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Workshop report

164th ENMC International workshop: 6th workshop on centronuclear (myotubular) myopathies, 16–18th January 2009, Naarden, The Netherlands

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1. Introduction and overview

17 clinicians and basic scientists from 11 centres in France, the UK and the USA convened from the 16th to the 18th of January 2008 in Naarden, The Netherlands, for the 164th ENMC sponsored Workshop on centronuclear/myotubular myopathies (CNM/MTM); in addition, the workshop was also attended by Anne Lennox and Melanie Spring as representative of the Myotubular Trust, a European patient support group for patients affected by myotubular (centronuclear) myopathies.

Following welcomes and introduction from Annette Boersen, ENMC representative and the chairpersons of the workshop, *Heinz Jungbluth* (London, UK) (on behalf of Carina *Wallgren-Pettersson*, Helsinki, Finland) gave an overview (for review, [1]) over the centronuclear/myotubular myopathies and developments in the field since the most recent, fifth ENMC workshop dedicated to these conditions held in September 2003 [2]. Whilst mutations in the myotubularin (*MTM1*) gene on chromosome Xq28 have been known to cause X-linked myotubular myopathy since the mid 90s [3], more recent years have seen at least partial genetic resolution of the autosomal forms of centronuclear myopathy following identification of mutations in genes encoding functionally-related proteins, the dynamin 2 (*DNM2*) gene on chromosome 19p13.2 and the amphiphysin 2 (*BIN1*) gene on chromosome 2q14. Dominant *DNM2* mutations were initially identified in a number of families with a mild form of CNM [4] but may also give rise to a severe form with neonatal onset if they involve the pleckstrin homology (PH) domain of the dynamin 2 protein [5]. Recessive homozygous missense or truncating *BIN1* mutations associated with an early-onset form of CNM have been identified in three families to date [6] but the frequency of this form is currently unknown. In addition, identification of a mutation in the skeletal muscle ryanodine receptor (*RYR1*) gene in one patient [7] and inactivating variants in the novel phosphoinositide phosphatase hJumpy in two patients [8] as well as a large number of patients without known molecular defect suggest further genetic heterogeneity.

The aim of the present workshop was therefore to establish clearer phenotype–genotype correlations for known forms of centronuclear myopathy, to identify common features of CNM without confirmed genetic defects, to characterize the function of the proteins implicated in various forms of CNM, to review animal models of the condition and to discuss potential therapeutic approaches. In addition, one session focussed on currently available mutational databases for the various genes implicated in CNM and a proposal for establishing a patient registry for CNM/MTM with a view to future therapeutic trials.

2. Genotype–phenotype correlations for known centronuclear myopathies

Caroline Sewry (Oswestry, UK) summarized the pathological features seen in neonates with X-linked myotubular myopathy due to myotubularin *(MTM1)* mutations.

The typical pathological feature in these patients is a variable number of large central nuclei but peripheral nuclei may also be present. In longitudinal sections the nuclei are regularly spaced down the length of the fibre (not in chains) and often appear in parallel in adjacent fibres. Multiple internal nuclei in cross sections are not a feature of *MTM1*-related myotubular myopathy and the distinction between central and internal nuclei is therefore of diagnostic importance. Necrosis is not a feature of X-linked myotubular myopathy and fibrosis is rare. Fibre size variation can give the impression of two populations of fibres as the smaller fibres are

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often type 1 and may be predominant, and the larger fibres are type 2; this is, however, not universal and fibre size variation may not be marked and can affect both fibre types. There are several different abnormal patterns of mitochondrial distribution, but radial strands commonly associated with *DNM2*-related centronuclear myopathy are not a feature of the *MTM1*-related form. A characteristic feature is the central accumulation of mitochondria and glycogen. Mitochondria may also accumulate in a focal loop-like manner which has been described by Dr Norma Romero as a 'necklace' (see paragraph below); this may be a particular feature of older cases but can also occur in neonates. Although a feature of *MTM1*-related cases necklace fibres are not specific and have occasionally been observed in association with other gene mutations. Pale peripheral halos representing peripheral zones containing myofibrils but no mitochondria are also found in many fibres. Holes which are devoid of all organelles but are not membrane bound occur in several fibres and core-like areas devoid of mitochondria may also be observed.

Immunolabelling of myosins shows that fibres with central nuclei may have fast or slow myosin. Neonatal myosin is not present in all fibres with central nuclei indicating that maturation occurs; similarly the presence of desmin and vimentin in fibres with central nuclei is not a universal feature.

The importance of excluding congenital myotonic dystrophy *(DM1)*, pathologically very similar to X-linked myotubular myopathy, is well known. Caroline Sewry confirmed published findings of a reduced number of fibres with slow myosin in cases of congenital myotonic dystrophy, and in addition showed that these can also be distinguished from X-linked myotubular myopathy with antibodies to muscleblind 1 isoform which bind to the triplet repeat expansion found in *DM1*.

Norma Beatriz Romero (Paris, France) summarized the morphological data of muscles biopsies from centronuclear myopathy (CNM) patients with and without known genetic defect. Her data were based on a large series of more than a hundred biopsies with a diagnosis of CNM reviewed at Institute of Myologie, Paris, before the *DNM2* gene as a cause of CNM had been identified. After the identification of *DNM2* mutations as the cause of the classical, severe and intermediate forms of autosomal dominant CNM [4–5,9] patients with CNM could be classified in two groups: those with *DNM2* mutations and those in whom such mutations were excluded (there were about 50% patients in each group). A triad of morphological abnormalities was consistently and almost exclusively observed in CNM *DNM2*-mutated patients and consisted in: (a) typical aspects of radiating sarcoplasmic strands, (b) significant nuclear centralization and c) type 1 muscle predominance.

Additional structural alterations within muscular fibres could be a useful criterion for suggesting or discarding *DNM2*-related CNM. Amongst the CNM patients unrelated to *DNM2*, at least five different subgroups could be identified based on muscle biopsies: (1) CNM characterized by the presence of "necklace" fibres; (2) Congenital myopathy with prominent nuclear internalisation and large areas of myofibrillar disorganization; (3) CNM having sarcomeric central disorganization with "rimmed" subsarcolemma; (4) CNM characterized by the presence of "core-targetoid" fibres; (5) CNM without any additional morphological abnormalities. In addition, CNM patients with mutations in the amphiphysin 2 (*BIN1)* gene still constitute a small group for which it was not yet possible to define a morphological pattern. "Necklace" fibres in muscular biopsies were observed only in sporadic late-onset *MTM1*-related centronuclear myopathy and characterized by a basophilic ring deposit, evenly spaced 3 μm beneath the sarcolemma, in which myonuclei were aligned, and which was clearly visible with routine histological stains. Ultrastructurally, these necklaces consisted of myofibrils of smaller diameter, in oblique orientation, surrounded by mitochondria, sarcoplasmic reticulum (SR) and glycogen granules. Immunolabeling of necklaces was positive for the SR proteins SERCA1 and 2, and the intermediate filament proteins, αβ-crystalline and desmin. These peculiar fibres should be considered as a helpful morphological indicator of adult *MTM1*-related patients [10] but are not specific. Congenital myopathy with prominent nuclear internalisation and large areas of myofibrillar disorganization were characterized by a significant nuclear centralization or internalisation, type 1 predominance or uniformity and in many fibres "pale zones" corresponding to a disorganization of the normal structure, devoid of oxidative activity and ATPase reaction; about 50% of the patients in this group had recessive forms linked to mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene. This emphasises a conclusion from the previous workshop that the *RYR1*gene has to be considered in cases with central nuclei. Sarcomeric central disorganization with "rimmed" subsarcolemma was observed in muscular biopsies from a few CNM patients, and "core-targetoid" fibres were seen in another small group of CNM patients. In these two groups, molecular genetics analysis excluded mutations of both *RYR1* and *SEPN1* genes. Finally, no gene mutation has been identified so far in the last group comprising CNM without additional morphological abnormalities. In conclusion, according to these studies, proper consideration of morphological criteria is very helpful to focus molecular analysis of the different CNM subgroups.

Meriel McEntagart (London, UK) summarized her previously published observations on genotype–phenotype correlations in X-linked myotubular myopathy [11].

X-linked myotubular myopathy is a severe congenital myopathy that presents in the neonatal period with profound hypotonia and an inability to establish spontaneous respiration. Usually death occurs in infancy from respiratory failure. However, there is phenotypic variability, as a number of affected boys have achieved respiratory independence and have become ambulatory. Disease-causing mutations have been identified throughout the *MTM1* gene on Xq28. The main objectives of this study were to establish whether the nature or site of the mutation in the *MTM1* gene could predict severity of the disease and to investigate whether early intensive clinical intervention facilitated survival until spontaneous improvement occurred. An association was demonstrated between the presence of a non-truncating *MTM1* mutation and the mild phenotype. However, many non-truncating mutations were also seen in association with the severe phenotype and these were not confined to recognized functional domains of the protein. This suggests that the use of mutation analysis to predict prognosis in the early period following diagnosis is limited. Of those boys ventilated at birth only 6.9% later established respiratory independence; in contrast, 52% of boys who did not require ventilation at birth maintained respiratory independence. The data confirmed that survival in X-linked myotubular myopathy is dependent on intensive medical intervention in the majority of cases.

Pascale Guicheney (Paris, France) presented an overview over genotype–phenotype correlations in Dynamin 2 *(DNM2)*-related centronuclear myopathy.

DNM2 mutations cause autosomal dominant centronuclear myopathy, a congenital myopathy characterized by limb muscle weakness, ptosis, ophthalmoplegia, and centrally located nuclei in muscle fibres, as well as dominant intermediate and axonal forms of Charcot-Marie-Tooth disease (CMT), a peripheral neuropathy.

Pascale Guicheney's group identified 14 CNM-related *DNM2* mutations in 3 of the 5 functional domains of the protein: 5 concerning the middle domain (MD), 7 within the Pleckstrin Homology domain (PHD), where most of the CMT mutations occurred, and 2 in GTPase effector domain (GED). Of their 95 genotyped CNM patients, 85% presented with the mild late-onset form without loss of ambulation, and most of them harboured one of the 3 MD mutations (p.R369W, p.R369Q, p.R465W) but mutations in other domains rarely caused a similar phenotype. A more severe

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form with neonatal onset and restrictive respiratory syndrome was found to be caused by *de novo* mutations, occurring in the C-terminus of the PHD at specific residues (A618, S619, V625), or in the MD domain (E368). An absence of typical muscle morphological features of CNM was found in the biopsy of a CMT2 patient carrying the p.K559deletion; this clearly suggests distinct morphological phenotypes between CNM and CMT, which could result from disruption of specific protein interactions in muscle and nerve. The typical radial strands of *DNM2*-related cases may be an age-related feature as they were not present in neonatal cases.

These data highlight the clinical and morphological heterogeneity of *DNM2* mutations and indicate some emerging phenotype–genotype correlations, which may be helpful for genetic counselling.

Jocelyn Laporte (Illkirch, France) reviewed the relationship between centronuclear myopathy and the Myotubularin–Amphiphysin–Dynamin (MAD) pathway. These three proteins are known to be implicated in membrane trafficking: myotubularin is a phosphatase that potentially regulates the level of specific phosphoinositides at discrete membrane domains, amphiphysin 2 can induce membrane bending and remodelling, and dynamin 2 is implicated in membrane tubulation and fission during endocytosis. Jocelyn Laporte's group reported a number of novel families with mutations in *BIN1* and *DNM2*, and preliminary data on their impact on the functions of these proteins in membrane remodelling and trafficking. 21 novel families with *DNM2* mutations were identified, confirming that amino acid residues 368, 369, 465 and 619 are mutation hot spots. A novel mutation in the PH domain was also reported in 5 unrelated families. Taken together, variability of onset, severity and muscle groups involved was noted depending on the underlying mutation: Several cases with neonatal onset were mostly associated with the p.E368K, p.A618T and p.619W substitutions, while the other mutation hot spots in the middle domain (p.R369W, p.R369Q, p.R465W) were associated with a milder phenotype with later onset. Concerning autosomal recessive CNM, two novel homozygous mutations were reported in *BIN1* in unrelated patients from consanguineous families with childhood onset. These mutations impact on either the membrane tubulation properties of amphiphysin 2, or on the recruitment of dynamin 2 to these tubules, as studied in cells overexpressing these proteins. These data indicate possible genotype–phenotype correlations between the severity and onset of autosomal dominant CNM and suggest that membrane remodelling and trafficking functions of amphiphysin 2 and dynamin 2 are affected in these diseases.

Heinz Jungbluth (London, UK) presented data on centronuclear myopathy related to mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene. *RYR1* mutations have been implicated in a wide range of phenotypes including the malignant hyperthermia susceptibility (MHS) trait, Central Core Disease (CCD) and Multiminicore Disease (MmD). The presence of central and internal nuclei and core-like areas as pathological findings in patients with MmD and ophthalmoplegia suggested the *RYR1* gene as a candidate also for CNM. This was subsequently confirmed by identification of a *de novo* heterozygous *RYR1* missense mutation (c.12335C>T; Ser4112Leu) in a girl with clinical and predominant pathological features of CNM on the initial muscle biopsy [7]. Additional clues to *RYR1* involvement in this patient were provided by the finding of external ophthalmoplegia, suggestive muscle MRI findings and the presence of cores (but no radial strands) on a second muscle biopsy. Preliminary findings currently awaiting confirmation suggest *RYR1* involvement in additional CNM patients with similar features. In addition to predominance of central nuclei as the diagnostic hallmark of the condition, the presence of multiple internal nuclei (not usually observed in other forms of CNM), type 1 predominance, type 1 hypotrophy and subtle Z-line disarray on electron microscopy may indicate *RYR1* involvement in these patients.

3. Cases of centronuclear myopathies without confirmed genetic defect

Heinz Jungbluth and Caroline Sewry (both London, UK) presented clinical and pathological findings from 8 sporadic patients with features of CNM in whom mutations in known CNM genes had been largely excluded. This cohort included three males with clinical features suggestive of X-linked myotubular myopathy but no *MTM1* mutation identified and two girls featuring prominent respiratory impairment with additional dilated cardiomyopathy and cataracts, respectively. Two patients with typical histopathological features of CNM had additional findings of type 1 predominance and cores suggestive of *RYR1* involvement but no *RYR1* mutation had been identified. These findings suggest the possibility of unusual mutations in known genes not detectable with currently applied techniques, or additional genes implicated in CNM not yet identified. In addition, a case with the genetically unresolved multisystem disorder Vici syndrome [OMIM 242840] (featuring callosal agenesis, cardiomyopathy, cataracts, hypopigmentation and combined immunodeficiency) together with histopathological features of centronuclear myopathy was presented; a contiguous gene syndrome as well as *MTM1*, *DNM2* and *BIN1* mutations had been excluded and the molecular basis for this association remains currently uncertain [12].

Jocelyn Laporte (Illkirch, France) presented his approach to the molecular basis of genetically unresolved forms of centronuclear myopathy. Mutations in myotubularin appear to be mainly loss-of-function and often lead to a decrease in the protein level, as assessed with specific antibodies [3,13]. Mutations in *BIN1* are expected to lead to partial loss-of-function, as the protein is detected at normal level, even in a patient with a C-terminal truncating mutation [6]. Lymphoblastoid and fibroblastoid cell lines of patient with *DNM2* mutations show a normal level of the protein. Mutations in *MTM1* represent to date the most common cause of CNM/MTM phenotype, while sequencing of 50 families without *MTM1* mutations revealed mutations of *DNM2* in 33% of cases and mutations in *BIN1* in 10%, leaving half of this cohort without any pathological variants. Mutations in hot spots previously found in these genes have been excluded in another 50 CNM families. Homozygosity by descent from 13 consanguineous families suggested the presence of other genes involved in autosomal recessive CNM forms. High throughput mutation screening is in progress to identify these novel implicated genes.

4. A database for centronuclear (myotubular) myopathies

This session was dedicated to already existing locus-specific databases (LSDB) for the various genes implicated in CNM with a particular focus on establishing a patient registry for CNM/MTM.

Hanns Lochmüller (Newcastle, UK) gave a short introduction on the network of excellence TREAT-NMD (www.treat-nmd.eu) and on patient registries for rare neuromuscular disorders which have been the topic of a recent ENMC workshop [14]. The TREAT-NMD ultimate goal is better treatments for patients with neuromuscular disorders and this requires testing of new treatments in clinical trials. Developing experimental therapies for neuromuscular disorders depends on the precise mutations in the individual patient; one of the prerequisites for clinical trials are therefore databases or patient registries listing patients, precise mutations, their phenotypes, clinical contacts and other details. Neuromuscular disease patients benefit from patient registries in various ways, including feedback on standards of care and research developments, belonging to a broader community, not being left behind as clinical trials develop and having a link to the research community. The benefits of registries to industry are facilitated access to appropriate patient

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cohorts, improved feasibility and planning of clinical trials. The TREAT-NMD patient registries for Duchenne muscular dystrophy and spinal muscular atrophy are nationally based, with national curators that feed into global databases. The TREAT-NMD registry effort will expand to include rarer neuromuscular diseases, such as myotubular and centronuclear myopathies and related disorders.

Meriel McEntagart (London, UK) presented the Cardiff database for X-linked myotubular myopathy. This database previously used to collect data on X-linked myotubular myopathy contains 168 entries including 138 males with proven *MTM1* mutations. Data collected include 20 items covering various aspects of the family history, perinatal history, early development and current cardiorespiratory status. Although this database is currently not in active use, patients in the database could be considered for inclusion in the new patient registry by contact via their clinician.

Valerie Biancalana (Strasbourg, France) presented the UMD database for X-linked myotubular myopathy due to mutations in the *MTM1* gene currently being set up in Strasbourg, France, in collaboration with the UMD developer Christophe Béroud, Montpellier, and clarified its objectives. The aim of this database is to facilitate research into the epidemiology, mutational spectrum, genotype–phenotype correlations and natural history of X-linked myotubular myopathy. The UMD database provides a set of analytic tools aimed at achieving various clinical and molecular goals: from a molecular point of view, UMD software is associated with a UMD-predictor® tool that allows the prediction of the pathogenic impact of substitutions, in particular synonymous or non-synonymous changes. A large set of analysis tools allows establishing phenotype–genotype correlations and obtaining data regarding distribution and frequency of mutations as well as epidemiological information. From a clinical point of view, the UMD software includes an optimized structure to allow the input of a wide range of clinical data.

As it will be crucial to allow queries and comparisons between different locus-specific databases considering the genetic heterogeneity of centronuclear myopathy, a minimal set of common clinical mandatory items will be defined and agreed for the different LSDBs concerning CNM/MTM and for the envisaged patient registry proposed by the Myotubular Trust, the parent organization for myotubular/centronuclear myopathy. The aim of the UMD database for X-linked myotubular myopathy will be to facilitate feasibility studies for and recruitment into future clinical trials and to be a valuable source of information for the improvement of daily management. To reach these different objectives, the stored data will need quality control (“curation”) and regular updates; this will be done by curators, who validate the data and standardize clinical and biological descriptions. The *MTM1* UMD database is currently running in a local version but the long-term objective will be wider web-based availability.

Anne Lennox (London, UK), parent and trustee of the Myotubular Trust, the charity for myotubular and centronuclear myopathy, gave an overview over the aims of her organization and goals achieved to date. The Myotubular Trust was established as the only dedicated charity for myotubular and centronuclear myopathy, with the stated aim of ‘*The relief of disability and the extension of life for those suffering from myotubular myopathy, by promoting the study of, and research into, the treatment and cure of the muscle weakness caused by myotubular myopathy*’. Secondary aims of the Myotubular Trust include becoming a well respected resource of funds of research that ultimately leads to a treatment or cure for myotubular myopathy, expedite research and to communicate new information to patients, families and the wider medical community. The Trust is currently in contact with 67 families affected by myotubular/centronuclear myopathy, 44 from Europe and 23 from overseas.

One of the major medium-term objectives of the Myotubular Trust is to promote a Patient Registry for patients with myotubular/centronuclear myopathy, corresponding to similar patient registries with comparatively more common neuromuscular conditions such as spinal muscular atrophy (SMA) or Duchenne muscular dystrophy (DMD) under the auspices of the TREAT-NMD network of excellence. Heinz Jungbluth (London, UK) outlined the background, principal objectives, aims and proposed format for a patient registry for myotubular/centronuclear myopathy. Patient registries containing patient information and, most importantly, mutational data are a prerequisite for future clinical trials in neuromuscular and other genetically determined disorders. The primary objective of a patient registry for MTM/CNM would therefore be the identification of a patient cohort for future clinical trials with secondary objectives of (i) establishing precise genotype–phenotype correlations for specific genes implicated in MTM/CNM, (ii) gathering information on the natural history and longitudinal data, (iii) compiling robust epidemiological data on MTM/CNM (iv) pooling of currently genetically unresolved cases for identification of further genes and (v) assisting the neuromuscular community with the development of recommendations and standards of care.

Data to be collected will include a set of 15–20 mainly self-reported mandatory items at the entry point for the patient registry and a more extensive set of data linked to the locus-specific databases (LSDBs) for specific genes implicated in MTM/CNM. Entry point for the patient registry will be a histopathologically confirmed diagnosis of MTM/CNM but inclusion into one of the LSDBs will by definition require genetic confirmation of the diagnosis. Strong emphasis will be placed on harmonization of data items, both between LSDBs but also in comparison with other TREAT-NMD databases.

Presentations on existing and planned databases/registries for MTM/CNM were followed by further discussion regarding the aims of a MTM/CNM patient registry, necessary steps to implement those and the relationship between the patient registry and already existing LSDBs. It was agreed by the workshop participants that wherever feasible elements of the MTM/CNM patient registry and the LSDB’s should be designed as part of an international collaborative effort in order to achieve maximum harmonization and to avoid duplication of efforts.

With regards to the planned MTM/CNM patient registry, registry objectives as outlined above (achieving readiness for clinical trials and obtaining data on the epidemiology, natural history, phenotype–genotype correlations and mutational spectrum of MTM/CNM) were agreed upon. Anne Lennox, the Myotubular Trust representative stressed the importance of providing information to families including the most severely affected and achievement of clinical trial readiness; accordingly, consenting of patients will be required. It was agreed that the patient registry should include both patients with confirmed mutations in currently known MTM/CNM genes (*MTM1, DNM2, BIN1, RYR1*) as well as patients with histologically confirmed MTM/CNM without known mutation. Whilst in the majority of cases histological confirmation of MTM/CNM should be straightforward, criteria for evaluation and confirmation of doubtful cases ought to be determined (Caroline Sewry, Heinz Jungbluth). A core group of members of the MTM/CNM workshop (Valerie Biancalana, Marc Bitoun, Jim Dowling, Heinz Jungbluth, Norma Romero) agreed to design a set of 10–20 mandatory clinical items as the key clinical core data set for entry into the MTM/CNM registry within the 2–3 months following the workshop and will aim to achieve as much harmonization as possible between items collected on the

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LSDBs. Annual updates of clinical core data was felt to be feasible and desirable.

Already existing locus-specific databases for MTM/CNM were discussed again (Leiden – *MTM1*; McEntagart, Cardiff database – *MTM1*; Biancalana – UMD *MTM1*; Bitoun – UMD *DNM2*) and it was emphasized that in cases where more than one LSDBs concerning the same gene existed (e.g., for MTM1) a strong effort should be made to avoid duplication of efforts.

5. Function of known genes involved in centronuclear (myotubular) myopathies

Helene Tronchere (Toulouse, France) presented her work on phosphoinositide (PI) metabolism with a particular emphasis on three different pathways (Myotubularin, PIKfyve and IpgD) involved in the production of PtdIns5P.

Phosphoinositides (PIs) are described as major regulators of key signalling pathways controlling proliferation, apoptosis, cytoskeleton remodelling and membrane trafficking, through their capacity to bind protein specific domains (PH, PX, FYVE, and others). The PI metabolism is complex and tightly regulated by lipid kinases and phosphatases whose deregulation is often associated with bacterial, neoplastic and genetic diseases. Among the eight members of the PI family, Dr Tronchere's main interest focuses on the more recently discovered PtdIns5P. Roles for PtdIns5P have been identified in the survival pathway or in the nucleus, however, many questions regarding its precise cellular functions remain currently unresolved. In addition to production from PtdIns(4,5)P_2 by 4-phosphatases (the bacterial IpgD or mammalian type 2 4-phosphatases), PtdIns5P is also produced from PtdIns(3,5)P_2 by the 3-phosphatase myotubularin (MTM1), mutated in X-linked myotubular myopathy [15]. Finally, another PtdIns5P pathway involves the phosphorylation of PtdIns by the 5-kinase PIKfyve. Myotubularin and PIKfyve functions are closely linked as myotubularin also dephosphorylates PtdIns3P to PtdIns and PIKfyve is responsible for the production of PtdIns(3,5)P_2 from PtdIns3P. Together these enzymes regulate converging pathways to control the level of PtdIns5P from the plasma membrane to internal membranes. These observations suggest that, in addition to the well described function of PtdIns3P and PtdIns(3,5)P_2 in the regulation of membrane trafficking, PtdIns5P plays an important role in endocytosis/secretion pathways and in cytoskeleton remodelling, therefore affecting important cellular functions such as survival and migration. Identification of the protein targets regulated by PtdIns5P is an important issue that will help to understand the function of this lipid in cells and its implication in the etiology of the pathologies linked to mutations in myotubularins.

Amy Kiger (San Diego, USA) presented her work suggesting cellular roles for *mtm* in a fly model for centronuclear myopathy. Her group has shown that *Drosophila myotubularin* (*mtm*), the single homolog of human MTM1 and MTMR2, is essential in muscles for adult fly viability and myofiber morphology. The characteristic nuclei displacement and myofibril disorganization share similarities with the human myotubular myopathy, suggesting that *Drosophila* could serve as a genetic model to study the cellular basis of the disease. Defects in muscle cell organization were detected with the disruption of both integrin complex and T-tubule membrane domains important in muscle contraction. An accumulation of PtdIns3P on membrane compartments was consistent with an *mtm* function for its regulation. In addition, her group discovered that RNAi of the *Drosophila* Class II PI3-kinase, *PI3K68D*, could fully rescue *mtm* mutant lethality. This suggests that PI3K68D acts on an antagonistic cellular process to Mtm, or that a PI3K68D-generated subpool of PtdIns3P may serve as a functional Mtm substrate. Importantly, these results suggest that PI3KC2 could be a candidate drug target for treatment of myotubular myopathy, as well as a candidate locus for activating mutations that could be associated with other centronuclear myopathies.

Marc McNiven (Rochester, USA) gave an overview of Dynamin-based cytoskeletal membrane dynamics. His presentation focused on defects in the large GTPase dynamin that have been shown to contribute to Centronuclear Myopathy (CNM). Dynamin is a polymeric enzyme that assembles into large, contractile structures along membranes via a PH domain and interacts with various cytoskeletal effectors by a C-terminal proline rich domain (PRD). Dynamin is known to mediate the deformation of biological membranes both by *in vitro* assays and *in vivo* by the expression of dominant-negative constructs, microinjection of inhibitory antibodies, siRNA knockdowns, and addition of a recently developed drug called 'dynasore' that inhibits its GTPase activity. MarcMc Niven's group believes that dynamin forms a complex with the non-receptor tyrosine kinase src and the actin binding protein cortactin, which together can modulate vesicle membrane dynamics at a variety of different cellular sites, including the plasma membrane, endosomal compartments, the Golgi apparatus, and more recently and surprisingly, lamellipodia and the centrosome. The presentation reviewed the identified point mutations in dynamin that reside in the middle domain and appear to be responsible for the centronuclear myopathy (CNM) phenotypes, in contrast to the nearby point mutations in the pleckstrin homology (PH) domain implicated predominantly in Charcot-Marie-Tooth (CMT) disease. Two bodies of information were presented supporting the concept that the identified mutations contribute to the disease state through an alteration in cellular organization and polarity, mediated in particular through their effect on the Golgi apparatus and microtubule organization. The Golgi apparatus is an essential organelle important in the processing and trafficking of nascent polypeptides to the cell surface. It is normally juxtanuclear, while the organization and positioning of the Golgi aids in distributing vesicles to the appropriate cellular sites, thus playing an important role in cell polarity. Dynamin has been shown by multiple investigators to participate in vesicle formation from the trans-Golgi network (TGN). In his presentation Marc McNiven demonstrated that activation of dynamin by src kinase leads to a pronounced vesiculation and disorganization of the Golgi and a reduction in secretory vesicle transport; this can be prevented by inhibitors of src kinase or expression of dominant-negative dynamin proteins which are unable to be phosphorylated on key tyrosines. These findings lend support to the concept that *DNM2* mutations in CNM may alter Golgi organization and distribution, thus altering cell polarity.

In addition, although dynamin has not traditionally been viewed as a microtubule (MT) associated protein there is substantial evidence in the literature that it can crosslink microtubules via an interaction with its PRD. A study published in 2004 by the McNiven group [16] showed that dynamin interacts with the centrosome in cultured cells via a novel domain in the middle region of the protein exactly where the mutations are found in CNM patients. This middle domain of dynamin appears to bind the centrosome nucleating protein gamma-tubulin and affects centrosome cohesion, which again would have profound effects on cell polarity in both epithelium and muscle cells. Recent studies by the McNiven group have shown that specific point mutations in the spliced inserts between the middle domain and the PH domain of dynamin significantly alter microtubule organization and appear to increase its affinity for cytoplasmic microtubules vs. the centrosome, thus converting the normally cytoplasmic or membrane distribution of this enzyme onto MTs. Thus, these findings suggest that this region of dynamin is essential for normal microtubule interaction, and mutations may again lead to significant alterations in cell polarity and potentiate the CNM and CMT disease phenotypes.

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Anne Toussaint (Illkirch, France) gave an overview over the pathophysiology of different forms of centronuclear myopathies. The severe neonatal X-linked form (myotubular myopathy) is due to mutations in the gene encoding the phosphoinositide phosphatase myotubularin *(MTM1)*, while mutations in the membrane tubulating GTPase dynamin 2 *(DNM2)* have been found in some autosomal dominant cases [17]. In addition, Anne Toussaint's group has also identified homozygous mutations in the amphiphysin 2 (or *BIN1*) gene in three families with autosomal recessive CNM [6]. Although myotubularin, amphiphysin 2 and dynamin 2 are ubiquitously expressed, mutations in the genes encoding these proteins are predominantly associated with a muscle phenotype.

Considering that amphiphysin 2 has been shown to localize to T-tubules and that a role in T-tubule assembly is suggested by anomalies in the localization of markers of T-tubule biogenesis and organization in patients harbouring *BIN1* mutations, the role of amphiphysin 2 and dynamin 2 in the biogenesis and organization of these structures in skeletal muscle is currently being investigated. Initial results using specifically designed antibodies indicate that amphiphysin 2 co-localizes with markers of the T-tubule in murine skeletal muscle but not with dynamin 2. In addition, abnormal localization of three markers of the triad (DHPR, RyR1 and SERCA) was observed in *MTM1* knockout mouse muscle compared to wild type. Further studies on the effect of *MTM1*, *BIN1* and *DNM2* mutations on the localization of the three proteins and on triad organization in the different forms of centronuclear myopathies are currently in progress.

6. Animal models of centronuclear myopathies

Jim Dowling (Michigan, USA) presented work from his laboratory detailing the generation and characterization of a zebrafish model of myotubular myopathy [18], using morpholino antisense technology to achieve knockdown of myotubularin in the zebrafish embryo. Embryos with myotubularin knockdown (called myotubularin morphants) had obvious functional motor deficits and morphologic changes. Histopathologic analysis revealed myofibre hypotrophy/atrophy, perinuclear disorganization, and alterations in nuclear size and position. In total, myotubularin morphant zebrafish shared many of the clinical and histopathologic features of the human disease. Further work in their zebrafish model of myotubular myopathy revealed abnormalities in T-tubule ultrastructural organization and excitation-contraction coupling; importantly, similar defects were found in muscle from myotubular myopathy patients. Based on these findings, Jim Dowling hypothesized that structural/functional T-tubule abnormalities significantly contribute to the weakness seen in myotubular myopathy patients and that these results link the pathogenesis of myotubular myopathy with that of other congenital myopathies, in particular core myopathies. More importantly, further work of his group on myotubularin-related proteins (MTMRs) suggests that MTMR1 and MTMR2 can rescue the myotubularin morphant phenotype. In addition, observations on the morpholino knockdown of zJUMPY/MTMR14 (a gene associated with autosomal centronuclear myopathy) [8] indicate that knockdown of MTMR14, whilst not associated with severe muscle phenotype by itself, may significantly exacerbate the myotubularin knockdown phenotype, suggesting that MTMR14 may function as a disease modifier of the CNM phenotype.

Marc Bitoun (Paris, France) presented his preliminary data on a knock-in mouse model of a dynamin 2 mutation found in autosomal dominant centronuclear myopathy.

DNM2 mutations have been identified in autosomal dominant centronuclear myopathy and in intermediate or axonal forms of Charcot-Marie-Tooth (CMT) neuropathy [4,19]. In COS7 cells transfected by *DNM2* mutants associated with CNM and CMT, his group have shown that the transferrin uptake, a marker of the clathrin-dependent receptor-mediated endocytosis, is decreased. In these cells, the same mutants also alter the EGF-induced ERK1/2 activation by phosphorylation. To understand better the impact of *DNM2* mutation *in vivo*, his group has recently constructed a knock-in mouse model expressing the most frequent mutation identified in human CNM patients. The first results indicate that this mutation in a homozygous state is associated with rapid neonatal mortality and that the heterozygous mutation is compatible with life. Characterization of this model is currently in progress to determine the cause of death in homozygous animals and to investigate a possible muscular phenotype in heterozygous animals.

Laurent Tiret (Maisons-Alfort, France) presented the work of his and Stéphane Blot's teams on the canine model of centronuclear myopathy. To date, the only known spontaneous model of human centronuclear myopathy is a congenital autosomal-recessive myopathy which has been described in the Labrador retriever, occuring sporadically and described in several countries for the last 30 years. Pups are normal at birth but develop weakness, muscle atrophy and exercise intolerance within the first 6 months of life and are usually euthanized before 1 year, when they ultimately develop severely disabling locomotor and feeding difficulties.

Since the mid-1990s, breeding of a pedigree of CNM dogs at the Alfort School of Veterinary Medicine, France, has allowed clinical description of the disease from birth to adulthood and identification of the disease locus. Under attentive medical care, these dogs have a normal lifespan of 14 years. Locomotor difficulties and muscular atrophy increase during the first 12 months of life but tend to stabilize thereafter if the dogs are kept in a stable temperature environment, as cold conditions exacerbate weakness and may result in crises. As the canine esophagus is composed of striated muscles, megaoesophagus is a major canine-specific CNM problem affecting some dogs, requiring acute gastrostomy insertion to avoid a fatal pneumonia secondary to food regurgitation.

Histopathological features in the dog are reminiscent of human MTM/CNM and long-term evaluation allowed to establish their typical chronological sequence: Earliest histopathological changes are seen at 3 months of age and comprise increased fibre size variability with both rounded, atrophied and hypertrophied myofibers within the same fascicle. Subsequently, signs of cytoplasmic disorganization such as the presence of radial strands of oxidative materials, a hallmark of *DNM2*-related human AD-CNM, and type 1 predominance due to loss of fast type 2 fibres are noticed. From 7 months of age, fibres with centrally located myonuclei are observed and their number increases linearly to reach 70% at 10 years. Finally, from 1 year of age, fatty infiltration of muscle fascicles is noted and evolves with intra- and interindividual variability. Discrete neuromuscular junction abnormalities are noticed at all ages and remain to be fully documented. In addition, the canine model also allows evaluation of the effects of the condition on other organs such as the heart.

Linkage analysis lead to the identification of a homozygous mutation in the *PTPLA* gene in affected dogs, resulting in a functional knockdown of the 2 main transcripts detected in skeletal muscles. Further extensive haplotype analysis demonstrated that every Labrador affected by canine CNM is homozygote for a single mutation due to a strong founder effect. The protein product of PTPLA has recently been identified as an enzyme involved in the synthesis of very long chain fatty acids (VLCFA), key components or regulators of molecules including phosphoinositides that act in signalling and trafficking in cells, indicating that CNM in Labradors may represent a unifying model of defective membrane trafficking in human CNMs; this is supported by the finding of T-

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tubule disorganization in canine CNM muscle cells. A central question is therefore to understand how PTPLA is connected to the existing molecular models, based on the analysis of human, murine, fly and zebrafish CNM. Ongoing projects therefore include study of the expression levels and patterns of myotubularin, dynamin 2, amphiphysin 2 and RyR1 in muscles of affected dogs as well as a more global proteomic comparison of normal and affected muscles biopsied during the earliest phase of the disease. It is expected that the differentially expressed proteins will be direct and indirect PTPLA-interactors or will play a role in convergent functional pathways altered in CNM/MTM; identification of such proteins will allow to better understand the pathophysiological mechanisms leading to the absence of a normal muscle development or maintenance and will pinpoint new candidates to screen CNM patients with no mutations in the presently known CNM-associated genes.

7. Potential therapeutic approaches in centronuclear (myotubular) myopathies

Anna Buj-Bello (Illkirch, France) presented her work on therapeutic approaches for X-linked myotubular myopathy in a mouse model of the condition.

Myotubular myopathy (XLMTM, OMIM 310400) is the X-linked and most severe form of centronuclear myopathies, characterized by the presence of hypotrophic myofibres with central nuclei in skeletal muscle. No specific treatment exists to date for patients with myotubular myopathy. Her group has generated

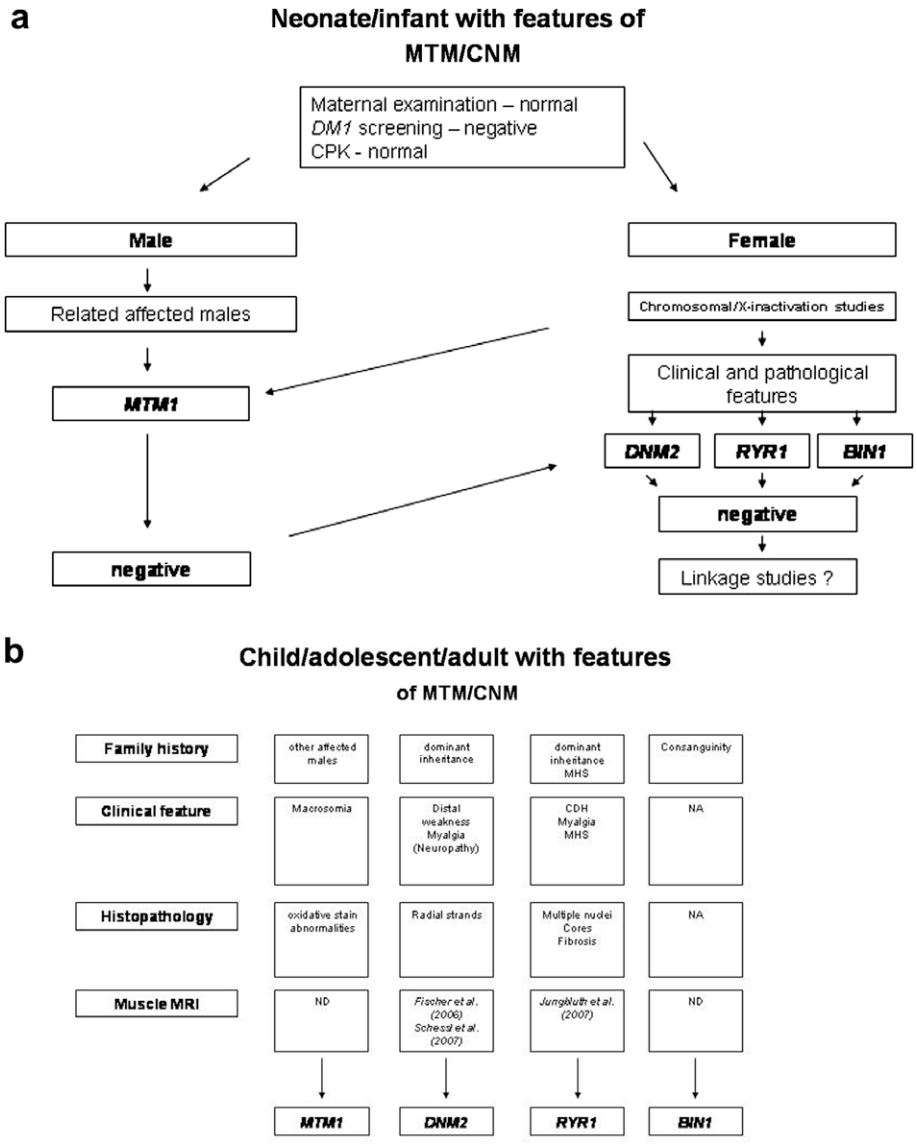

Fig. 1. Diagnostic approach to Paediatric and adult patients with myotubular/centronuclear myopathy (MTM/CNM). In neonates or infants with clinical and histopathological features of MTM/CNM (a), myotonic dystrophy must be appropriately excluded before further genetic testing is considered. Following exclusion of myotonic dystrophy, *MTM1* mutation screening should be performed initially, particularly in males with a family history suggestive of X-linked inheritance, before other genes are considered based on clinical and histopathological findings. Identification of *MTM1* mutations in females should prompt a search for associated X chromosomal abnormalities and/or skewed X-inactivation. In older patients with MTM/CNM (b), family history and/or the presence of certain clinical and histopathological findings may aid molecular genetic testing in some cases; however, none of these are invariable. Muscle MRI may be particularly useful in the *DNM2*- and *RYR1*-related forms where distinct patterns of selective muscle involvement have been reported [7,24–25]. *MTM1*, myotubularin gene; *DNM2*, dynamin 2 gene; *RYR1*, skeletal muscle ryanodine receptor gene; *BIN1*, amphiphysin 2 gene; MHS, malignant hyperthermia susceptibility; CDH, congenital dislocation of the hips.

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two mouse models of the disease, a constitutive (KO) and a muscle-specific (mKO) *Mtm1*-deficient line that reproduce clinical and pathological aspects of the disease [20]. Studies of these rodent mutants have revealed that skeletal muscle is indeed the primary tissue involved in the pathogenesis of XLMTM and that myotubularin is essential for muscle growth and proper distribution of organelles in myofibres. Myotubularin knockout (KO) mice develop a centronuclear myopathy, starting at around 3–4 weeks of postnatal age, with progressive muscle weakness that severely reduces life expectancy to about 6–12 weeks. The molecular mechanisms responsible for these structural abnormalities and force deficit are currently unknown but under investigation. By using novel antibodies against myotubularin and overexpression studies in skeletal muscle, Anna Buj-Bello's group has recently shown that myotubularin, which is located at triads, may regulate plasma membrane/T-tubule homeostasis and/or remodelling in myofibres [21].

Gene therapy represents the hope of a cure for many human diseases, in particular, for genetic disorders affecting skeletal musculature. Numerous successes have already been achieved in mouse models of human diseases. Due to the lack of pathogenecity, recombinant adeno-associated viruses (rAAV) are the vectors of choice for replacement therapy *in vivo* and several serotypes, such as the AAV1 serotype, transduce with high efficiency skeletal muscle [22]. Anna Buj-Bello's group has recently reported that a single intramuscular injection of a rAAV1 vector containing the *Mtm1* cDNA under the CMV promoter in symptomatic muscle-specific *Mtm1*-deficient mice ameliorates the pathological phenotype of targeted muscles [21]. Myotubularin replacement largely corrects nuclei and mitochondria positioning in myofibres, and leads to a strong increase in muscle volume and contractile force. Future experiments will investigate if this approach is also efficient in muscle of constitutive KO mice. Furthermore, since XLMTM muscle is characterized by the presence of hypotrophic fibres, one may speculate that therapies aiming at increasing muscle mass, such as myostatin blockage and insulin-like growth factor (IGF1) overexpression [23], may also be beneficial for patients.

8. Conclusions and future perspectives

In conclusion, the present workshop did address a number of key points concerning centronuclear (myotubular) (CNM/MTM) myopathies. In particular, participants discussed possible phenotype–genotype correlations for genetically resolved forms of these conditions and proposed a flowchart to guide molecular investigations, the diagnostic gold standard, based on histological and clinical findings in Paediatric and adult patients with MTM/CNM (Fig. 1). In addition, it clearly appears that a subset of patients with centronuclear myopathy do not have mutations in the coding sequence of any of the known genes implicated in the condition to date, suggesting that better characterization of this cohort will be useful to search for novel genes involved in CNM. Already existing locus-specific databases (LSDBs) and plans for a patient registry for MTM/CNM were discussed. A list of 15–20 mandatory and highly desirable items will be defined by a subgroup of participants to achieve greater harmonization and consistency. A patient registry for MTM/CNM should lead to better genetic counselling and, as a means of collecting a well characterized patient cohort, will be useful for future therapeutic approaches. Several animal models for MTM/CNM have now been reported and were discussed in detail; those should prove useful both for linking the different implicated genes in a common pathway and for testing different approaches aiming to rescue biochemical and histological phenotypes , with the ultimate goal of ameliorating muscle weakness in patients.

Workshop participants

Valerie Biancalana (Strasbourg, France)
Marc Bitoun (Paris, France)
Anna Buj-Bello (Illkirch, France)
James Dowling (Ann Arbor, USA)
Victor Dubowitz (London, UK)
Pascale Guicheney (Paris, France)
Heinz Jungbluth (London, UK)
Amy Kiger (San Diego, USA)
Jocelyn Laporte (Illkirch, France)
Anne Lennox (London, UK)
Hanns Lochmüller (Newcastle, UK)
Meriel McEntagart (London, UK)
Marc McNiven (Rochester, USA)
Norma Romero (Paris, France)
Caroline Sewry (Oswestry, UK)
Melanie Spring (London, UK)
Laurent Tiret (Paris, France)
Anne Toussaint (Illkirch, France)
Helene Tronchere (Toulouse, France)

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