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Workshop report

118th ENMC International Workshop on Advances in Myotubular Myopathy. 26–28 September 2003, Naarden, The Netherlands. (5th Workshop of the International Consortium on Myotubular Myopathy)

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1. Introduction

XLMTM (X-linked myotubular myopathy) is a rare recessive disorder. The *MTM1* gene is localized on Xq28 and it encompasses 100 kb at the genomic level, with 15 exons coding for a 603 aa protein named myotubularin. Mutations in myotubularin result in the human disease XLMTM, which is characterized by the persistence of muscle fibres that retain an immature phenotype [1].

MTM1 gene is the founder member of the myotubularin family (14 genes in human) which constitutes a large group within the tyrosine/dual specificity phosphatases super

family (PTP/DSP). Members are present in almost all Eucaryotic organisms, including yeasts and plants. Two other members were more recently found mutated in 2 forms of demyelinating Charcot-Marie-Tooth (CMT) neuropathy type 4B1 and 4B2[2]. In the last 4 years, after the past ENMC Consortium on Myotubular myopathy, which was held in 1999, a great number of findings have emerged concerning the function of myotubularin and myotubularin related proteins. Accordingly, the Consortium was extended to many new participants with expertise in biochemistry and cell signalling. The ENMC Consortium on advances in Myotubular Myopathy (MTM) held its 5th Workshop in Naarden, the Netherlands, the weekend of 26–28th September 2003. It was attended by 19 active participants from Australia, Finland, France, Germany, Italy, Switzerland, the United Kingdom and the USA.

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2. Genotype–phenotype correlation

2.1. Molecular diagnosis

The meeting opened with an overview by Wolfram Kress and Valérie Biancalana on the genotype–phenotype correlation of XLMTM

A mutational review by Valérie Biancalana, including 7 not reported cases found in the Strasbourg group, counted a total of 196 different mutations in 340 families. 154 mutations were ‘private’ mutations and were found only in one family [3,4].

Eighty five percent of the mothers that were tested were mutation’s carriers, a fact that is important for genetic counselling. In addition, germline mosaicism has been described in several cases, therefore prenatal diagnosis should be proposed to each mother, even if she does not carry the mutation.

Most XLMTM patients were ventilator dependent while 16% of them were classified as having either a moderate or a mild phenotype based on the level of the ventilatory support required [5], that is to say with no chronic ventilator dependence.

In most cases mutation leads to the inactivation or absence of myotubularin. Out of the 196 mutations the percentage of mutation types observed was 29% missense mutations, 21% nonsense mutations, 23% small insertion/deletion; 20% splice site mutations and 7% large deletion. These are widespread through the gene but nearly half of the patients have mutations in exons 12, 8 and 4. These 3 exons should then be tested in first intention in a diagnostic laboratory (with exon 9 in case of a patient with a mild phenotype).

In fact, 7 mutations account for 25% of all MTM1 mutations. In the order of frequency in the total set of mutations there are the splice site mutation c.1261-10A > G ensuing in the insertion of three amino acid FIQ at position 420 (7.3%), R241C (3.8%), c.141-144 delAGAA resulting in a frameshift at amino acid 48 (3.8%), R37X (2.7%), P205L (2.4%), R421Q (2.4%) and R421 X (2.4%). They are mainly associated with a severe form of the disease, except R241C, which is often found associated with a mild or intermediate phenotype.

Myotubularins all share the same protein domains core, which consists of the following four domains: GRAM, RID, PTP/DSP active site and SID. The 100 disease causing missense mutations have been found in each of the domains, suggesting that they all participate in the function of myotubularin. Missense mutations are, however, predominant in the RID and in the PTP/DSP homology region.

Wolfram Kress summarized his experience on molecular genetic diagnosis in Germany. The number of new confirmed diagnosis of patients with congenital MTM1 is about 7–8/year and this reflects very roughly the German incidence of severe MTM1, because routine molecular diagnosis in Germany is being performed only in his lab.

Mutation analysis is performed by direct sequencing of all fifteen exons of the myotubularin gene, and this method should be considered as the gold standard because it improves molecular diagnosis in relation to other screening methods. However, the impression is that the number of mutations found in severe MTM1 patients has not decisively increased and do not get beyond 60–70%. Probably some mutations localized in the promoter or intron regions of the *MTM1* gene are still missing. Kress reported that he had analysed about the same number (7–8/year) of new mild sporadic cases with a centronuclear myopathy (older than two years), and one third of them were females. The number of mutations found in the mild cases is low and never exceeds 20%.

Norma Romero from Paris presented a particular three-generation family with XLMTM. The grandfather (67 years) and his brother (48 years) were considered as affected by centronuclear myopathy (CNM) until his two grandsons (13 and 5 years) presenting with severe hypotonia at birth, respiratory mechanic assistance, and latter muscle weakness, long thin face, ptosis and ophtalmoplegia were diagnosed as having X-linked myotubular myopathy (their mothers, obligated carriers, were healthy). A common *MTM* gene mutation (N180K) was identified in all patients [3]. These patients are the oldest alive patients with myotubular myopathy known so far.

2.2. Manifesting carriers in XLMTM

Manifesting carriers of MTM1 are rare (3 out of 43 studied). To find out whether clinical manifestations in a proportion of carriers was due to skewed X-inactivation, a recent study was presented by Carina Wallgren-Pettersson who reported the X-inactivation pattern in blood DNA compared with the clinical phenotype in 43 carriers of X-linked myotubular myopathy [6]. Three overtly affected carriers had skewed X inactivation with the mutated X as the predominantly active X for at least two of them. Four females with mild symptoms had random X inactivation. The unaffected XLMTM carriers had either skewed X inactivation in favor of expression from the normal X or random X inactivation. Thus, there appeared to be a correlation between overt manifestations and skewed X inactivation, but the correlation did not reach significance, possibly due to the small patient numbers in this series, or to the tissue under study being blood and not the muscle tissue itself. Skewing of X inactivation in XLMTM is likely to be due to genetic factors influencing the X-inactivation process rather than to chance or to a selection against cells expressing the X chromosome carrying the mutated *MTM1* gene.

Wolfram Kress also presented his experience on one symptomatic female patient with a MTM1 mutation. In a five years old congenitally affected girl showing muscle hypotonia, proximal weakness, a waddling gait, joint laxity and a myopathic face a frameshift mutation in exon 8 was

found. She had a skewed X-inactivation [7]. There were at least two families in the last few years with manifesting carrier mothers and grandmothers (weakness in early adulthood, myopathic face, ptosis). X-inactivation was not enlightening in any case.

2.3. Functional studies of myotubularin on MTM1 defects

In addition to mutation analysis, Jocelyn Laporte presented results on his studies with protein analysis to verify if this method can improve the diagnostic workup of XLMTM [8]. The protein status can be studied by immunoprecipitation but not yet routinely. He characterized antibodies against myotubularin and monitored the level of the protein in XLMTM patients cell lines. The vast majority of mutations, including some missenses, lead to a decrease in protein level. All suspected autosomal cases had normal level suggesting that CNM is not due to an indirect decrease in MTM1 protein level [8]. Laporte also used a probe specific to PI3P provided by Harald Stenmark [9]. While overexpression of myotubularin clearly remove the PI3P from early endosomes, he failed to detect any differences in PI3P (and EEA1, an early endosome marker) in fibroblasts and myoblasts cell lines from patients with XLMTM or suspected autosomal CNM (Tronchere et al., submitted) [10].

3. Myotubularin function and myotubularin related proteins

3.1. Metabolic pathways and subcellular localization

X-linked myotubular myopathy is a muscle disorder caused by mutations on the MTM-1 gene, coding for myotubularin—a 65 kDa polypeptide similar to protein phosphatases. Myotubularin is a dual-specific phosphatase that dephosphorylates phosphatidylinositol 3-phosphate (PtdIns(3)P), and phosphatidylinositol (3,5)-bisphosphate (PtdIns(3,4)P₂).

The establishment of a causal relation between mutations on the MTM-1 gene and the development of myotubular myopathy, prompted the search for a biochemical function of myotubularin [1].

Phosphoinositide 3-kinase (PI3-kinase)-derived [11,12] membrane-anchored phosphoinositides regulate diverse cellular processes, including proliferation, cell survival, vesicular trafficking, cytoskeletal remodeling, and metabolism [13]. Lipid products produced by PI 3-kinase activity include the following: [PtdIns(3)P], [PtdIns(3,4)P₂], phosphatidylinositol 3,5 bisphosphate [PtdIns(3,5)P₂], and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃). Diverse proteins are regulated by binding to the D3-phosphorylated phosphoinositides PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, including the serine/threonine kinase Akt, a potent suppressor of apoptosis [14]. PtdIns(3)P, the most

abundant product of PI 3-kinase activity in mammalian cells, specifically interacts with a protein module designated the 'FYVE domain' and functions as a regulator of endosomal trafficking [15].

Initial experiments by using synthetic substrates suggested a function for myotubularin as a protein phosphatase. However, more compelling evidence from both biochemical and in vivo studies now define myotubularin as a lipid phosphatase capable of hydrolysing the 3' inositol position of phosphatidylinositol 3-phosphate [PtdIns(3)P] and [PtdIns(3,5)P₂] [2]. Although there is now information on the biochemical function of myotubularin, the rationale whereby mutation on MTM-1 leads to myotubular myopathy is still elusive.

A whole morning was focused on studies that try to unravel the subcellular localization and the function of MTM1 and myotubularin related proteins (MTMRs), highlighting the specific substrates, the catalytic activities and the crystallographic structure of MTMR2. Using a bioinformatic approach, Laporte characterized the 14 human members which include 6 inactive phosphatases [2]. He reported the chromosomal localisation for all of them as there are candidate for human diseases. Indeed mutations in MTMR2 and MTMR13 have recently been found, respectively, in CMT4B1 [16,17] and CMT4B2 [18,19]. There is no co-evolution of catalytically active and inactive myotubularins looking at the sequenced genomes [2]. Although all myotubularins tested localized as a dense cytoplasmic network and are recruited to plasma membrane upon Rac1 GTPase induction, differences exist in the gradient of localisation from the nucleus membrane to the plasma membrane suggesting different substrate pools [20].

The group from Australia conducted by Harshal Nandurkar had previously described the purification of a rat [PtdIns(3)P] 3-phosphatase, comprising a heterodimer of a 78-kDa adapter subunit in complex with a 65-kDa catalytic subunit [20,21]. In addition, they cloned and characterized the cDNA encoding the human 3-phosphatase adapter subunit (3-PAP) [22]. Sequence alignment showed that 3-PAP shares significant sequence similarity with the protein and lipid 3-phosphatase myotubularin, and with several other members of the myotubularin gene family [22]. Nandurkar's group also had previously described that unlike myotubularin, 3-PAP does not contain a consensus HXX₅R catalytic motif, and is thus a catalytically inactive member of the myotubularin gene family, which coprecipitates lipid phosphatidylinositol 3-phosphate-3-phosphatase activity from lysates of human platelets [22].

Nandurkar reported that he recently identified myotubularin as the catalytically active 3-phosphatase subunit interacting with 3-PAP [23]. A 65-kDa polypeptide, coprecipitating with endogenous 3-PAP, was purified from SDS/PAGE, subjected to trypsin digestion, and analysed by collision-induced dissociation tandem MS. Three peptides derived from human myotubularin were identified. Association between 3-PAP and myotubularin was confirmed by

reciprocal co-immunoprecipitation of both endogenous and recombinant proteins expressed in the human K562 cell line. Recombinant myotubularin localized to the plasma membrane, causing extensive filopodia formation. However, co-expression of 3-PAP with myotubularin led to attenuation of the plasma membrane phenotype, associated with myotubularin relocalisation to the cytosol. Collectively these studies indicate that 3-PAP functions as an 'adapter' for myotubularin, regulating myotubularin intracellular location and thereby altering the phenotype resulting from myotubularin overexpression. In collaboration with Jocelyn Laporte of Professor Mandel's group (Strasbourg, France), Nandurkar has demonstrated that certain myotubularin mutations that are associated with disease phenotype, but retain enzyme activity, do not interact with 3-PAP. This observation supports his hypothesis that any perturbation of interaction between 3-PAP and myotubularin may be sufficient to cause myopathy. Nandurkar is generating mice with tissue-specific targeted null mutation of the 3-PAP gene. The phenotype of these mice will contribute to the understanding of 3-PAP in muscle development and maturation.

The X-ray crystal structure of human MTMR2 was presented by Matthew Wishart. The structure revealed that the previously described GRAM sequence is actually a part of larger pleckstrin-homology domain, and that SID and RID are, in fact, sequence motifs within a larger lipid phosphatase domain. Moreover, the so-called 'substrate-trapping' aspartic acid does not play a role in catalysis, but rather is important for maintaining the structural integrity of the phosphatase domain. One of the aspartic acids within the active site CX5R loop likely functions as the catalytic acid. The structure also provided a framework for understanding the functional consequences of mutations associated with human disease.

Helene Tronchère focused her presentation on myotubularin and phosphoinositides pathways. Phosphoinositides represent 10% of the total phospholipids pool of cell membranes, where they do not play a structural role but have been shown to be essential component of the signal transduction machinery. Their role of second messenger controls many key cellular functions like proliferation, apoptosis, vesicular trafficking and cytoskeleton remodeling. Phosphoinositides levels in cells are tightly controlled by a set of lipid phosphatases and kinases, some of them have been linked to human diseases, like the tumor suppressor PTEN. Tronchère's laboratory has previously shown that myotubularin, encoded by the *MTM1* gene, which is mutated in the X-linked genetic muscular disorder myotubular myopathy (XLMTM), is a lipid phosphatase hydrolyzing PtdIns(3)P. PtdIns(3)P is localized in the membranes of internal vesicles, the early endosomes and is implicated in vesicular trafficking regulation. Tronchère's group has therefore looked for the implication of MTM1 in intracellular vesicular trafficking by comparing PtdIns(3)P levels and localization in XLMTM patients or control

derived myoblasts and fibroblasts. They did not see any differences between the patients and control cell lines suggesting that MTM1 could act on small pools of PtdIns(3)P, but also that the phosphatase could have other(s) function(s) in the cell. Therefore, they pushed further the in vitro characterization of its phosphatase activity by using fluorescent lipids. Thus, they could show that MTM1 and also other members of the MTM family (MTMR1, MTMR2, MTMR3) can hydrolyze, in addition to PtdIns(3)P, PtdIns(3,5)P₂, leading to the production of phosphatidylinositol 5-phosphate (PtdIns(5)P). It was confirmed that myotubularin hydrolyzes phosphatidylinositol (3,5)P₂ in vivo and induces an increase of PtdIns(5)P. Future developments are devoted to studying the function of PtdIns(5)P whose function in the cell is still not known and trying to determine the importance of the lack of production of PtdIns(5)P in the etiology of XLMTM [10].

3.2. Molecular function of MTM1 and related proteins

Michael Clague presented data demonstrating that several members of the myotubularin family utilize PtdIns(3,5)P₂ in addition to PtdIns3P as substrate [24]. Expression of MTM1 and MTMR3 in yeast demonstrated that hydrolysis of PtdIns(3,5)P₂ provides a biosynthetic pathway to PtdIns5P [25]. PtdIns5P is an allosteric activator of MTM1 and MTMR3 and a mutation in MTM1 (R69C), which leads to a moderate disease phenotype, shows both reduced enzyme activity and dampened response to PtdIns5P. Enzyme activity of MTM is non-linear within a narrow concentration range. Incubation of a catalytic site mutant of MTM1 with PtdIns5P and substrate (PtdIns3P) leads to the formation of a 12.5 nm diameter heptameric ring structure, which can be visualised by negative stain electron microscopy. It is proposed that this structure represents a stabilised form of an otherwise unstable intermediate that dissociates upon hydrolysis. This may explain the observed co-operativity of enzyme activation.

Luciano Pirola investigated the function of myotubularin in its most physiologically relevant context, i.e. in differentiated muscle cell lines. As differentiated muscle cell lines are hardly transfectable, he used an adenoviral vector to efficiently express wild type myotubularin and the two catalytically inactive mutants C375S and D278A.

Metabolic labelling of L6 and C2C12 myotubes followed by purification of total phosphoinositides and profiling of the differently phosphorylated forms by HPLC demonstrated an increase of PtdIns(3)P upon expression of catalytically inactive myotubularins, suggesting they may have a dominant negative action. In keep with this observation, overexpression of GFP-myotubularin disrupted the endosomal punctuated staining of early endosomal antigen 1 (EEA1), a FYVE-domain-containing PtdIns(3)P binding protein.

The modulation of PtdIns(3)*P* levels by myotubularin associated to its capability to displace EEA1 from an endosomal localisation prompted Luciano Pirola to search for a cell function affected by PtdIns(3)*P*. As this lipid is involved in the trafficking of internal membranes [9], he enquired whether ectopic expression of myotubularin might affect insulin-induced glucose uptake, which is mediated by the translocation of the glucose transporter GLUT4 from intracellular insulin-responsive membranous compartments to the plasma membrane [12], a fusion process possibly requiring PtdIns(3)*P*. Indeed, expression of *wt* myotubularin while not affecting the activation of proximal insulin signalling targets such as PKB and MAPK-induced a decrease in insulin-induced glucose uptake. Moreover, overexpression in 3T3-L1 adipocytes of myotubularin impaired insulin-induced translocation at the plasma membrane of GFP tagged GLUT4. Together, his observations indicate that PtdIns(3)*P* is required for the proper trafficking of GLUT4 and thus myotubularin might intervene in the regulation of this cellular process [26]. An important question to be further addressed is whether the impact of myotubularin on glucose transport has a significant physiological link to the development of myotubular myopathy.

3.3. The myotubularin deficient mouse

A mouse model for X-linked myotubular myopathy has been generated by homologous recombination in order to understand the physiopathological mechanism of the disease. Anna Buj-Bello showed that absence of myotubularin in mouse leads to a generalized and progressive myopathy starting at around 4 weeks of age [27]. The life span of knockout mice is severely reduced and death occurs at 59 ± 19 days, probably due to cachexia and respiratory difficulties. The histopathological analysis of *Mtm1* deficient mice reveals lesions only in the skeletal muscle with a progressive accumulation of paracentral and central nuclei in myofibres, fibre size variation and atrophy. Degenerative changes appear progressively in fibres but signs of necrosis are very limited. The analysis of these mice shows that myotubularin is essential for skeletal muscle maintenance but not for the differentiation of myotubes into mature myofibres, contrary to prior hypothesis on human XLMTM pathogenesis. Anna Buj-Bello used a mouse conditional targeting strategy to determine whether a defect in proper innervation or nerve trophic supply to developing skeletal muscle could play a role in the pathogenesis of the disease. Two tissue specific knockout lines were generated by crossing an *Mtm1* conditional line with transgenic mouse lines overexpressing the Cre recombinase under either an HSA (human skeletal muscle α -actin) or NSE (human neuron specific enolase) promoter. The analysis of these mutant mice reveals that deletion of *Mtm1* specifically in skeletal muscle is sufficient to induce a severe centronuclear myopathy with clinical and pathological signs similar to that

observed in constitutive knockout animals. *Mtm1* knockout mice will be used to dissect the molecular events leading to the myopathy and represent an important tool for developing therapeutic approaches.

4. Charcot-marie-tooth and MTMR related proteins

Mutations in the gene on chromosome 11p15 encoding myotubularin-related protein 2 (MTMR2) are linked to autosomal recessive Charcot-Marie-Tooth disease type 4B1 (CMT4B1), a severe hereditary motor and sensory neuropathy characterized by focally folded myelin sheaths and demyelination [16,17]. More recently, mutations in the MTMR13/SBF2 gene were identified by two different groups [18,19] in patients with CMT4B2 showing myelin outfoldings in the sural nerve biopsy, and occasionally associated with infantile glaucoma. Both MTMR2 and MTMR13 belong to the Myotubularin-related (MTMR) family of dual specificity PTP-like protein phosphatases [2]. MTMR2 shows specific activity towards PI(3)P, both in vitro and in vivo as well as PI(3,5)P₂, as recently demonstrated in vitro. However, how abrogation of this lipid phosphatase activity is leading to the specific disease phenotype has not yet been demonstrated.

The group of Ueli Sutter analyzed the biochemical properties of the MTMR2 protein. Both, phosphatidylinositol-3-phosphate (PI(3)P) and PI(3,5)P₂, are substrates for MTMR2 and are dephosphorylated by this enzyme at the D3 position with high efficiency and peak activity at neutral pH [17]. Furthermore, disease-associated MTMR2 mutations lead to dramatically reduced phosphatase activity, indicating that the MTMR2 phosphatase activity is crucial for the proper function of peripheral nerves in CMT4B1. Functional dissection of different domains of the MTMR2 protein revealed that its membrane association is mediated by a pleckstrin homology-GRAM domain and a coiled-coil dimerization module [28]. In hypoosmotically stressed COS cells with increased levels of PI(3,5)P₂, MTMR2 is recruited to the membrane of vacuoles. The pleckstrin homology-GRAM and the coiled-coil domains are crucial mediators of this membrane targeting. The pleckstrin homology-GRAM domain binds directly to PI(3,5)P₂ and PI(5)P, a substrate and a product of the MTMR2 enzyme, respectively. In addition, MTMR2 forms dimers and the coiled-coil is responsible for homodimerization. Thus, phosphoinositide-protein interactions together with protein-protein interactions, are required for correct regulation of MTMR2.

On the cellular level, expression analysis of MTMR2 suggests particularly high levels in neurons with some expression also by myelinating Schwann cells. Thus, the demyelinating neuropathy CMT4B1 might be triggered by the malfunction of neural membrane recycling, membrane trafficking, and/or endocytic or exocytotic processes, combined with altered axon-Schwann cell interactions [17,29].

Alessandra Bolino and her group analysed the cellular and subcellular distribution of *Mtmr2* (mouse *MTMR2*) in nerve. *Mtmr2* was detected in all cytoplasmic compartments of myelin-forming Schwann cells, as well as in the cytoplasm of non-myelin forming Schwann cells and both sensory and motorneurons. In contrast, *Mtmr2* was detected in the nucleus of Schwann cells and motorneurons, but not in the nucleus of sensory neurons [30]. As *MTMR2* is diffusely present also within the nerve, a specific function could derive instead from nerve-specific interacting proteins. We performed two yeast two-hybrid screenings, using either fetal brain or peripheral nerve cDNA libraries. The neurofilament light chain protein, *NF-L*, was identified repeatedly in both screenings, and found to interact with *MTMR2* in both Schwann cells and neurons. Interestingly, *NF-L*, encoding *NF-L*, is mutated in *CMT2E* and in severe forms of *CMT* often diagnosed as Dejerine-Sottas syndrome. These data may provide a basis for the nerve-specific pathogenesis of *CMT4B1*, and further support for the notion that hereditary demyelinating and axonal neuropathies may represent different clinical manifestations of a common pathological mechanism.

Jan Senderek reviewed in detail *CMT4B2*. So far, six families with *SBF2/MTMR13* mutations were identified. Affected individuals have early-onset severe sensorimotor neuropathy with slow nerve conduction velocities. In some families, patients had congenital glaucoma or elevated intraocular pressure. Nerve biopsies showed signs of demyelination and characteristic myelin outfoldings. Preliminary expression studies suggest ubiquitous expression in mouse and human adult tissues, which is similar to other *MTMRs*. This clearly awaits more detailed studies, at different developmental stages, especially in neural and ocular tissues. The encoded *SBF2/MTMR13* protein belongs to the catalytically inactive antiphosphatase branch of the myotubularin group. As shown for other active and inactive members of this family [23,31,32], *SBF2/MTMR13* may physically interact with *MTMR2*. This would be a plausible explanation of how mutations in an active phosphatase (*MTMR2*) and an inactive phosphatase (*SBF2/MTMR13*) result in the same phenotype.

5. Centronuclear myopathy

A whole session was dedicated to autosomal CNM [33].

First of all Caroline Sewry discussed the pathology of a variety of cases with an abundance of central nuclei. These included severe and infantile cases of X-linked myotubular myopathy, 2 fetuses with a mutation in the *MTM1* gene and 5 females and one male in whom mutations in *MTM1* and expansions of the gene for myotonic dystrophy had been excluded. A young female case presented at the previous workshop had been identified as a manifesting *MTM1* carrier [34]. Severe male, X-linked cases showed large, well spaced central nuclei and peripheral nuclei were rare.

Necrosis and endomysial fibrosis were not present and oxidative enzymes were abundant in the centre of fibres and were surrounded by a pale peripheral halo. In infantile cases central nuclei were often confined to small type 1 fibres and some nuclei were internal rather than central. Again, nuclei at the periphery of the fibre were rare. Some fibres were hypertrophic and type 1 atrophy/hypotrophy was also apparent. Oxidative enzyme stains showed pale peripheral halos but the dark centres were less obvious than in the severe cases. The possibility that this difference relates to age could not be excluded. Infantile cases can show lines of glycogen radiating from the centre of the fibre but this was not a universal feature in the cases reviewed. Immunolabelling of myosin isoforms showed that the maturational switch occurs in myotubular myopathy and that fibres with central nuclei lack neonatal myosin with most fibres expressing the mature slow or fast isoform. Fibres with high levels of desmin and vimentin were not a universal feature. Electron microscopy of severe X-linked cases often showed clusters of dense tubules. Analysis of skeletal muscle from 2 fetuses, aged 13 weeks of gestation, with an *MTM1* mutation, showed that myotube formation occurs and that there was a possible increase in the number of central nuclei. Myofibril and sarcomere formation had also occurred but myofibrils were sparse in some fibres. Some fibres also showed non-membrane bound spaces of unknown origin. An artefact of processing could not be excluded. The mutation therefore seems to relate to maintenance of the myofibres rather than to myogenesis. The 5 female and one male case without *MTM1* mutations showed both peripheral and central nuclei and some showed mild endomysial fibrosis. Core-like lesions devoid of oxidative enzymes were a common feature in these cases. In view of these pathological findings and the clinical features, that included facial involvement and neck weakness, Dr Sewry suggested that the gene for the ryanodine receptor 1 (*RYR1*) should be screened before considering them as autosomal cases of myotubular/centronuclear myopathy. The pathological spectrum associated with defects in *RYR1* is now known to be broad and includes the features found in these cases, and it was agreed that at least the 'hot spot' mutations in *RYR1* should be excluded.

Heinz Jungbluth from London presented clinical, radiological and genetic data from 5 sporadic cases with histopathological features of myotubular (centronuclear) myopathy. Four of these cases were female and one was male. Age range was 4–14 years. All were cytogenetically normal.

In addition to a full clinical assessment, T1-weighted muscle MR images were obtained from the pelvis, thigh and lower legs in 4 patients. Mutational screening of the *MTM1* gene (Dr Biancalana, Strasbourg) was performed following exclusion of the CTG repeat expansion associated with myotonic dystrophy. All affected females had X-inactivation studies in lymphocytes (Dr Kristiansen, Oslo).

A heterozygous mutation C1315T in exon 12 of the *MTM1* gene was identified in this patient resulting in

a R421X substitution [34], and previously reported in severely affected males. This patient had presented at birth with marked hypotonia. She did not achieve independent walking and required a gastrostomy and nighttime ventilation because of persistent feeding and respiratory difficulties. Urinary and stool incontinence were additional features, suggesting smooth muscle involvement. Marked facial weakness with bilateral ptosis and external ophthalmoplegia, a mild scoliosis and severe axial and proximal weakness were prominent clinical findings. Height and weight were above the 90th centile. The clinical phenotype of this female was of moderate severity and associated with an extremely skewed X-inactivation pattern (97:3) in favour of the X-chromosome harbouring the mutation. This case emphasizes that investigation of the *MTM1* gene and X-inactivation studies are indicated in isolated females with histopathological and clinical findings suggestive of myotubular myopathy.

MTM1 mutations could not be identified in three female patients and one male patient. The only male patient without confirmed *MTM1* mutation was more severely affected than other patients in this cohort and had presented antenatally with reduced fetal movements. There was a marked chest deformity and severe respiratory impairment. There were no axial antigravity movements with associated severe wasting of neck muscles. Feeding and respiratory difficulties were progressive and he died from respiratory failure in his second year of life.

All female patients without confirmed mutation had a normal X-inactivation pattern, providing further evidence against involvement of the *MTM1* gene. These patients presented at birth with marked hypotonia and two girls each required gastrostomy insertion and nocturnal ventilation for persisting feeding and respiratory difficulties. Motor development was delayed but all achieved independent ambulation. Delayed maturation of bladder control was a consistent finding, suggesting smooth muscle involvement. In two patients myasthenia gravis had been initially suspected because of a positive Tensilon test, consistent improvement following a trial of pyridostigmine and a suggestive single fibre EMG in one girl. Marked axial weakness and wasting of the lower leg with associated foot deformities were prominent findings on examination. Eye movements were preserved in all but one patient. All female patients shared a consistent appearance on muscle MRI characterized by marked and diffuse signal increase within the thigh and severe and diffuse involvement of the lower leg with relative sparing of the gastrocnemii. Muscle bulk within the thigh was better preserved compared to the X-linked form and muscle involvement was more diffuse compared to other congenital myopathies. Muscle MRI may therefore provide an additional tool in the assessment of patients with myotubular (centronuclear) myopathy.

Norma Romero from Paris presented the clinical and morphological data from a group of 29 patients from 12

unrelated families affected by autosomal centronuclear myopathy [35]. The clinical course of CNM is nonspecific and the diagnosis is based on the morphological findings: centrally located nuclei in a large portion of muscle fibers, most often associated with fiber type 1 predominance and hypotrophy. The typical radial arrangement of sarcoplasmic strands around the central nuclei on NADH-tetrazolium reductase staining, initially described by Goulon et al. in 1976 [36], is an hallmark of CNM. It was always found in our series of muscle biopsies. The aim of our study is to better define the clinical and morphological characteristics of autosomal CNM and identify potentially homogeneous subgroups for genetic analysis.

Pascale Guicheney from Paris presented molecular studies performed on CNM. A canine autosomal recessive centronuclear-like myopathy has recently been reported [37]. It involves Labrador Retrievers, which exhibit early occurrence of muscle weakness, hypotonia, proximal muscle wasting and abnormal neck posture, mimicking the clinical evolution of the human centronuclear myopathy. The muscle pathology is also similar to that seen in human CNM, and consists in with centralized myofiber nuclei associated with predominance and hypotrophy of type 1 fibers. By homozygosity mapping, a locus has been identified on canine chromosome 2, in a 18 cM interval including the *VIM* gene encoding vimentine [38]. The human locus, syntenic to canine *cnm*, is located in 10p13-14.

The group of Pascale Guicheney used four microsatellite markers, D10S547, D10S1653, D10S548, and D10S197, to screen this human locus in two large autosomal dominant (AD) CNM families and one putative autosomal recessive family by linkage analysis. In one AD family, an haplotype was cosegregating with most of the affected subjects. In this interval, *PTPLA* gene was thus considered as a putative candidate gene. This gene encoded a Tyrosine Phosphatase-Like Protein with a proline instead of arginine in its catalytic site, presenting an early expression at embryonic day 8.5 in murin muscle progenitors and later in differentiated muscle types. In murin and human adult tissues it is expressed in heart, skeletal muscle, liver, testis and kidney, but its exact role in myogenesis or cardiogenesis is not known [39,40]. The seven exons corresponding to the coding sequence were studied in 6 CNM probands but no mutation was found [41].

Besides, a genome screening was performed in the two French AD CNM families by the French National Center of Genotyping (CNG, Evry) with 400 microsatellite markers set with an average spacing of 10 cM, and a putative locus common to these two families was found. Confirmation of this locus will be undertaken by the analysis of additional markers and family members and by analysis of additional CNM families.

Jocelyn Laporte used a candidate gene approach testing MTMRs for mutation in autosomal CNM. He searched for mutations in an interacting partner of *MTM1*. No mutations were found in 17 patients with suspected autosomal CNM

(dominant, recessive and sporadic cases), nor in 11 patients with suspected XLMTM where no MTM1 mutations have been found. Other functional candidate genes implicated in the same functions as myotubularin are currently being sequenced and additional CNM patients are collected.

6. X-linked myotubular myopathy and trophic factors

6.1. Insulin related trophic factors (IGF1 and IGFII) in patients with myotubular myopathy

Finally the last session was devoted to recent findings emerged on the reported growth abnormalities in XLMTM. It is well known that the majority of the patients with XLMTM have linear growth that remains above the 50th percentile [5]. Moreover, in the large series of 55 males reported by Herman et al. [5] five patients, ranging in age from 3 to 9 years, had documented bone ages >1 standard deviation above their chronologic age in comparison with radiographic standards. Enrico Bertini reported the detailed study on 2 patients affected by a severe XLMTM confirmed by 2 missense mutations (F438S and R69C). These 2 patients had typical clinical signs of XLMTM, showed respiratory failure from birth and were still ventilator dependent at the age of 1 year. In both babies serum levels of IGF1 were not quantifiable at the age of 6 months, while the serum levels of IGFII, IGFBP2, and GH were in normal ranges for age. These 2 patients had no signs of overgrowth and the bone age corresponded to the anagraphic age by the age of 12 months. Another patient with a mild form of XLMTM and a de novo missense mutation (T197I), who is still walking at the age of 23 years, had an overgrowth syndrome with accelerated bone age [42]. In this patient IGF1 serum levels were increased (345 ng/ml, normal ages: 50–150), IGFII were at the higher limits (1622 ng/ml; normal range: 1100–1700), and IGFBP3 was markedly increased (7.5 ng/ml; nv: 1.9–4.5). Anna Buj-Bello reported a study on 2 patients with severe XLMTM carrying *MTM1* deletions (a 46 bp intronic deletion encompassing exon 7 splice site and a large deletion from exon 4 to 15). One patient had an overgrowth syndrome and the other had an accelerated bone age. The bone age of the patient with overgrowth has not been calculated yet. IGF1 serum level was increased in both cases and IGFBP3 was at the upper limit in one patient (2.6 mg/ml, normal range: 1.1–2.5 mg/ml). GH blood level was normal for the age and IGF2 was not analysed in these patients. From this work we can demonstrate that by measuring systematically insulin related trophic factors in serum we may subdivide XLMTM patients in 2 groups: one with markedly reduced IGFI in serum and a second group with overgrowth and increased serum IGFI. Further confirmation studies are needed in a larger series of patients.

6.2. Insulin signaling pathways and related trophic factors in skeletal muscle. Lessons from the transgenic mouse for IGF1

Antonio Musaro presented an overview on IGF-I and muscle. Among growth factors, the insulin-like growth factor-1 (IGF-1) has been implicated in the control of skeletal muscle growth and differentiation in embryonic development, while in the adult it plays a central role during muscle regeneration [43]. Since IGF-1 levels decline with age in both rodents and human muscle, this growth factor has been considered a promising therapeutic agent in staving off advancing muscle weakness during ageing and promoting regeneration.

Skeletal muscle regeneration involves the activation of quiescent satellite cells, which participate in the reconstitution of damaged tissue. However, in several muscle pathologies the regenerative process is compromised, leading to a progressive loss of muscle mass and function. In this context the goal of our research was to characterize the role of factors involved in muscle regeneration and repair.

His group reported that muscle-specific expression of a local IGF-1 isoform (mIGF-1) promotes and improves regeneration in senescent muscle [44]. In addition they have recently reported that high levels of mIGF-1 transgene expression in the mdx mouse model of muscular dystrophy also preserves muscle function in the absence of dystrophin, inducing significant hypertrophy and hyperplasia at all ages observed, reducing fibrosis and myonecrosis, and elevating signaling pathways associated with muscle survival and regeneration [45]. Proliferation of desmin-expressing cells in the injured area of mIGF-1 transgenic muscle, suggested that mIGF-1 induces a local increase in the myoblast population in response to damage. In addition, they analyzed whether muscle regeneration involved the recruitment of uncommitted cell populations, and they have been able to demonstrate that the capacity of the transgenic muscle to regenerate is also associated to an increase in the recruitment of circulating stem cells expressing Sca1 and c-Kit, general markers of hematopoietic stem cells. The recruited uncommitted cells homing the damage muscle, contribute to muscle regeneration and guarantee a reserve of muscle stem cells.

IGF-1 can therefore act, in combination with other regenerative factors, as a homing signal attracting circulating stem cells to repair injured muscle.

7. Conclusions

This workshop has been particularly useful in giving some guidelines for the molecular diagnosis of XLMTM and to start clarifying some points on the function of myotubularin and myotubularin-related proteins. The presentations related to unravel the function of myotubularin

have given prerequisites for accelerating future therapeutic directions. The workshop have also been fruitful in describing different strategies directed to solve the genetic characterization of several forms of autosomal centronuclear myopathies, and will surely lead to new collaborations in this field.

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Appendix A. List of participants

Enrico Bertini (Rome, Italy)
 Valerie Biancalana (Strasbourg, France)
 Alessandra Bolino (Milan, Italy)
 Anna Buj Bello (Strasbourg, France)
 Michael J. Clague (Liverpool, UK)
 Pascale Guicheney (Paris, France)
 Heinz Jungbluth (London, UK)
 Wolfram Kress (Wurzburg, Germany)
 Antonio Musaro' (Rome, Italy)
 Harshal Nandurkar (Melbourne, Australia)
 Luciano Pirola (Nice, France)
 Norma Romero (Paris, France)
 Jan Senderek (Aachen Germany)
 Ueli Suter (Zurich, Switzerland)
 Caroline Sewry (London, UK)
 Helene Tronchere (Toulouse, France)
 Carina Wallgren-Pettersson (Helsinki, Finland)
 Matthew J. Wishart (La Jolla, CA, USA)
 Jocelyn Laporte (Strasbourg, France)

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