathogenesis of NMD. Mutations in muscle-specific transcription factors may modify the phenotype and hence become relevant in the molecular genetic diagnosis of MD and myopathies.

The MD-NET linkage facility at the Technical University Dresden has developed DNA microsatellite marker sets for 84 gene loci (including MD, MDC, CMS and ion channel muscle diseases) which is ready for use and can be applied for linkage and haplotype analyses in families with unclassified NMDs. This group represents the largest linkage centre specifically dedicated to neuromuscular diseases and has made a substantial contribution to the detection rate of disease-causing mutations in patients and families with yet unclassified NMDs. Quick and large scale linkage and haplotype analyses are provided for most of the currently known neuromuscular diseases. The facility provides a high level of flexibility to include novel genes and gene loci into the screening sets just within a couple of days. The method is based on family analyses of the inheritance of about six genetic markers (microsatellites) in close proximity to each of the candidate genes. Dependent on the size of the families, approximately 40–50 families per year have been investigated so far. This marker set has been proved highly effective for the narrowing of the number of differential diagnoses and for the characterisation of the molecular defect in informative families with extensively prediagnosed but yet unclassified NMDs. So far, 390 informative families were investigated and it was possible to reduce the number of candidate genes to one or two gene loci in 40% of the families, while in about 30% of all families the disease-causing mutation was subsequently identified by direct sequencing. Informative families in whom all known candidate genes are excluded have been transferred to a genome wide linkage scan and in one family the genome scan allowed the identification of a new candidate gene, filamin c (*FLNC*), for a novel myofibrillar myopathy. The method is

universally applicable and can be extended to other diseases with overlapping phenotypes. So far, the investigation is done on a collaborative basis (free of charge) as the project is supported by a grant of the German Federal Ministry of Education and Research (BMBF). In conjunction with this approach other modern genomic approaches were discussed as well, in particular the possibility of sequencing the entire or partial exome in a single patient. This approach would be greatly facilitated by prior haplotype analysis narrowing down the genomic regions to be covered, by affected and unaffected family members to compare discovered alterations, by independent clearly similarly affected patients with similar morphology who likely have the same disease and who therefore then also should have a mutation in the same genes suspected in the initial case; and by clear ideas about possible candidate genes, facilitated by careful morphological and immunohistochemical examination.

Caroline Sewry (London, UK) discussed specific aspects of immunohistochemistry that need to be considered when studying biopsies from patients within this group of disorders. Antibodies to numerous muscle proteins localizing to various subcellular regions, are widely available, however, immunohistochemistry likely will only be of value if the structures are numerous and already visible at the light microscopic level with histochemical techniques. Immunohistochemical reduction or absence of a protein caused by a primary gene defect in the gene encoding for the protein is well recognised in recessive disorders (e.g. dystrophin), and secondary abnormalities, such as a reduction, accumulation, or post-translational modification, are also of diagnostic importance. Accumulation of a primarily affected mutant protein can also occur (e.g. actin and desmin). It is not always known if the immunoreactivity of certain structures such as inclusion with a particular antibody accurately reflects the presence of the protein the antibody is supposed to recognise, or if non-specific binding to unusual structures could also occur. Laser capture of specific structures, followed by mass spectrometry could help answer this. Other aspects to consider when applying immunohistochemistry were also discussed. These included studies of interacting proteins, fibre type differences in the localisation of proteins (some structures under consideration are fibre type-specific), and studies of microtubules. The use of different types of microscopic techniques useful for the characterisation of histological phenomena was also discussed, in particular fluorescence methods, as some structures such as cylindrical spirals are autofluorescent. In addition, neither the menadione-NBT technique that stains reducing bodies, nor the stain for myoadenylate deaminase which stains tubular aggregates requires the use of the enzyme substrate and are thus useful additional stains to use in the full histological characterisation of unusual inclusions and bodies found in a muscle biopsy.

Carsten Bönnemann (Philadelphia, USA) described the laser microdissection mass spectrometry approach used by his laboratory in biopsies from two sporadic patients to identify FHL1 as the most prominent component of reducing bodies, leading to the identification of mutations in FHL1 as underlying the disorder. Reducing body myopathy is the first example for which this type of approach has been successful, potentially making it a model for analysis of other myopathies with prominent inclusions. Several features of reducing body myopathy made this an ideal situation for the laser microdissection mass spectroscopy approach to work: The inclusions are readily visible by light microscopy on

eosin staining as condensed material, even without the application of a cover slip and they were frequent and quite uniform. Laser microdissection in the reducing body analysis was done using a Zeiss microscope and P.A.L.M. catapult system on 10 micron frozen sections stained with eosin but not coverslipped. 3000–5000 “shots”, i.e. individual laser punches, were obtained from two sections and captured in 40 µl water, digested with trypsin after intermediary steps, and run on C18 reversed phase HPLC and submitted for mass spectrometry analysis first obtaining a full scan MS, followed by the acquisition of raw MS/MS spectra (tandem mass spectrometry), which were then assigned and annotated. Carsten Bonnemann’s lab has now also verified the technique on sections stained with modified Gomori trichrome, obtaining clean spectra for expected muscle proteins. It was also found that modifications of this stain that result in crosslinking of proteins will interfere with the mass spectrometry analysis. Further developments in the lab will be directed at decreasing the material needed by increasing sensitivity of the mass spectrometry, expanding the technique to other histochemical stains and to immunostained material, exploration of different proteases to capture trypsin resistant proteins, and to consider work on archival material.

Hans H. Goebel (Mainz, Germany) reported on the use of electron microscopy to facilitate direct gene analysis, which is possible in certain CM. Cytoplasmic bodies are the crucial morphological hallmark of cytoplasmic body myopathy and other protein aggregate myopathies. They consist of intermediate filaments, separated from a rather electron-dense amorphous core. Another morphological hallmark of protein aggregate myopathies is granulofilamentous material originally considered a morphological equivalent of a distinct type of CM, but now known to be less specific. The granular component of this material is more conspicuous, mixed with myofilaments and beneath the plasma membrane while filaments are often difficult to identify. Nevertheless, the typical components of intermediate filament aggregation, i.e. granular amorphous material and intermediate filaments, are present in such granulofilamentous material as well. Hence, it is of no surprise that certain patients with granulofilamentous material in their diseased muscle fibres have been found to carry heterozygous mutations in the desmin *DES* gene itself. Rosenthal fibres represent accumulation of granular amorphous electron-dense material and GFAP-positive intermediate filaments of astrocytes. Mutations in the *GFAP* gene, occurring in Alexander disease, are morphologically marked by innumerable Rosenthal fibres. While filamentous bodies consisting of actin filaments within muscle fibres are a non-specific feature in many different neuromuscular conditions, larger aggregates of actin filaments, are demonstrated by positivity to antibodies to sarcomeric actin at both the electron and light microscopic levels, are found in patients who have heterozygous mutations in the *ACTA1* gene. Nemaline myopathies are characterized by sarcoplasmic rods on the basis of possible mutations in genes of Z-band-related proteins such as sarcomeric actin (*ACTA1*), and tropomyosins 2 and 3 (*TPM 2* and *3*), and nebulin (*NEB*), while the marker protein of Z disks, alpha-actinin 2, has not been found to be mutant in nemaline myopathies. Moreover, a subgroup of nemaline myopathies is marked by the presence of intranuclear rods with or without sarcoplasmic rods. These intranuclear rods if not demonstrating a criss-cross pattern of Z-disks may be identified by antibodies against sarcomeric actin by light microscopy and at the ultrastructural level. So far, nemaline myopathies with intranuclear rods have almost

exclusively been demonstrated to show heterozygous dominant mutations in the *ACTA1* gene.

3. Rare structural congenital myopathies

Ana-Lia Taratuto (Buenos Aires, Argentina) reported on familial cylindrical spirals myopathy. Cylindrical spirals (CS) have been reported in a few sporadic cases, five of these were associated with myalgia and/or cramps, while two cases, belonging to one family, were associated with a myotonic disorder. Cylindrical spirals were the main pathological finding in muscle biopsies from a 72-year-old mother (case 1) and her 52-year-old son (case 2) who both exhibited myopathic facies and diffuse weakness of late onset [2]. At least 10 other family members spanning five generations were variously affected by muscular weakness, gait disorders, motor impairment, and/or scoliosis suggesting an autosomal-dominant trait with variable expression, although these additional family members had not undergone a muscle biopsy yet. In the two biopsies cylindrical spirals were observed in type-2 fibres as sub-sarcolemmal or intermyofibrillar clusters. They appeared bluish with haematoxylin, bright red with Gomori and strongly reactive for non-specific esterase and myoadenylate deaminase. Cylindrical spirals stained faintly with NADH-TR and were non-reactive for succinate dehydrogenase and myofibrillar ATPase. Immunostaining was negative for desmin, actin, and dystrophin. Electron microscopy revealed concentrically wrapped lamellae of 1–2 nm in diameter merging into tubular vesicular structures, some closely resembling tubular aggregates. Dilatation of adjacent lateral sacs suggests an origin of the cylindrical spirals from the sarcoplasmic reticulum. In an innervated muscle tissue culture obtained from case 2 cylindrical spirals were identified within lateral sacs [1,3]. A cousin of case 2 with three daughters and one son had been identified more recently, all of them clinically presenting facioscapulohumeral (FSH) involvement of variable severity. A muscle biopsy of the more severely affected daughter at the age of 26 years (case 3, 6th generation) also showed cylindrical spirals together with myopathic features and inflammatory infiltration [1]. Frozen tissue from this case is available for possible laser capture micro dissection. DNA studies (Marc Jean-Pierre Cochin/Paris, personal communication) performed in both parents and 3 out of the 4 siblings, showed in all of them a pathologic fragment of 5 D4Z4 repeat units (except the father who had more than 20) consistent with FSHD. After a 13-year follow-up of the clinical evolution in this family, a repeat haplotype analyses at the FSHD1A locus on chromosome 4q35.2 by Angela Hübner, Dresden/Germany involving mother and her children (paternal DNA was not available) showed that the affected proband female (Case 3, 6th generation) had a haplotype that was identical to that of her affected mother and affected siblings, thus supporting linkage to the FSHD locus. Interestingly, a biceps muscle biopsy (although other muscles were clinically more severely affected) of the affected male sibling showed dilatation of the sub-sarcolemmal sarcoplasmic reticulum as well as some vesicles and tubules suggestive of early CS formation, thus supporting the notion of a sarcoplasmic reticulum origin of the the spirals.

This family raises the enticing notion, that CS formation could be a direct result of the FSHD mutation. It was thus suggested to study other members of this large family from a morphologic as well as genetic point of view.

Janice Holton (London, UK) presented a review of tubular aggregates in skeletal muscle. Tubular aggregates were first described in hypokalaemic periodic paralysis and myotonia congenita. Subsequently four clinical syndromes have been described in which tubular aggregates are the major abnormality in the muscle biopsy: weakness with exercise-induced cramps, pain and stiffness; autosomal recessive limb girdle myasthenia with or without cardiomyopathy; progressive limb girdle weakness which may be sporadic or inherited with an autosomal dominant or recessive pattern; gyrate atrophy of the retina and choroid due to autosomal recessive inheritance of mutations in the gene encoding ornithine aminotransferase. In addition tubular aggregates may be observed in the context of hypokalaemic periodic paralysis, myotonia congenita, inflammatory myopathies, malignant hyperthermia, alcoholic myopathy, Whipple's disease and porphyria cutanea tarda [4]. A number of case reports have also been published describing tubular aggregates as a feature in other clinical syndromes. These include distal myopathy in association with multiple mitochondrial DNA mutations in which both ragged red fibres and fibres containing tubular aggregates were a pathological feature [5]. Cases associated with pupillary abnormalities suggest that smooth muscle dysfunction may occur in some cases in which tubular aggregates are present in skeletal muscle [6,7]. The characteristic morphological appearances of tubular aggregates were described and illustrated. In haematoxylin and eosin preparations tubular aggregates are represented by basophilic regions of varying size, often with an angular profile in the muscle fibre. These may have a sub-sarcolemmal location or be more centrally placed within the fibre. Tubular aggregates are typically bright red in the Gomori trichrome preparation, are darkly stained in the reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) histochemical reaction and also show strong activity for myoadenylate deaminase. They show no histochemical activity for cytochrome oxidase, succinic dehydrogenase or ATPase. In most cases tubular aggregates are found in type-2 fibres although they have been described in type-1 fibres in familial cases. Ultra-structural studies have demonstrated that tubular aggregates arise from the sarcoplasmic reticulum. In the most commonly observed form they are composed of regular arrays of tubules 50–70 nm in diameter with a smaller central tubule although a number of different forms have been described [8]. Immunohistochemical studies have confirmed the origin of tubular aggregates from the sarcoplasmic reticulum by the demonstration of a number of proteins derived from the sarcoplasmic reticulum within tubular aggregates [9]. Preliminary data from a review of the archive of the Division of Neuropathology, UCL Institute of Neurology were also presented. Examination of the database containing details of muscle biopsies performed from 1975 to 2009 identified 27 muscle biopsies from 24 cases in which tubular aggregates were recorded as a feature. The biopsies were reviewed and it was observed that there was a wide range in the number of affected fibres between different cases. Tubular aggregates were present in type-2 fibres in all cases examined. Future analysis will be undertaken to ascertain whether there is any correlation between the clinical syndrome and pathological features such as the proportion of affected fibres and the ultrastructural features of the tubular aggregates. In view of the rarity of tubular aggregates myopathy, a multi-centre approach may facilitate future studies.

Caroline Sewry (London, UK) presented data from the original case of zebra body myopathy published in 1975 by Lake and Wilson [10]. The clinical phenotype was consistent with a

congenital myopathy and she had suggested the *ACTA1* gene as a candidate gene after the observation that patients homozygous for null *ACTA1* mutations showed a higher number of zebra bodies than usually encountered in diseased muscle. In addition, the case of Lake and Wilson also showed nemaline rods, as did the only other reported case of zebra body myopathy [11]. Molecular studies of the Lake and Wilson case have now confirmed a novel *ACTA1* mutation. Thus zebra body myopathy is not a separate disease entity but is part of the nemaline, *ACTA1* spectrum.

She also presented a review of the literature on fingerprint body myopathy that was first published in 1972 by A G Engel [12]. All reported cases had hypotonia from birth, and most were sporadic cases, although a few familial cases suggest a possible genetic basis. The fingerprint bodies are only visible with electron microscopy and can occur in association with other pathologies, such as central cores, rimmed vacuoles and type-1 hypotrophy. They can also occur in association with various disorders and in normal foetal muscle. One paper suggested that they may be restricted to type-1 fibres, based on ultrastructural measurements of Z and M lines.

Kristl Claeys (Paris, France) reported four unrelated patients with a myopathy with hexagonally cross-linked tubular arrays [13,14]. Two patients had a familial history, one of which was suggestive of dominant inheritance, and two occurred sporadically. Age at onset varied between 13 and 56 years. Patients experienced exercise intolerance with exercise-induced muscle pain and weakness, without rhabdomyolysis. One patient additionally presented mild permanent pelvic girdle muscle weakness. Serum creatine kinase levels varied between normal and five times the normal value. Electromyography showed myopathic changes in one patient, and was normal in the others. Muscle imaging and respiratory function were normal. Minor cardiac abnormalities were found in two patients. The inclusions were eosinophilic and slightly refractile at haematoxylin and eosin, and bright red after modified Gomori trichrome staining. They were selectively present in type-2 fibres, with a frequency varying from 13% to 28% on transverse sections. The inclusions revealed immunoreactivity exclusively for the antibody directed against caveolin-3. Ultrastructurally, the inclusions showed a highly organised, hexagonally cross-linked crystalloid structure at transverse sections. Mutations in the caveolin-3 encoding gene were excluded. Biochemical assessment of glycogenolysis in muscle of two patients did not reveal any abnormality. This myopathy should be differentiated from a myopathy with tubulin-reactive crystalloid inclusions [15,16], and from nonspecific myopathic changes with crystalline aggregates of protein–glycogen complexes, also called virus-like particles [17,18]. We concluded that myopathy with hexagonally cross-linked crystalloid inclusions is associated with a homogeneous clinical and histopathological phenotype.

Joachim Schessl (Munich, Germany) reported on reducing body myopathy (RBM), a rare structural disorder of muscle, first described more than 35 years ago as a severe progressive myopathy in two girls [19], and subsequently in other sporadic and small familial occurrences. The typical muscle histopathological findings are intracytoplasmic inclusions staining strongly with the menadione-NBT stain. Working in Carsten Bonnemann's group at the Children's Hospital of Philadelphia he recently established the X-chromosomal four and a half LIM domain gene *FHL1* as the causative gene for RBM by using laser

microdissection of the inclusions out of biopsy material followed by proteomic analysis [20]. Twenty-one sporadic patients and eight familial cases with RBM have been documented in the literature so far, at this point mostly without molecular confirmation. All mutations underlying RBM have so far been located in the second LIM domain of FHL1. The mutations reported have been seen in patients with variable, but more often with severe disease symptoms. While females can be severely affected, males show even more severe symptoms compared to females carrying the same mutation. He also demonstrated in this meeting a family originally reported with reducing body myopathy with cytoplasmic bodies and prominent spinal rigidity [21]. In this family a novel *FHL1* LIM2 domain mutation (p.C150R) was detected as the underlying genetic cause for the disease in this family [22]. Cytoplasmic bodies and reducing bodies appear to coexist very frequently [23]. There are also reports on patients with milder forms of reducing body myopathy with mutations in the last half zinc finger at the end of the second LIM domain that may cause less severe disruption of the structure of the domain and so a less severe phenotype [23,24]. It was also pointed out, that many unsolved cases may be due to mutations in the FHL1 gene as often the important staining with menadione-NBT is not performed or a less severe muscle is biopsied. The authors expect the phenotype to broaden even further, shedding additional light on the role of FHL1 and aggregate formation in disorders of muscle.

Hans H. Goebel (Mainz, Germany) alerted the participants to a growing list of additional rare congenital myopathies based on the suitability of the two main investigative techniques to approach molecular identification of rare congenital myopathies: linkage/haplotype analysis and exome analysis, and laser capture microdissection with subsequent mass spectrometry analysis. These rare CM could not further be the object of this workshop's aims. They can be separated into those suitable for laser capture microdissection analysis because they presented with inclusions or circumscribed lesions recognisable at the light microscopic level and those defying laser capture microdissection analysis. Each of these rare congenital myopathies occurred in single patients or rarely in siblings, making the laser microdissection approach attractive for the suitable cases, since it has the potential to succeed in single cases.

4. Patient/tissue archives

Elena Pegoraro (Padova, Italy) had screened about 8000 consecutive muscle biopsies from the tissue bank of the Neuromuscular Unit of the University of Padova for patients showing tubular aggregates at muscle histopathology. Forty-one patients were selected: 33 sporadic cases and 8 familial cases belonging to two families (Table 1).

They then studied individuals from two large three-generation families from Northern Italy. In family # 1, 10 family members were clinically evaluated: 6 females and 4 males with ages ranging from 4 to 63 years. Only one patient was symptom-free and had normal neurological findings, but was very young (aged 4 years). Nine individuals complained of muscle weakness commencing in childhood (range: 5–13 years) and, at clinical presentation, they all showed symptoms of weakness such as difficulty in running, keeping up with peers, and climbing stairs. Progression of the disease was relatively slow in all patients, and all patients are still ambulatory at ages ranging between 4 and 63 years. The oldest patient (63 years of

age) uses a cane for walking. CK was significantly elevated (range between 1000 and 5000 U/l).

The pattern of muscle weakness was symmetrical and included proximal and, to a lesser extent, distal muscles both in the upper and lower limbs. In the upper limbs, the distribution of predominant muscle weakness included periscapular muscles resulting in mild winging of scapulae, deltoid, and triceps though rather sparing the biceps. The distal upper extremity muscles were also involved, with both finger extensor and flexor weakness. In the lower extremities, there was pelvic girdle weakness involving the hip flexors with sparing of the quadriceps, and the anterior compartment of the distal legs. Four patients complained of myalgia and cramps during effort and at rest.

In family # 2, seven family members in two generations were evaluated: 1 female and 6 males at an age ranging from 34 to 74 years. Three patients experienced onset of clinical symptoms in their 50s, including mild proximal weakness in the upper and lower limbs, while four patients were unaware of clinical symptoms. CK was mildly elevated up to 1000 U/l. The pattern of muscle weakness showed mild proximal muscle weakness in the upper and lower limbs. Muscle weakness in the lower limb included iliopsoas, and knee flexors. In the upper limb, weakness was greater in deltoid, triceps, and shoulder external rotators. Mild winging of the scapula and calf hypertrophy were present in 5 patients. No patients in families # 1 and # 2 showed facial weakness, cognitive impairment, respiratory or cardiac failure.

In both families, muscle histopathological examination showed the presence of multiple basophilic, sub-sarcolemmal, or centrally located collections of granular material. The granular material had the characteristics of tubular aggregates in showing a strong reaction on NADH-TR histochemistry, but reacting negative for SDH and remained unstained for ATPase. In family # 1, the aggregates occurred in type-2 fibres in 4 individuals and in type-1 fibres in a single patient. In family #2, the aggregates were more frequent in type-1 fibres. Moreover, in family # 2, multiple vacuoles in up to 40% of muscle fibres were observed. Electron microscopy was performed only in muscle tissues of family # 1 and showed a collection of single- and double-membraned tubules and single-membrane sacs containing electron-dense material.

Nigel Clarke (Sydney, Australia) presented three patients from the Children's Hospital at Westmead. The first was a young boy with delayed early motor milestones, moderate generalized muscle weakness, ptosis and progressive scoliosis associated with a *de novo* heterozygous *RYR1* mutation and central cores on oxidative stains of deltoid and quadriceps biopsies. In addition, electron microscopy showed extensive membrane-bound vacuolation of muscle fibres suggestive of sarcotubular myopathy. No abnormalities were found on sequencing of *TRIM32*. Therefore sarcotubular-like changes can be associated with mutation of *RYR1*.

He further presented two patients, a newborn and a 12-month-old child with crystalline inclusions similar to those reported by Kristl Claeys (Paris, France), and also seen by Steven Moore (Iowa City, USA). These two patients and their tissues will enlarge the repertoire of

cases to further elucidate the significance, origin, and genetic association of crystalline inclusions in crystalline body myopathy.

Caroline Sewry (London, UK) presented two sporadic cases from the UK with cylindrical spirals. The clinical phenotype of one case, identified by the neuropathologist in Bristol, suggested a metabolic disorder based on the clinical presentation but various biochemical investigations had proved negative. Some immunohistochemistry of the spirals had been performed but this had been limited by the autofluorescence of the structures. Peroxidase labelling, however, showed that they bound antibodies to SERCA1. The autofluorescence of other structures was discussed, including curvilinear bodies seen in Batten's disease, the granular material characteristic of vitamin E deficiency, and lipofuscin. The muscle biopsy of the second case, identified by colleagues in Birmingham, was also characterized by similar cylindrical spirals and some of these also appeared to merge with small aggregates of tubules, as reported in the literature.

Tuomo Polvikoski (Newcastle, UK) presented a family with hyperkalaemic periodic paralysis based on a most common point mutation T704N in the sodium channel gene *SCN4A* affecting the father, one son and a male grandchild. The son experienced pain and cramps in his limbs since the age of 12 years, such episodes usually lasting for some 15 min. Also since the age of 12 years, he had mild weakness of leg flexors and limb girdle muscles, and a CK value of 277 U/l. A biopsy revealed scattered atrophic type-2 fibres, a mild type-1 fibre predominance, and patches of increased NADH activity in some 10% of the muscle fibres which, by electron microscopy, turned out to be tubular aggregates composed of about 20–50 tubules, 200 at the most, located either internally or, peripherally in muscle fibres.

Martin Lammens (Nijmegen, The Netherlands) reported from his institutional archives a child with a new clinical presentation of hyaline body myopathy and a new mutation in *MYH7* was presented.

Furthermore, a young adult with a progressive proximal and later also distal muscle weakness and cramps was demonstrated. In consecutive muscle biopsies characteristic sarcoplasmic inclusions were found which on electron microscopy partly consisted of lysosomes resembling those found in vitamin E deficiency. It was concluded that the inclusions closely resembled findings in a hereditary sarcoplasmic body myopathy which is not yet genetically characterized [25]. Similar cases will be retrieved by other members of the workshop.

Three patients with a myopathy characterized by the presence of multiple tubular aggregates on muscle biopsies were also presented. In one patient aggregate of three types were present: aggregates consisting of tubules with homogenous dense material, almost empty tubules, and more granular tubules with filaments. The clinical presentations were very heterogenic with uveitis, familial mental retardation and cryoglobulinemia. In another patient a progressive loss to complete disappearance of tubular aggregates was seen in consecutive biopsies with at first biopsy having shown a classical picture of tubular aggregate myopathy. These observations emphasize the likely aetiological heterogeneity of tubular aggregates.

Dominique Figarella-Branger (Marseille, France) found that in her experience such unusual structured congenital myopathies are exceptionally rare. In Marseille, only 21 cases out of the 13,987 muscle biopsies performed during the last 35 years were on record (0.15%). These include 15 cases of tubular aggregate myopathy, 3 cases of fingerprint myopathy, 2 cases of crystalline inclusion body myopathy and one case of cylindrical spirals myopathy.

Among the group of tubular aggregate myopathy three different phenotypes were observed:

1. Progressive muscle weakness affecting predominantly the proximal muscles (4 cases, i.e. 2 males and 2 females). The onset was before the age of 15 years in all patients (mean age 6 years).
2. Three patients suffered from myalgia, with a CK value (available in 3 patients) elevated in one. In addition one patient suffered from cardiomyopathy and another from mental retardation [26]. Exercise-induced cramps, pain and stiffness were present in 5 cases, all males, mean age of onset 45 years.
3. Clinically asymptomatic patients with presentations related to malignant hyperthermia (6 cases, all males). Among these, two patients had exertional heat stroke, and an abnormal in vitro contracture test (IVCT), was recorded in one of them. The three other patients (father and two sons) demonstrated elevated CK levels, and the IVCT, performed in two of them, was abnormal.

The number of fibres affected by tubular aggregates varied from one case to another. Although tubular aggregates occur largely in type-2 muscle fibres, some type-1 fibres were affected in all cases.

Clinical features of the three sporadic patients presenting with a fingerprint body myopathy at muscle biopsy were heterogeneous. Fingerprint body myopathy was observed in a 72-year-old male suffering from pelvic weakness and bent spine syndrome, in an 8-year-old girl suffering from difficulties in walking until the age of 7 years and in a 22-year-old male presenting in infancy clinical features mimicking spinal muscular atrophy. IVCT was performed in this third case and was abnormal [27].

In addition to typical fingerprint bodies, an abnormal intermyofibrillar network with features of multiminicores was recorded in two patients.

Hexagonally cross linked tubular arrays were recorded in two patients. The first one, a 38-year-old woman, presented with ataxia and gait disturbances for 3 years. CK level was normal. The other, a 57-year-old woman, suffered from muscle weakness and exertional myalgia. In both cases, inclusions selectively affected type-2 muscle fibres.

Cylindrical spirals myopathy was observed in a 52-year-old male presenting with myalgia, diffuse weakness and increased CK level. His brother also suffered from myalgia and fatigue; however, a muscle biopsy was not performed in him.

Enrico Bertini (Rome, Italy) presented three patients affected by heterogeneous myopathies in which the morphological common and characteristic feature was the proliferation of rimmed vacuoles in the muscle biopsy.

In all patients acid maltase deficiency was excluded biochemically in the muscle biopsy, and LAMP2 deficiency was ruled out by immunofluorescence. Moreover mutations in *VMA21*, responsible for XMEA were excluded by collaboration with Berge A. Minassian, Toronto, Canada.

These 3 sporadic patients showed peculiar and different clinical features and had a limb-girdle distribution of weakness rather than signs of distal myopathy, which is known to be frequently associated with proliferation of rimmed vacuoles [28].

The first patient is a now 4-year-old girl. She presented with neonatal hypotonia with swallowing and sucking difficulties, and was born to healthy non-consanguineous parents. She developed respiratory failure and needed a tracheotomy at the age of 2 years. She was able to sit alone at the age of 1 year and has never been able to walk unaccompanied, but was only able to stand with support after the age of 18 months. CK has always been elevated (600–800 IU/l). Muscle biopsy showed a massive proliferation of rimmed vacuoles that were mostly negative for acid phosphatase and for LAMP2 antibodies by immunofluorescence. Histochemistry and biochemistry for myophosphorylase were reduced, while electron microscopy showed marked proliferation of concentric laminated myelin-like bodies suggesting autophagic bodies. Molecular genetics analysis excluded mutations in *TRIM32* and *PYGM*. This condition appears to be a peculiar form of congenital myopathy characterized by a massive proliferation of rimmed vacuoles. A similar patient has been published by Goebel and coworkers in 1986 [29]. Remarkably we could not observe any typical aspects of autophagic vacuoles in the muscle biopsy of this patient.

The second patient is now 10 years old and presented with progressive limb girdle muscle weakness from the age of 6 years. At the age of 8 years the neurological examination showed marked proximal weakness of the lower limbs (MRC = 3) and of upper limbs (biceps brachii and deltoid MRC = 4). Serum CK was elevated persistently at around 4000. Serum acylcarnitines and urine organic acids were normal.

The muscle biopsy showed numerous fibres with glycogen storage and vacuoles that stained positive for acid phosphatase and non-specific esterase. Ultrastructural examination confirmed the presence of big vacuoles circled by a membrane with glycogen and organelle storage suggesting autophagic vacuoles. Acid maltase activity in muscle was normal and molecular genetic analysis for *VMA21* mutations was negative. The child had marked clinical improvement with Coenzyme Q10 supplementation, 400 mg/daily. Coenzyme Q10 in muscle was at the lower range of normal: 23 mmol CoQ10/g muscle. A candidate gene approach for genes along the pathway of coenzyme Q10 biogenesis is on the way to detect the gene responsible for this peculiar condition. Activation of autophagy has been reported in conditions with a defect of coenzyme Q10 biogenesis [30].

Finally the third patient, a 9-year-old boy, started at the age of 4 years with difficulty in jumping and a waddling gait. Serum CK was normal. The muscle biopsy demonstrated the

presence of some hypertrophic myofibres surrounded by sub sarcolemmal vacuoles positive for acid phosphatase reaction. Acid maltase activity and mitochondrial respiratory chain activities on muscle homogenate were normal. Glycolytic enzymes in the skeletal muscle were biochemically normal.

A brain MRI at the age of 8 years demonstrated bilateral and diffuse hyperintensity of the white matter in T2-images. The child had no mental retardation and no clinical signs of central nervous system involvement. Leukocyte activity for α -galactocerebrosidase and arylsulfatase was normal and LAMP2 deficiency was ruled out by immunofluorescence in muscle. Molecular genetics for *VMA21* yielded no mutations.

Montse Olivé (Barcelona, Spain) presented a summary of clinical and pathology findings in two patients studied at the Institute of Neuropathology in Barcelona, Spain.

The first one was a 32-year-old woman recently diagnosed with cylindrical spirals myopathy. She was the second child of healthy unrelated parents, born after an uneventful pregnancy by normal delivery. No other members of the family were affected by the disease. Her motor milestones were mildly delayed and she presented with a very slowly progressing proximal and distal muscle weakness during infancy. She did not complain of cramps or myalgia. Over the following years she developed a progressive scoliosis and restrictive ventilatory insufficiency requiring nocturnal non-invasive ventilatory support. On examination, the patient had short stature, short-neck, bilateral pes cavus, prominent scoliosis and distal joint hypermobility. There was mild muscle weakness in four limbs. The facial and extraocular muscles were spared. Muscle CT scan of pelvis and lower extremities revealed mild involvement of gluteus maximus, as well as posterior thigh and leg muscles. CK levels were within normal levels, an EMG examination was consistent with a myopathy.

Muscle biopsy showed variation of fibre size, large numbers of internal nuclei, and moderate endomysial fibrosis. Several fibres contained small faintly basophilic inclusions that stained bright red in the modified trichrome preparations. They were located under the sarcolemma, near the myo-nuclei. On NADH and SDH reactions, some fibres showed central areas partially devoid of oxidative enzyme activity. ATPase reaction and myosin immunohistochemistry revealed normal fibre type distribution. By electron microscopy the inclusions were revealed to be cylindrical spirals. They were clustered in the sub sarcolemmal space and only occasionally they were seen in close proximity to tubular aggregates. The sarcomere structure was well preserved except in some fibres that showed streaming and widening of Z-lines. The causative gene is not known.

The second patient was a 56-year-old man who presented at the age of 12 years with increasing dyspnea on exertion and was subsequently diagnosed with nonobstructive hypertrophic cardiomyopathy. He later developed slowly progressive weakness of arms and legs. One of his sisters, born from his father's first marriage, died at the age of 40 years from cardiac failure due to a hypertrophic cardiomyopathy, with no apparent muscle weakness. Clinical examination revealed hypertrophy of some limb muscles, marked atrophy of paraspinous muscles, winging of the scapulae, proximal weakness of the four limbs and rigidity of the spine. Muscle biopsy showed sub sarcolemmal structures in virtually all

type-1 fibres. They were eosinophilic on HE stain, faint green with modified trichrome, and they displayed ATPase but not oxidative enzyme activity. Additionally, some fibres contained collections of very small rod bodies in the cytoplasm or within the inclusions. Using a panel of antibodies they showed strong reactivity for slow myosin, myomesin, M-protein, MuRF3 and faint reactivity for FHL1 but absent immunoreactivity for α -actinin, telethonin, actin, tropomyosin, desmin, α B-crystallin, dystrophin, filamin C, myotilin or ubiquitin. On electron microscopy the structures corresponded to areas of disorganised myofibrils that were mainly composed of thick filaments. Some fibres showed aberrantly oriented myofibrils which otherwise consisted of fragments of sarcomeres with preserved A band and M line, but absent I band and Z lines. Collections of small rods were seen in some disorganised myofibrils. The molecular basis of the disease is currently under investigation. So far, mutations in MYH7 and FHL1 genes have been excluded.

Ikuya Nonaka (Tokyo, Japan) reported on a congenital myopathy with extreme muscle fibre immaturity.

Although muscle fibre immaturity can be seen in congenital myopathies, especially in the severe infantile forms, there are some patients with clinical features of a congenital myopathy with no characteristic diagnostic morphologic abnormalities or selective type-1 fibre atrophy but with striking muscle fibre immaturity as the only defining feature. We tentatively refer to this condition as “congenital myopathy with extreme muscle fibre immaturity”. The diagnostic criteria of this disease include (1) floppy infant with developmental delay with the characteristic clinical features of a congenital myopathy with facial muscle involvement, (2) absence of intracytoplasmic abnormalities such as nemaline bodies, cores, central nuclei, or peripheral halo structures, (3) no selective type-1 fibre atrophy, (4) presence of extreme muscle fibre immaturity; reflected by numerous small fibres with prominent nuclei but with scant cytoplasm and poorly organised myofibrils and (5) presence of many atrophic fibres with positive anti-neonatal myosin antibodies.

1. Clinical characteristics: six patients fulfilled the diagnostic criteria of this disorder. The clinical symptoms are summarized in Table 2. All patients had difficulty breathing at birth and 4 required respiratory support. All had generalized muscle weakness with marked facial involvement with high arched-palate. Two patients had joint contractures at birth. Except for patient 1, all had progressive muscle weakness leading to early death in 2 patients.
2. Morphologic features: the most striking finding was the presence of numerous small fibres with prominent nuclei with scant cytoplasm. The findings varied from fascicle to fascicle. In some fascicles there was intermingling of normal to hypertrophic type-1 fibres, but in most fascicles there were extremely small fibres embedded in dense connective tissue. There were no typical nemaline bodies on modified Gomori trichrome stain. On electron microscopy, the fibres measured 3–5 microns and the myofibrils were poorly organised, occasionally forming small rod-like structures. Satellite cells were not increased in number. Interestingly, there were numerous remnants of the basal lamina.

3. Molecular genetic analysis: We have examined the ACTA1, MTM1, TPM3 and cofilin (CFL2) genes. Patient 3 was found to have a mutation in the ACTA1 gene.

Congenital myopathy with extreme muscle immaturity probably represents a group of heterogeneous disorders because only one of our patients had a mutation in *ACTA1* gene whereas the others were negative for *ACTA1* mutations. The patient who was found to carry an *ACTA1* gene mutation, however, displayed no typical nemaline bodies or actin aggregates on electron microscopy. Unlike congenital myotonic dystrophy which can also show striking muscle fibre immaturity, there was no increase in the number of satellite cells suggesting that there was no defect in myotube formation. Since the myofibrils were not organised into band structures or showed neonatal myosin expression, there appears to be no doubt that these muscle fibres were immature and defective in myofibrillar organization. However, numerous remnants of basement membrane on electron microscopy indicate that the muscle fibres had once attained a certain size and then become atrophic leaving only remnants of the basal lamina. Such findings for instance are frequently seen in Werdnig–Hoffmann disease. Etiologic analysis in the *ACTA1* negative cases is ongoing.

Steven Moore (Iowa City, USA) searched the diagnosis field from an archive of approximately 3000 muscle biopsy records at the University of Iowa. Nine cases of “myopathy with tubular aggregates” were identified. Glass slides were available for review on eight of these cases and frozen tissue was available of the same eight cases. Clinical data were limited to information submitted on biopsy requisitions. All the cases were adults, and only one case was from a female patient. This female patient represented the only example of familial disease, in that a family history of probable autosomal dominant LGMD was present. This particular muscle biopsy had tubular aggregates in a relatively small percentage of fibres. The remaining seven cases were all from males with clinical histories of myalgia and mild to moderately elevated CK; clinical indication for all these biopsies was to look for myositis. Among these biopsies, two had tubular aggregates in a relatively small percentage of fibres, while five had aggregates in numerous fibres. The morphology and enzyme histochemistry of the aggregates in cryosections did not vary among the cases reviewed. None of the cases had evidence of myositis.

Steven Moore also presented a case of neonatal hypotonia with lactic acidosis in which ultrastructural pathology included dilated membranous structures, perhaps sarcoplasmic reticulum, and numerous rod shaped, electron dense, crystalline inclusions. The inclusions were comprised entirely of well-ordered granular material, 15–18 nm in diameter spaced at ~20 nm intervals forming a lattice. No tubular or filamentous structures were identified in the inclusions. A small number of glycogen granules decorated the periphery of each inclusion. Inclusions were found in sub sarcolemmal regions and between sarcomeres. No clusters of inclusions were observed.

Ana-Lia Taratuto (Buenos Aires, Argentina) reported on a congenital myopathy with abnormal nuclei. A 39-week gestation newborn female developed respiratory distress and required nasal O₂ for 10 days. Oedema from hips down involving lower limbs developed while cardiac and renal involvement were ruled out and ultrasound heart and brain studies

were normal as was brain MRI. Her motor milestones were quite delayed; she was able to maintain a sitting position at 8 months of age. She showed a torticollis-like position of the neck to the left at the age of 10 months. At 22 months she was able to crawl, get to standing and walk with support. Creatine kinase was 1037 UI/l units when 1 year old and 759 UI/l when 3 years old. Her muscle weakness did not increase, and she is now able to walk with support. A muscle biopsy performed at the age of 17 months showed rounded transverse muscle fibres with variation in fibre size within the same fascicles with atrophy and hypertrophy of both type-1 and type-2 fibres, slight endomysial and perimysial fibrosis, and a chequerboard pattern of both fibre types with ATPase. Immunohistochemistry for dystrophin, sarcoglycans, laminin alpha 2 and alpha-dystroglycan was normal. The myonuclei were enlarged, hyperchromatic, and extremely abnormal, while the myofibrils seemed not to reach the periphery of the fibre in some areas. On electron microscopy, nuclei had condensed chromatin, some of them were surrounded by a felt-like rim and there was also nuclear fragmentation. Tubular-profile pseudoinclusions were very prominent in some of the nuclei. Due to some resemblance to myonuclear degeneration in LMNA null mice [31], lamin A/C DNA studies are in progress at Pitié Salpêtrière/Paris. If negative, nesprin has also been proposed as a candidate gene at this ENMC workshop.

Carsten Bönnemann (Philadelphia, USA) reported on other cases referred for the laser microdissection approach. Most interesting and suitable amongst these was a family referred by Dr. Menachem Sadeh (Wolfson Medical Center, Holon, Israel). The family included two adult brothers with cerebellar ataxia, optic atrophy, cataract, and cardiomyopathy leading to lethal heart failure. Muscle biopsy revealed abundant and large sub sarcolemmal accumulations of material that was eosinophilic red on haematoxylin and eosin, and dark blue green on modified Gomori trichrome, but remained unstained using NADH, PAS, Sudan black, ATPase, Congo red, desmin, alpha actinin and heavy-chain myosin. On electron microscopy the accumulations contained masses of parallel filaments, not unlike Hirano bodies. These inclusions are an excellent candidate to the laser microdissection approach because of their visibility on H&E and modified Gomori trichrome stains, their abundance and their apparent uniformity.

Janbernd Kirschner (Freiburg, Germany) screened 4170 biopsy specimens and presented two patients, one with congenital muscle weakness and contractures, and a muscle biopsy tissue specimen which contained unidentified material. He further communicated data on a 4-year-old child whose muscle specimen contained unidentified “idiopathic” inclusions.

5. Conclusions and future perspectives

The workshop concluded that there are a number of additional myopathies with characteristic morphological findings that should be amendable to contemporary gene identification strategies. Complicating such efforts are the facts that these cases are rare, often sporadic and that there is compelling evidence that most of the possible entities discussed in this workshop likely are genetically heterogeneous. The molecular tools at disposition to solve these cases include: (1) haplotype analysis in informative families to exclude known loci or if the family is large enough or of suitable genetic structure to establish linkage to a novel locus. This will be particularly successful in consanguineous

families in which homozygosity by descent mapping it feasible, requiring much smaller family sizes. (2) Whole or partial exome sequencing in sporadic patients. This approach would be greatly facilitated or indeed only made possible by the conditions mentioned earlier in this report (3) laser capture microdissection followed by proteomic analysis. This approach is feasible in biopsies with inclusions visible on light microscopy in non-coverslipped slides that are of reasonable homogeneity and frequency. This analysis could help in pointing to the actual disease gene if the mutant protein is the major one accumulating, or it could help in generating candidate genes or pathways that could be useful in supplementing information from the first and second approach. Expression profiling of the biopsy has the potential of being helpful also, in particular in cases in which there may be total loss of the transcript of the mutated gene, and also in establishing a hypothesis about possible disease pathway that then could inform the candidate selection for the first two approaches.

The consortium felt that gene identification even in very rare disorders is of great importance as the phenotypic and morphological spectrum of any condition is bound to expand, and also because rare disease genes frequently are very informative for the understanding and treatment of more common disorders of muscle.

To facilitate further characterisation of these conditions as well as gene discovery within them, it was decided to form subinterest groups within the consortium focusing on the major entities identified as promising for further investigation:

- Crystalline bodies: Kristl Claeys;
- Cylindrical spirals: Ana-Lia Taratuto;
- Fingerprints: Dominique Figarella-Branger;
- Tubular aggregates: Janice Holton und Elena Pegoraro;
- “Immature fibre” myopathy: Ikuya Nonaka;
- Sarcoplasmic bodies: Martin Lammens;
- Excessive autophagic vacuoles: Enrico Bertini;
- M-line caps – Montse Olivé;
- “New” myopathies: Hans-Hilmar Goebel.

Researchers with patients or families showing these appropriate morphologies are encouraged to contact the appropriate subinterest group.

6. List of participants

- Bertini, Enrico (Rom, Italy);
- Bönnemann, Carsten G. (Philadelphia and Bethesda, USA);
- Boersen, Annette (ENMC, Baarn, The Netherlands);
- Claeys, Kristl (Paris, France and Aachen, Germany);

Clarke, Nigel (Sydney, Australia);
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 Sewry, Caroline (London, UK);
 Taratuto, Ana Lia (Buenos Aires, Argentina).

(Complete addresses of the individual workshop participants are available from the ENMC workshop organisers.)

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Table 1

Tubular aggregates in muscle specimens from Padova/Italy.

Clinical diagnosis	Histopathological diagnosis	Number of cases
Familial tubular aggregates myopathy	Myopathy	8 (2 families)
Periodic paralysis	Vacuolar myopathy	10
Myotonic dystrophy	Myopathy	1
LGMD-myasthenia	Myopathy	1
ALS	Myopathy	1
Malignant hyperthermia	Vacuolar myopathy	1
Myopathy	Myopathy	16
Myopathy	Vacuolar myopathy	3

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Table 2

Clinical summary.

	Age/sex	Respiratory failure	Respirator	Joint contractures	Facial involvement	Head contractures	Outcome
1	5 m/F	+	-	-	+	-	improved
2	1 m/F	+	+	knee, hip, ankle	+	-	died 40d
3*	6 m/M	+	-	±	+	-	?
4	8 m/F	+	+	-	+	-	?
5	3 m/F	+	+	-	+	-	died 3 m
6	5 m/M	+	+	multiple	+	-	bed-ridden, on a respirator, (3 yrs. old)

* : Mutation in *ACTA1* gene.