



## Workshop report

# 51st ENMC International Workshop: Nemaline Myopathy 13–15 June 1997, Naarden, The Netherlands

## 1. Introduction

The second ENMC international workshop on nemaline myopathy was held in Naarden, The Netherlands 13–15 June 1997 in the presence of thirteen participants from eight countries.

Since the first workshop [1], a review paper on nemaline myopathy has been compiled [2], a number of new families have been studied, nemaline myopathy has been established as a cause for the fetal akinesia sequence [3], a new mutation in the gene for slow tropomyosin TPM3 has been discovered [4], the linkage region for autosomal recessive nemaline myopathy on chromosome 2 has been refined and a candidate gene has been assigned to this region [5].

## 2. Clinical session

Martin Lammens (Leuven, Belgium) presented eight cases from four families where nemaline myopathy was the cause of severe fetal akinesia deformation sequence, including arthrogryposis, lung hypoplasia and craniofacial dysmorphism. The eight patients and one sibling presented with fetal akinesia. Multiple nemaline bodies were found in muscle biopsies of a fetus of 22 gestational weeks. Five out of seven children of a Turkish family died at birth with fetal akinesia sequence. Muscle biopsies of four of them showed multiple nemaline bodies. In one child of a Dutch family with fetal akinesia sequence nemaline myopathy was confirmed on archival paraffin blocks using  $\alpha$ -actinin antibodies. Two children of consanguineous Moroccan parents had neonatally severe nemaline myopathy. Molecular genetic studies of this family indicate no linkage to the region for autosomal recessive nemaline myopathy, NEM2, on chromosome 2q. There were no intranuclear rods present in any of the patients.

Martin Lammens concluded that nemaline myopathy can cause the fetal akinesia sequence with arthrogryposis, that it can be present at 22 weeks of gestation, and that there is a neonatally severe form without intranuclear rods. In the discussion following on Dr. Lammens' presentation, Caro-

line Sewry reported that 3 out of 50 fetuses with fetal akinesia she had screened had been found to show nemaline bodies in their muscle tissue. It was concluded that screening muscle samples from fetuses and neonates with arthrogryposis should include immunohistochemical studies of  $\alpha$ -actinin to detect cases of nemaline myopathy.

Martin Lammens also presented two cases diagnosed as adults, one with facial and limb-girdle weakness from childhood, likely to represent the mainstream form of autosomal recessive nemaline myopathy, and another who presented at the age of 73 years with muscle pain, proximal muscle weakness and elevated inflammation parameters. Muscle biopsies from both patients showed nemaline bodies and atrophy of type 1 fibres.

Norma Romero (Paris, France) reported three families with autosomal dominant inheritance. In one, the patients had a combination in their muscle tissue of central cores, nemaline bodies and positive *in vitro* tests for susceptibility to malignant hyperthermia. Linkage analysis in this family showed results compatible with linkage to the ryanodine receptor on chromosome 19. Further studies are underway.

Isabelle Penisson-Besnier (Angers, France) presented a large kindred in which females in one branch had mild proximal muscle weakness but no weakness of the facial muscles or the neck flexors. A muscle biopsy showed mild dystrophic changes and there were no nemaline bodies. One had a high CK. In another branch of the same kindred, related to the first one through an asymptomatic male, there were three brothers with a clinical and histological picture consistent with nemaline myopathy and with normal CK values. The workshop participants thought it likely that in this kindred, a muscular dystrophy and nemaline myopathy are segregating as separate traits. Further studies of the family are underway.

Alan Beggs (Boston, USA) summarised data of 56 patients from 45 families. Out of the familial cases, there were two kindreds showing autosomal dominant inheritance and five autosomal recessive. A segregation analysis of the sporadic and autosomal recessive cases of this North American series gave a segregation ratio of only 0.15. The possible reasons for this include avoidance by the families of

further pregnancies, partial penetrance, phenocopies and new mutations. Out of the families for which details of clinical presentation were known, there were two families with a neonatally lethal form and 17 families with congenital onset of a static or slowly progressive form, including one with autosomal dominant inheritance, and one sporadic case with childhood onset.

Carina Wallgren-Pettersson (Helsinki, Finland) presented data on four new multiplex families. One sib pair with the clinically mainstream form of autosomal recessive nemaline myopathy, NEM2, showed an unusual histological picture (see section on Pathology). This family and all the others with the mainstream form showed linkage results compatible with linkage to the NEM2 region on chromosome 2 [6]. A son of consanguineous parents whose brother had died neonatally of unknown causes was said to be normal at birth, but his motor development was severely impaired and delayed, he never sat, and he died at 21 months from pneumonia [4]. This boy had unusual muscle biopsy findings and was found to have a previously undescribed TPM3 mutation (see below under Pathology and Molecular Genetics). Mutations in the genes for other sarcomeric proteins expressed only in type 1 fibres might give rise to a similar histological picture.

### 3. Session on pathology, immunohistochemistry and pathophysiology

Kathryn North (Sydney, Australia) presented recent results of her pathological and immunocytochemical analysis of muscle biopsies from patients with TPM3 mutations. There are a number of variable features in the pathology of muscle from patients with nemaline myopathy including the percentage of fibres with rods in them, proportions and relative sizes of fibre types, distribution of rods among different fibre types, expression of fetal myosin isoforms and loss of correlation between  $\alpha$ -actinin-3 and myosin heavy chain (MHC) fast isoform expression ( $\alpha$ -actinin-3 is normally expressed only in a subset of fast type 2 fibres). A biopsy from a patient with autosomal dominant nemaline myopathy who is heterozygous for the missense TPM3 mutation Met9Arg [7] exhibited apparently normal type 2 fibres with type 1 fibre hypotrophy or atrophy and rods exclusively in the smaller type 1 fibres. Anti- $\alpha$ -actinin-2 stained the Z lines in all fibres and the rods in type 1 fibres but anti- $\alpha$ -actinin-3 stained only Z lines in the normal-appearing type 2 fibres. Examination of a biopsy from a 21-month-old deceased patient with nemaline myopathy who was homozygous for a nonsense mutation at codon 31 of TPM3 (see Laing's report below) revealed a similar pattern of pathology although the changes were even more striking. In contrast, the majority of biopsies from other patients with nemaline myopathy exhibit marked predominance of type 1 fibres (including sometimes exclusively fibres expressing slow MHC isoforms) with little or no fibre size variation and

rods that react with both anti- $\alpha$ -actinin-2 and anti- $\alpha$ -actinin-3 antisera.

A review of 14 biopsies previously studied by North revealed four with the proposed "TPM3 pattern" (mild type 2 predominance, normal diameter of type 2 fibres, type 1 atrophy and rods only in type 1 fibres) suggesting that analyses of these patient's TPM3 genes should be a high priority.

Caroline Sewry (London, UK) described her survey of pathology and immunohistochemistry among cases of nemaline myopathy studied between 1973 and 1997 in London. These included 24 patients from 21 families. Six cases had severe neonatally lethal courses while 18 represented the slowly or non-progressive congenital or childhood-onset form. Fibre size variation was a common feature seen in 16 biopsies and the percentage of fibres containing rods varied widely even within families, ranging from 7 to 100%. Three of these cases had rods only in a population of small fibres, similar to the "TPM3 pattern" described by North.

The six neonatally lethal cases were characterised by very small fibres, presence of satellite cells, a poor number of myofibrils and many heterochromatic nuclei, perhaps suggesting an apoptotic process of cell death. None of them showed intranuclear rods. Fibres expressing fetal myosin were common and were often small.

The severe, neonatally lethal cases, in common with the milder slowly progressive form of nemaline myopathy, show predominance of slow, type 1 fibres. It is unclear whether this represents poor development of fast fibres or some active process of degeneration or apoptosis leading to loss of type 2 fibres or fibre type conversion of the faster fibres.

Studies with isoform-specific  $\alpha$ -actinin antisera from Beggs revealed that  $\alpha$ -actinin-2-positive rods were present in virtually all rod-containing fibres but  $\alpha$ -actinin-3-positive rods were only seen in a subset of fibres. In many of these instances, the  $\alpha$ -actinin-3-positive rods were seen in fibres that expressed slow MHC isoforms and variable quantities of  $\alpha$ -actinin-3 at the Z lines. This lack of correlation between  $\alpha$ -actinin-3 and MHC expression was also seen in biopsies studied by North and Beggs. These observations support the notion that the paucity of fast fibre types may reflect a loss of fast-specific isoforms and conversion to expression of genes for slow type proteins. The presence of residual  $\alpha$ -actinin-3-positive rods in slow fibres may indicate that isoform turnover in rods is less than in Z lines.

Finally, immunocytochemical studies of nebulin revealed no demonstrable differences between control and chromosome 2-linked cases of nemaline myopathy. One nemaline myopathy biopsy did exhibit slight variability in the intensity of nebulin staining, but it was unclear whether this reflected a real difference or was simply an artefact of specimen preparation or condition.

Alan Beggs (Boston, USA) discussed recent preliminary results from yeast two-hybrid screens using fragments of  $\alpha$ -actinin as bait. Since  $\alpha$ -actinin is a primary constituent of

both Z lines and nemaline rods, characterisation of new proteins that interact with  $\alpha$ -actinin may provide candidate genes for NM and should shed light on mechanisms of rod formation.  $\alpha$ -Actinin-2 and  $\alpha$ -actinin-3 are muscle-specific isoforms localised at the Z lines of all fibres ( $\alpha$ -actinin-2) or a subset of fast, type 2 fibres ( $\alpha$ -actinin-3) [8,9]. Subclones containing the  $\alpha$ -actinin-2 rod (clone 3c) or the  $\alpha$ -actinin-3 EF hand domain (clone 6c) were used to screen a human skeletal muscle cDNA library. Twenty-five to 50 HIS3-positive yeast colonies were characterised for each bait and 10–11 clones from each screen contained prey inserts that specifically activated both HIS3 and lacZ genes only when co-expressed with the 3c or 6c bait clones. DNA sequence analysis revealed that one of the 3c-interacting clones contained a partial  $\alpha$ -actinin-2 transcript, consistent with the fact that  $\alpha$ -actinin forms head to tail dimers [10]. Three novel bait clones were also identified of which one (3c8) was independently isolated eight times. 3c8 encodes a previously unidentified 1.8 kilobase muscle-specific transcript. Of the ten 6c-interacting clones, four contained  $\alpha$ -actinin-2 which suggests that portions of  $\alpha$ -actinin-2 and  $\alpha$ -actinin-3 may form heterodimers. Three additional new and uncharacterised transcripts were also identified, including one (6c6) that was selected six times.

To confirm these putative interactions, portions of each of the six new genes were cloned into an expression vector to allow in vitro transcription and translation. Preliminary experiments with 3c8 demonstrated that it co-immunoprecipitates with the  $\alpha$ -actinin-2 rod domain in these in vitro studies. Further characterisation of this and the other new proteins is continuing.

Kathryn North also presented preliminary results on the generation of transgenic mouse models for nemaline myopathy. In collaboration with Edna Hardeman, Peter Gunning and Nigel Laing, two lines of transgenic mice carrying the Met9Arg TPM3 mutation ([0,1995]) are being produced, one with the TPM3 gene under control of the troponin I slow (TnISLOW) promoter and the other using the fast fibre-specific skeletal actin promoter. The TnISLOW-Met9Arg TPM3 transgenes should be expressed in slow fibres, similar to the pattern of native TPM3 expression. In contrast, the fast-skeletal actin promoter is expected to cause expression of the mutant TPM3 in fast type 2 fibres which are particularly abundant in many mouse muscle groups. Since the Met9Arg TPM3 mutation is inherited in a dominant fashion, it is hoped that expressing mice will have similar patterns of pathology to human patients with this mutation.

To date, there have been 18 pups born with the TnISLOW construct and 7 born carrying the fast skeletal actin transgene. Preliminary rtPCR expression studies of soleus muscles from the TnISLOW-Met9Arg-positive mice have demonstrated that most, if not all, are expressing the transgene. The mice are currently only five weeks old and it remains to be seen what clinical and pathological phenotypes they will express.

#### 4. Molecular genetics session

Nigel Laing (Perth, Australia) reviewed results on screening nemaline myopathy patients for mutations in the  $\alpha$ -tropomyosin slow, TPM3 gene. To date 53 nemaline myopathy families have been screened for mutations in the TPM3 gene by single-strand conformational polymorphism (SSCP) analysis of each of the 10 exons which make up the muscle-specific isoform cDNA by amplification of genomic DNA. Only one further patient has shown a mutation in the TPM3 gene and this is a patient who died at 21 months of age from respiratory infection and who had apparently recessively inherited nemaline myopathy. The parents of the boy were first cousins and the boy showed homozygosity for a nonsense mutation at codon 31 of the TPM3 cDNA, approximately 20 codons 3' of the missense mutation identified in the Australian family with autosomal dominant inheritance. This result makes TPM3 nemaline myopathy similar to rhodopsin retinitis pigmentosa where missense mutations may give rise to dominant disease and nonsense mutations to recessive disease. Dr. Laing also gave preliminary data on the investigation of nebulin cDNA from illegitimate transcription in lymphocytes of nemaline patients showing possible linkage to chromosome 2.

Carina Wallgren-Pettersson presented linkage data on five new multiplex families, four of which showed results compatible with linkage to the NEM2 region on chromosome 2. Two new key recombinations helped narrow the region down from 13 cM to 4 cM. The candidate region still encompasses the nebulin gene, which is thus a strong candidate as the gene causing the mainstream autosomal recessive form of nemaline myopathy. The fifth family, described clinically by Martin Lammens as showing a severe, arthrogryptic form of nemaline myopathy, did not appear to show linkage to this or any other known candidate loci.

Alan Beggs updated linkage results in US families with nemaline myopathy, including description of two families with autosomal recessive inheritance and features atypical of the mainstream form which did not show linkage to chromosome 2.

Katarina Pelin (Helsinki, Finland) gave the results of a radiation hybrid study localising nebulin within the linkage region for autosomal recessive nemaline myopathy on chromosome 2 and thus confirming nebulin as a candidate gene for this disease. She also gave preliminary results of a screen of part of the nebulin cDNA, in approximately 1.5 kb PCR fragments, up to 15.3 of the 21 kb of the cDNA, using Southern blots of a number of restriction enzyme digests. No abnormality was seen in 19 patients.

Stefania Millevoi (Heidelberg, Germany) described the nebulin cDNA and the start of investigation of the genomic structure of the nebulin gene. The C-terminal 500 residues of the nebulin protein are apparently embedded in the Z disc. Different length thin filaments and Z discs of different thickness occur in different muscle fibres. Investigation of the genomic structure should illuminate the differential pro-

cesses by which this can occur. Preliminary results indicate that the exon boundaries match the repeat structure of the cDNA.

Siegfried Labeit (Heidelberg, Germany) gave an overview of the structure of titin, now localised just telomeric of the recessive nemaline myopathy locus on chromosome 2, including characterisation of an  $\alpha$ -actinin binding site within the part of titin embedded in the Z disc.

## 5. Plans for collaborative efforts

Plans for collaborative ventures within the Consortium include addressing the question of the subclassification of nemaline myopathy in a number of ways: An international database has been set up in Helsinki to collect and analyse detailed clinical, histological and, later on, mutational data. A project is being initiated to find out how common nemaline myopathy is as a cause of neonatal arthrogryposis, with Caroline Sewry, Hammersmith Hospital, London, as the contact person. All new cases are being analysed for the known TPM3 mutations, and all new multiplex families are being tested for linkage to a number of candidate loci. In families showing linkage to the 2q locus, mutations are being sought in the nebulin gene.

## 6. Workshop participants

Professor Peter Barth, Amsterdam, The Netherlands  
 Dr. Alan H. Beggs, Boston, USA  
 Dr. Marc Fiszman, Paris, France  
 Dr. Siegfried Labeit, Heidelberg, Germany  
 Dr. Nigel G. Laing, Nedlands, Australia  
 Dr. Martin Lammens, Leuven, Belgium  
 Dr. Stefania Millevoi, Heidelberg, Germany  
 Dr. Kathryn North, Adelaide, Australia  
 Dr. Katarina Pelin, Helsinki, Finland  
 Dr. Isabelle Penisson-Besnier, Angers, France  
 Dr. Norma Romero, Paris, France  
 Dr. Caroline Sewry, London, UK  
 Dr. Carina Wallgren-Pettersson, Helsinki, Finland

## Acknowledgements

This workshop was made possible thanks to the financial support of the European Neuromuscular Centre (ENMC) and its main sponsors: Association Française contre les Myopathies, Italian Telethon Committee, Muscular Dystrophy Group of Great Britain and Northern Ireland, Unione Italiana Lotta alla Distrofia Muscolare, Vereniging Spier-

ziekten Nederland as well as its associate members: Schweizerische Stiftung für die Erforschung der Muskelkrankheiten, Deutsche Gesellschaft für Muskelkranke and Muskelsvindfondene. We are grateful to Professor Alan E.H. Emery, Research Director of the ENMC, for scientific advice and to Michael Rutgers and Janine de Vries for organisational support.

*Carina Wallgren-Pettersson*  
 Department of Medical Genetics  
 University of Helsinki  
 and the Folkhälsan Department of Medical Genetics  
 Helsinki, Finland

*Alan H. Beggs*  
 Genetics Division, Children's Hospital  
 Harvard Medical School  
 Boston, USA

*Nigel G. Laing*  
 Molecular Neurogenetics Laboratory  
 Queen Elizabeth II Medical Centre  
 Nedlands, Western Australia

## References

- [1] Wallgren-Pettersson C, Laing NG. Workshop report: 40th ENMC International Workshop: Nemaline Myopathy. 2–4 February 1996, Naarden, The Netherlands. *Neuromusc Disord* 1996;6:389–391.
- [2] North KN, Laing NG, Wallgren-Pettersson C and the ENMC International Consortium on Nemaline Myopathy. Nemaline myopathy: current concepts. *J Med Genet* 1997;34:705–713.
- [3] Lammens M, Moerman Ph, Fryns JP et al. Fetal akinesia sequence caused by nemaline myopathy. *Neuropediatrics* 1997;28:116–119.
- [4] Tan P, Briner J, Boltshauer E et al. Homozygosity for a nonsense mutation in the alpha-tropomyosin gene TPM3 in a patient with severe congenital nemaline myopathy. Submitted.
- [5] Pelin K, Ridanpää M, Donner K et al. Refined localisation of the genes for nebulin and titin on chromosome 2q allows the assignment of nebulin as a candidate gene for autosomal recessive nemaline myopathy. *Eur J Hum Genet* 1997;5:229–234.
- [6] Wallgren-Pettersson C, Avela K, Marchand S et al. A gene for autosomal recessive nemaline myopathy assigned to chromosome 2q by linkage analysis. *Neuromusc Disord* 1995;5:441–443.
- [7] Laing NG, Wilton SD, Akkari PA et al. A mutation in the alpha-tropomyosin gene TPM3 associated with autosomal dominant nemaline myopathy. *Nat Genet* 1995;9:75–79.
- [8] Beggs AH, Byers TJ, Knoll JH, Boyce FM, Bruns GA, Kunkel LM. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. *J Biol Chem* 1992;267:9281–9288.
- [9] North KN, Beggs AH. Deficiency of a skeletal muscle isoform of alpha-actinin (alpha-actinin-3) in merosin-positive congenital muscular dystrophy. *Neuromusc Disord* 1996;6:229–235.
- [10] Blanchard A, Ohanian V, Critchley DR. The structure of alpha-actinin. *J Muscle Res Cell Motility* 1989;10:280–289.