Muscle histopathology in *nebulin*-related nemaline myopathy: ultrastructural findings correlated to disease severity and genotype

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Abstract

Nemaline myopathy (NM) is a rare congenital myopathy characterised by hypotonia, muscle weakness, and often skeletal muscle deformities with the presence of nemaline bodies (rods) in the muscle biopsy. The nebulin (*NEB*) gene is the most commonly mutated and is thought to account for approximately 50% of genetically diagnosed cases of NM. We undertook a detailed muscle morphological analysis of 14 *NEB*-mutated NM patients with different clinical forms to define muscle pathological patterns and correlate them with clinical course and genotype. Three groups were identified according to clinical severity. Group 1 (n = 5) comprises severe/lethal NM and biopsy in the first days of life. Group 2 (n = 4) includes intermediate NM and biopsy in infancy. Group 3 (n = 5) comprises typical/mild NM and biopsy in childhood or early adult life. Biopsies underwent histoenzymological, immunohistochemical and ultrastructural analysis. Fibre type distribution patterns, rod characteristics, distribution and localization were investigated. Contractile performance was studied in muscle fibre preparations isolated from seven muscle biopsies from each of the three groups. G1 showed significant myofibrillar dissociation and smallness with scattered globular rods in one third of fibres; there was no type 1 predominance. G2 presented milder sarcomeric dissociation, dispersed or clustered nemaline bodies, and type 1 predominance/uniformity. In contrast, G3 had well-delimited clusters of subsarcolemmal elongated rods and type 1 uniformity without sarcomeric alterations. In accordance with the clinical and morphological data, functional studies revealed markedly low forces in muscle bundles from G1 and a better contractile performance in muscle bundles from biopsies of patients from G2, and G3.

In conclusion *NEB*-mutated NM patients present a wide spectrum of morphological features. It is difficult to establish firm genotype phenotype correlation. Interestingly, there was a correlation between clinical severity on the one hand and the degree of sarcomeric dissociation and contractility efficiency on the other. By contrast the percentage of fibres occupied by rods, as well as the quantity and the sub sarcolemmal position of rods, appears to inversely correlate with severity. Based on our observations, we propose myofibrillar dissociation and changes in contractility as an important cause of muscle weakness in *NEB*-mutated NM patients.

Keywords: Congenital myopathies, Nemaline myopathy, Nebulin, Muscle contractility

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Introduction
Nemaline myopathy (NM) is a congenital muscle disorder associated with hypotonia, muscle weakness, and often skeletal muscle deformities with the presence of numerous nemaline bodies (rods) in muscle biopsy [1]. Clinically the disorder has a marked clinical variability, ranging from neonatal lethal to mild non-progressive forms with onset in childhood and adulthood. NM has been classified into six clinical categories according to the severity of the disease, the age of onset and the pattern of muscle weakness [2]. To date at least nine genes have been implicated in NM (ACTA1, MIM#161800; NEB, MIM#256030; TPM2, MIM#609285; TPM3, MIM#609284; TNNT1, MIM#605355; KBTBD13, MIM#609273; CFL2, MIM#610687; KHLR40 MIM#615340; and KHLR41) encoding proteins of the thin filament of skeletal muscle sarcomere or the Kelch domain associated proteins [3-11]. ACTA1, TPM2 and TPM3 NM is inherited both as autosomal dominant or recessive trait, with de novo dominant mutations being common in all three genes. KBTBD13 NM is an autosomal dominant disorder. The other five genes present autosomal recessive mode of inheritance.

Nebulin is a sarcomeric structural protein crucial for the proper assembly and function of thin filaments [12]. One molecule spans nearly the entire length of the thin filament, making nebulin one of the largest polypeptides in nature. The nebulin (NEB) gene is the most commonly mutated and is thought to account for approximately 50% of genetically diagnosed cases of NM [12]. It is composed by 183 exons of which at least 17 [13] have been shown to be alternatively spliced, giving rise to several different nebulin isoforms in skeletal muscle [14]. Molecular diagnosis has mostly been based on dHPLC and confirmed by exon Sanger sequencing which are time-consuming, laborious, and expensive [15]. Recently next generation sequencing technology in combination with microarray methodology [16] has been demonstrated to be a fast and reliable tool for analysis of large genes such as NEB [15]. Patients are usually compound heterozygous for two different mutations [17]. The mechanisms leading to the alteration of muscle structure or rod formation are largely unknown.

Nemaline bodies are the pathologic hallmarks of congenital NM, even if these structures may sometimes be found associated with other conditions [18]. These are protein aggregates staining red with the modified Gomori trichrome technique. They can appear within the fibres as fine isolated/diffuse structures, compact subsarcolemmal clusters, or both [19]. On electron microscopy, nemaline bodies are electron dense and generally measure 1–7 μm in length and 0.3-2 μm in width. Due to their structural continuity with the Z-disk, and their resemblance to Z-disk lattice pattern, they are considered to be lateral expansions of the Z-disk [20-22]. In the case of nebulin mutation the rod formation could be due to a defect of the nebulin C-terminal, and serine-rich (SH3) domains. Concordantly, the nebulin C-terminal region, or part of it, may extend into the Z-disk [23]. Another common histologic finding of NM is type 1 predominance or type 1 uniformity [21,22]. Based on observations from consecutive muscle biopsies done in the same patient, a substitution of type 2 to type 1 fibres has been suggested to occur with increasing age [19,24]. All congenital NM patients seem to present a homogeneous morphological phenotype characterised by the presence of rods and type 1 predominance. However the largest series reporting on histologic NM findings were published before the identification of specific NM genes or they included patients harbouring mutations in other genes [22,25]. For this reason it is difficult to assess the existence of specific genotype-morphological phenotype correlations in the nine genetically identified forms of NM. A systematic morphological analysis of each entity is therefore pivotal in order to reveal pathogenetic mechanisms.

With the aim of characterising different patterns of muscle involvement, defining the relationship between morphological changes, genotype, and disease severity, we describe muscle morphology and functional studies of a large cohort of clinically heterogeneous NEB-mutated NM patients.

Material and methods
Patients
Fourteen patients from 13 unrelated families from France, the French Antilles, and Argentina were included in the present study. Patients were classified into three groups according to their clinical disease severity. P4 and P5 are brothers. P1 to P5 (Group 1) presented a severe/lethal congenital myopathy leading to death in the first days of life. Their muscle biopsy was performed between 2 days and 15 days of life. P6 to P9 presented an intermediate congenital myopathy and a biopsy effectuated between 2 and 10 months (Group 2); P10 to P14 presented typical or mild (P13, and P14) nemaline myopathy and a muscle biopsy performed during childhood or adolescence/early adult life (6 months-21 years; Group 3). The clinical data of these patients were systematically retrieved and retrospectively analysed. Patients were personally examined by one of 6 of the authors. Clinical and genetic characterization of P1, P2, P4, and P5 has been previously reported [4,15,26].

Mutation analysis
Patients or parents gave informed consent for the genetic analysis according to French legislation (Comité de Protection des Personnes Est IV DC-2012-1693). Genomic DNA was extracted from blood by standard methods. As nemaline myopathy (NM) is genetically heterogeneous and
as the immense size of the nebulin gene significantly impedes classical sequences approaches [12], we performed exome sequencing on 5 μg of genomic DNA from the patients and their parents as in Böhm et al. [15]. Exome sequencing was performed at the BGI (Shenzhen) on a Hiseq 2000 (Illumina) by using the Agilent 44 M v2 SureSelect Exon enrichment kit. Variant calling was done with the SOAP software. Variants filtering and prioritization were performed by comparison with SNP databases and with the VaRank program [27]. We discarded polymorphisms with a minor allele frequency (MAF) of more than 0.5% and excluded all variants with a frequency <20% of the total reads for a specific position. Additionally dHPLC and Sanger sequencing was performed in 7 patients as reported in Lehtokari et al. [17]. The mutations are reported according to the coding sequence of the nebulin cDNA reference sequence NM_001164508.1, and its translation.

RT-PCR
RNA was extracted from muscle biopsies with TRI-Reagent (Sigma), and cDNA was reverse transcribed using the SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers. The PCR fragments of selected cDNA regions were cloned into the pGEM-T easy vector (Promega) and transformed into E. coli DH5α cells. Plasmid DNA was then extracted from single colonies and Sanger sequenced.

Morphological studies
An open muscle biopsy was performed in all patients after informed consent. Age at biopsy varied from 29 weeks of adjusted gestational age to 21 years. The biopsied muscle is reported in Table 1 and was deltoid in 9 patients (P1, P2, P3, P5, P6, P9, P10, P13, and P14) and vastus lateralis in 5 (P4, P7, P8, P11, and P12). In order to make a precise and comparative study of muscle biopsy findings in Group 1 we standardized the age of new-borns calculating their ‘gestational adjusted age’ as described in Shichiji et al. [28] (Table 1). Samples were analysed in our research laboratory at the Myology Institute in Paris or in the Neuropathology laboratory of FLENI Institute and Garrahan Hospital in Buenos Aires, Argentina. For conventional histochemical techniques 10 μm thick cryostat sections were stained with haematoxylin and eosin (H&E), modified Gomori trichrome (mGT), Periodic acid Schiff technique (PAS), Oil red O, reduced nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH), cytochrome c oxidase (COX), and adenosine triphosphatase (ATPase) preincubated at pH 9.4, 4.63, 4.35. Digital photographs of each biopsy were obtained with a Zeiss AxioCam HRc linked to a Zeiss Axiosoplan Bright Field Microscope and processed with the Axio Vision 4.4 software (Zeiss, Germany). The fibre type pattern was determined by counting 1000 fibres from each patient in ATPase 9.4 and 4.35 reactions, and by calculating the percentage of type 1 and type 2 fibres.

Based on our experience, and the fibre type proportion reported in the literature regarding the muscle analysed, we considered type 1 fibres predominance to be present when there were more than of 60% type 1 fibre in deltoid muscles, and more than 40% in vastus lateralis muscle [29]. Fibre type distribution in G1 patients was analysed comparing the data on muscle fibre patterns during the main phases of skeletal muscle development obtained from individual with no neuromuscular disorder [30,31]. Moreover, where possible, we analysed fibre type proportion in age-matched control biopsies corresponding to G2, and G3 patients.

For the analysis of the proportion of fibres with rods, 800–1000 fibres of the muscle sections of each patient were analysed, and the percentage of fibres appearing with and without rods on the total number of fibres of a muscle sections was calculated; four consecutive, non-overlapping fields were counted. In addition, a classification of the rods and their pattern was effectuated. We defined the rods as being cytoplasmic when localised mainly inside the fibres sparing the subsarcosomal areas, scattered when they were randomly distributed in the muscle fibre, diffuse, when several small rods were distributed across the whole fibres homogenously occupying the majority of their area, central when distributed mainly in the centre of the cytoplasm, and subsarcolemmal when they were localized in a compact manner close to the fibre membrane (as clusters). We also evaluated the shape of nemaline bodies being mainly globular/ovoid, squared, or elongated.

Immunohistochemistry and immunofluorescence
Frozen muscle samples for immunohistochemical and immunofluorescence analyses were available for 7 patients (P1, P6, P7, P9, P10, P11, and P14). Myosin heavy chain fast (NCL-MHCF, Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom), myosin heavy chain slow (NCL-MHCS, Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom), myosin heavy chain developmental (NCL-MHCD, Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom), myosin heavy chain neontal (NCL-MHCn, Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom). Antibodies were visualized using immunoperoxidase techniques [28]. Myosin alpha and beta-slow heavy chain, fast 2A heavy chain, and 2X myosin heavy chain (BA-D5, SC-71, and 6H1, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA) immunofluorescence were assessed on 10-μm-thick cryosections over night at 4°C. Subsequently, sections were incubated with appropriate
| Patient sex | Ethnic origin | Age at onset (Gestational age) | Biopsied muscle | Morphological methods (Functional studies) | Clinical phenotype | Permanent mechanical ventilation/age | NEB mutation: nucleotide/protein change | Effect of the mutations | Reference |
|-------------|---------------|-------------------------------|------------------|------------------------------------------|---------------------|------------------------------------|---------------------------------------|----------------------------------|-----------|
| P1, F, deceased at 10 days | French Caucasian, Yes | Antenatal (38 weeks) | Deltoid | IHC, IF, EM (Yes) | Group 1/Severe congenital nemaline myopathy. Polyhydramnios, foetal akinesia. Severe global hypotonia, respiratory distress, arthrogryposis, hip hyperlaxity, club feet, and dysmorphic features. | Yes From birth | ex45; c.5574C > G; p.Tyr1858Stop; int122; c.19101 + 5G > A; p.Leu6333_Glu6367del | exon 45: nonsense mRNA decay (by RTPCR) and sequencing cDNA | Böhm et al., [15] |
| P2, F, deceased at 1 month | French Jewish (Ashkenazi) Yes | Antenatal (36 weeks) | Deltoid | IHC, EM (No) | Group 1/Severe congenital nemaline myopathy. Polyhydramnios, foetal akinesia. Severe global hypotonia, respiratory distress, arthrogryposis, club feet and dysmorphic features. | Yes From birth | homozygous deletion of exon 55; c.7432 + 1916_7535 + 372del p.Arg2478_Asp2512del | Ashkenazi founder mutation; deletion of exon 55 | Lehtokari et al., [26] |
| P3, M, deceased at 6 days | French Caucasian, No | Antenatal (38 weeks) | Deltoid | IHC, EM (No) | Group 1/Severe congenital nemaline myopathy. Polyhydramnios, foetal akinesia. Severe global hypotonia, respiratory distress, arthrogryposis, club feet, and dysmorphic features. | Yes From birth | ex86 (triplicated region); c.13066delT; p.Tyr4356Thrfs*18 ex110; c.17535G > A; p.Glu5845Glu | exon 86: frameshift mutation leading to either truncation or degradation. exon 110: splice site mutation | Present paper |
| P4, M, deceased at 5 days, Brother of P5 | French Caucasian, Yes | Antenatal (29 weeks) | Vastus lateralis | IHC (No) | Group 1/Severe congenital nemaline myopathy. Polyhydramnios, foetal akinesia. Severe global hypotonia, absence of spontaneous movements at birth, respiratory distress, macrosomy and macrocephaly. | Yes From birth | ex177; c.24686_24687del; p.Glu8229_Glu8230del ex163; c.23420_23421del; p.Arg7807Serfs*16 | both mutations truncating/degrading | Pelin et al., [4] |
| P5, M, deceased at 29 days, Brother of P4 | French Caucasian, Yes | Antenatal (36 weeks) | Deltoid | IHC, EM (No) | Group 1/Severe congenital nemaline myopathy. Polyhydramnios, macrosomy. Severe global hypotonia, respiratory distress, reduced spontaneous movements, ptosis, arthrogryposis, hypertrichosis, and macrocephaly. | Yes From birth | ex177; c.24686_24687del; p.Glu8229_Glu8230del ex163; c.23420_23421del; p.Arg7807Serfs*16 | both mutations truncating/degrading | Pelin et al., [4] |
| P6, M, deceased at 5 months | French Caucasian, No | Birth (39 weeks) | Deltoid | IHC, IF, EM (No) | Group 2/Intermediate congenital nemaline myopathy. Hypotonia and poor spontaneous | Yes From 1 month | ex6; c.300dup; p.Tyr1016*5 int49, c.6496-G > A; p.2166_2234del | exon 6: truncating/degrading exon 50 skipping by RT and sequencing cDNA | Present paper |
| Patient | Ethnicity, Sex | Age at Diagnosis | Clinical Features | Genetic Findings | Additional Information |
|---------|----------------|------------------|-------------------|----------------|------------------------|
| P7, F, African, Yes | 11 days (39 weeks) | Vastus lateralis 6 months | Movements at birth. At one month respiratory distress and deglutition problems. Elongated face. High-arched palate. Low-set ears. Facial diplegia. | Group 2/Intermediate congenital nemaline myopathy. Apparently normal at birth. Successively hypotonia and deglutition problems. At 1.5 months development of progressive respiratory failure followed by recuperated cardiac arrest. | Yes 2 months | trunctating/degrading | Present paper |
| P8, M 5 yrs, Argentinian, No | 6 months (40 weeks) | Vastus lateralis 6 months | Movements at birth. At one month respiratory distress and deglutition problems. Elongated face. High-arched palate. Low-set ears. Facial diplegia. | Group 2/Intermediate congenital nemaline myopathy Hypotonia, motor delay. High-arched palate. Proximal and distal muscle weakness. Retractions of fingers. Mild hyperlaxity. At 1 year development of progressive respiratory involvement necessitating tracheostomy. | Yes 1 month | RTPCR and Sanger sequencing of cDNA showed that instead of 105 nt, exon 139 contains only 34 nt: frameshift and a premature stop codon. Exon 172; truncating/degrading | Present paper |
| P9, M 11 yrs, French Caucasian, Yes | Birth (39 weeks) | Deltoid 10 months | Movements at birth. At one month respiratory distress and deglutition problems. Elongated face. High-arched palate. Low-set ears. Facial diplegia. | Group 2/Intermediate congenital nemaline myopathy. Shortly after birth severe respiratory failure. Tracheostomy and gastrostomy at five months. Facial diplegia, drooling, deglutition problems. Axial hypotonia and weakness of all limb muscles. | Yes 5 months | RTPCR and Sanger sequencing of cDNA showed that instead of 105 nt, exon 139 contains only 34 nt: frameshift and a premature stop codon. Exon 172; truncating/degrading | Present paper |
| P10, M 17 yrs, French Caucasian, No | 6 years (At term) | Deltoid 6 years | Movements at birth. At one month respiratory distress and deglutition problems. Elongated face. High-arched palate. Low-set ears. Facial diplegia. | Group 3/Typical congenital nemaline myopathy. Global hypotonia, deglutition problems. Proximal muscle weakness. Facial diplegia. Nasal voice. Mild respiratory problems. | No | intron 43: c.5343 + 5G > A p.Arg1747 Thr1778del ex153; c.22273del p.Val7425Serfs*49 | Present paper |
| Patient | Age | Ethnicity | Years Old | Years Old (At term) | Muscle Group | IHC, EM | Group | Clinical Features |
|---------|-----|-----------|-----------|--------------------|--------------|---------|-------|-----------------|
| P11, M, 19 yrs | 1 yrs | French Antillean | No | Vastus lateralis 6 yrs | IHC, IF, EM (Yes) | Group 3/Typical congenital nemaline myopathy. Hypotonia and feeding difficulties. Delayed motor milestones. Facial weakness with open mouth. Axial and limb girdle proximal weakness. Mild respiratory involvement treated with discontinuous non-invasive ventilation. | ex175; c.24579G > A, p.Ser8193Ser ex119; c.18676C > T, p.Gln6226* | Present paper |
| P12, M, 20 yrs | 2-3 yrs | French Caucasian | No | Vastus lateralis 6 yrs | IHC, EM (No) | Group 3/Typical congenital nemaline myopathy. Difficulties in running and rising stairs. Facial weakness with open mouth. Mild upper and lower limb girdle weakness. | int155; c.22591-3C > G; p.7531Val_Ser7564del; ex148; c.21796_21810delinsT; p.Pro7266fs*30 | Present paper |
| P13, F, 52 yrs | 6 yrs | French Caucasian | No | Deltoid 18 years | IHC, EM (No) | Group 3/Mild congenital nemaline myopathy. Difficulties in sport activities in school. Bilateral pes cavus. Presence of mild upper girdle muscle weakness. Diffuse muscle pain. | No | Present paper |
| P14, F, 37 yrs | 2 yrs | French Caucasian | No | Deltoid 21 yrs | IHC, IF, EM (Yes) | Group 3/Mild congenital nemaline myopathy. Frequent falls. Difficulties in running, rising stairs. Jaw contractures. Nasal voice. Elongated face. Respiratory involvement. Axial weakness with difficulties in neck flexion. Asymmetrical distal weakness with foot drop (right > left). Proximo-distal weakness. | No | Present paper |
conjugated secondary antibodies for one hour (Alexa Fluor-488 green goat anti-rabbit antibody, and Alexa Fluor-594 red goat anti-mouse antibody, Molecular Probes, Cergy Pontoise, France). A set of control slides was prepared with omission of the primary antibodies.

Electron microscopy
Detailed electron microscopy analysis was prospectively performed in thirteen patients. Small muscle specimens were fixed with glutaraldehyde (2.5%, pH 7.4), post fixed with osmium tetroxide (2%), dehydrated and embedded in resin (EMBed-812, Electron Microscopy Sciences, USA). Ultra-thin sections from at least three small blocks from each patient were stained with uranyl acetate and lead citrate. The grids were observed using a Philips CM120 electron microscope (80 kV; Philips Electronics NV, Eindhoven, The Netherlands) and were photo documented using a Morada camera (Soft Imaging System, France).

Muscle contractility experiments
To investigate whether the contractile performance is affected in muscle biopsies from patients with mutations in the nebulin gene, we performed skinned muscle fibre contractility experiments. Small strips were dissected from muscle biopsies of patients P1 (Group 1), P7, P8 and P9 (Group 2), and P10, P11 and P14 (Group 3) and were skinned overnight as described previously [32]. The skinning procedure renders the membranous structures in the muscle fibres permeable, which enables activation of the myofilaments with exogenous Ca2+. Preparations in the muscle fibres permeable, which enables activation were slacked to 70% of their original length followed by a rapid restretch to the original length after 30 milliseconds. This procedure allows the force to redevelop from zero [33]. The rate of tension redevelopment was calculated by fitting a bi-exponential through the force redevelopment curve. The first-order rate constant $k_1$ reflects crossbridge cycling kinetics and was therefore used in the analyses [34].

Myosin heavy chain composition of bundles used for contractility experiments
For determination of the myosin heavy chain isoform composition of the muscle fibre preparations we used specialized SDS-PAGE [32]. In brief, muscles fibres were denatured by boiling for 2 minutes in SDS sample buffer. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acrylamide (pH 8.7) with 30% glycerol (v/v). The gels were run for 24 h at 15°C and a constant voltage of 275 V. Lastly, the gels were silver-stained, scanned, and analysed with One-D scan EX software (Scanalytics Inc., Rockville, MD, USA).

The ethical committee of La Pitié-Salpêtrière Hospital (CCPPRB) approved this study.

Results
Clinical findings
Five patients were female and 9 were male. Clinical summary, laboratory features, a complete list of morphological methods, functional studies applied to muscle biopsies, and genetic characterization of all patients is provided in Table 1. Patient 6 and 7 were classified to be a part of intermediate congenital nemaline myopathy group because they were breathing and moving at birth. However, these patients developed soon after birth a severe clinical picture and they were never able to achieve respiratory independence and/or ambulation. They eventually deceased at 5 months, and 32 months respectively. They therefore present a phenotype in between a severe and an intermediate congenital nemaline myopathy.
Molecular data
To identify the genetic cause in this cohort of patients, we performed exome enrichment and sequencing on genomic DNA from the patients and their parents. Exome sequencing allows a rapid and parallel screening of most human genes, and is suitable and efficient for the diagnosis of neuromuscular diseases and the analysis of large genes such as NEB, frequently mutated in NM [15]. This approach also covers any newly discovered gene for the disorder. For all patients presented here we found known or novel variations in the NEB gene. These changes were confirmed by Sanger sequencing, and their familial segregation validated when parent DNA available. In all the patients, at least one of the two compound heterozygous mutations was a truncating mutation (frameshift or nonsense mutation) leading to protein truncation or degradation, or both (Table 1). The second mutations were, either a frameshift mutation, deletion of several amino acids in frame (splice site mutations), a non-conservative missense change close to an actin binding site (P13), or synonymous variants (frameshift or nonsense mutation) leading to protein truncation or degradation, or both (Table 1). The second mutations were, either a frameshift mutation, deletion of several amino acids in frame (splice site mutations), a non-conservative missense change close to an actin binding site (P13), or synonymous variants (frameshift or nonsense mutation) leading to protein truncation or degradation, or both (Table 1).

Morphological findings
Histological and histochemical features
Group 1
A similar morphological pattern characterised by marked fibre size variability was noted in all biopsies from G1. We constantly identified two populations of fibres; the first characterized by muscle fibres of predictably normal or slightly augmented size, and the second one consisting of severely atrophic fibres (Figure 1A; arrows). Small rounded/globular inclusions staining red by the mGT, corresponding to nemaline bodies, were present in less than half of muscle fibres (Figure 1A). Nemaline bodies occupied both normal-sized and atrophic fibres (Figure 1A). They presented a dispersed or, more often, a subsarcolemmal distribution (Figure 1A). Some atrophic fibres appeared completely occupied by nemaline bodies. The percentage of muscle fibres harbouring rods was to 22% to 33% (Figure 1D, Group 1, red). The oxidative enzyme reactions revealed some alteration of the intermyofibrillar network, probably corresponding to rod accumulation or myofibrillar disorganization with oxidative techniques. ATPase techniques showed type 1 fibre predominance in G1. The majority of fibres staining differently from type 1 are probably undifferentiated fibres (not shown).

Group 2
The morphological pattern found in this group was heterogeneous compared with G1 biopsies. While in P6, and P7 samples we noticed the presence of two populations of muscle fibres (predictably normal size and severely atrophic) (Figure 1B; arrows), P8, and P9 showed a mild variation of fibres size without any particular topography (not shown). Conversely to G1 nemaline bodies were present in the vast majority of fibres (mean: 95% to 100%; Figure 1D, Group 2, blue) and presented a variable shape varying from oval to elongated. The oxidative enzyme reactions revealed some alteration of the intermyofibrillar network, probably corresponding to rod accumulation or myofibrillar disorganization with oxidative techniques. ATPase techniques showed type 1 fibre predominance in P6, and P8. Type 1 uniformity was noted in P7 and P9.

Group 3
In this group we noticed mild variation of fibre size except in P12 where some atrophic rounded fibres were identified (not shown). Nemaline bodies presented a constant elongated shape and formed well separated clusters both in subsarcolemmal and cytoplasmic areas (Figure 1C). There was a large variability in the percentage of fibres harbouring rods. While P10, P13 and P14 presented rods in almost all fibres (97-99%), P11 had 60% and P12 31% of fibres with rods, respectively (Figure 1D, Group 3, green). ATPase techniques showed almost complete type 1 uniformity in G3 muscle biopsies. The areas of muscle fibres containing rods lacked ATPase staining.

Summary
Overall, severe NM was associated with a fibre size variability, presence of rods in about 1/3 of fibres, and a high percentage of undifferentiated fibres. By contrast a higher percentage of fibres with rods, and a type 1 fibre predominance/uniformity was noted in the intermediate and typical NM patients. The amount of nemaline bodies seems to be inversely related to clinical severity.

Immunohistochemistry and immunofluorescence
Group 1
In P1 we identified many fibres expressing developmental, neonatal, fast and/or slow myosin. Immunofluorescence studies showed absence of type 2X myosin.

Group 2
In P6 we identified many fibres co-expressing slow and fast myosins. Developmental and neonatal myosins were
expressed in a minority of fibres. In P8 we identified occasional fibres expressing developmental myosin and less than 5% of fibres expressing neonatal myosin; there was partial co-expression of fast and slow myosins. P9 showed unique expression of slow myosin both in immunohistochemical, and immunofluorescence studies. Group 3

P10, P11, and P14 showed slow myosin uniformity using both techniques. No fibres expressing developmental and/or neonatal myosins were noted.

Electron microscopy

Group 1

The prominent ultrastructural finding in all G1 patients was the diffuse myofibrillar dissociation. The myofibrils appeared thinner, and smaller than in age-matched controls (Figure 2A). The latter suggested either a defect in sarcomeric structure establishment either the lost of it. Remnants of sarcomeres were intermingled with organelles (e.g. mitochondria) or glycogen granules (Figure 2A). Globular/ovoid nemaline bodies were scattered or distributed in subsarcolemmal and perinuclear areas (Figure 2B, and C). Some fibres were completely occupied by them (Figure 2C). At higher magnification globular/ovoid rods presented thin filaments projecting from their thinnest edges (Figure 2D).

Group 2

This group presented a milder degree of myofibrillar dissociation accompanying dispersed or clustered nemaline bodies (Figure 3A, and 3B). Rods showed the typical lattice structure resembling Z-disc material at very high magnification (Figure 3C). In P9 we noticed the presence of some typical cytoplasmic bodies with a dense core, and a clear halo of fine filaments (not shown).

Group 3

We found a homogenous picture characterised by the presence of well-separated clusters of subsarcolemmal (Figure 4A), perinuclear, and less often cytoplasmic nemaline bodies (Figure 4B). The rods were always surrounded by thin filaments and amorphous material. The sarcomeric structure was overall conserved (Figure 4A, and 4B).

Summary

Taken together, our results suggest that myofibrillar dissociation correlated with clinical severity.
Muscle contractility experiments

The maximal force generation capacity of the muscle fibre preparations was normalized to their cross-sectional area (i.e. maximal active tension) (Figure 5). An overview of the maximal active tension and the rate of tension redevelopment of the muscle fibres are summarized in Table 2. Data are presented as mean ± SEM.

Myosin heavy chain analyses

The myosin heavy chain (MHC) gel electrophoresis experiments revealed that muscle preparations from biopsies from patient P1 contained both neonatal isoforms as well as type 1 and type 2A isoforms: (9.2 ± 2.8% MHC neonatal; 40.0 ± 3.2% MHC type 1; 50.8 ± 1.3% MHC type 2A). Muscle preparations from biopsies of P7 and P8 (group 2) contained both MHC type 1 and MHC type 2A isoforms: P7 (66.6 ± 6.6% MHC type 1 and 33.4 ± 6.6% MHC type 2A) and P8 (40.8 ± 12.0% MHC type 1 and 59.2 ± 12.0% MHC type 2A), respectively. All other patient biopsies (P9, P10, P11 and P14) showed exclusively myosin heavy chain type 1 isoforms.

Discussion

In our tertiary Center for Neuromuscular Disorders we perform a detailed clinical, morphological, and genetic analysis of large cohorts of patients presenting NM. Due to the genetic heterogeneity of NM, and the difficulties encountered in the molecular screening of the ‘giant’ NEB gene [15,35], in France and Finland an integrated approach combining next generation sequencing and dHPLC/Sanger sequencing was set-up. Our strategy allowed the identification of ten new families harbouring NEB mutations. All patients presented autosomal recessive pattern of inheritance and either homozygous or compound heterozygous pathogenic variants. Our results confirm that NEB is one of the most frequently mutated NM genes, accounting for almost half of the genetically identified NM patients screened for the known genes associated with NM [3-11].

We undertook a detailed clinical histological, and, when possible, muscle functional analysis in a cohort of fourteen NEB-mutated subjects whose muscle biopsy was available in our laboratory. We comment on relevant findings encountered.

NEB-mutated patients revealed a wide pathological spectrum and showed recurrent morphological pattern with some overlap among the clinical groups. Lethal/severe NM subjects (G1) presented: high degree of myofibrillar dissociation and smallness revealed by electron microscopy, scattered globular/ovoid nemaline bodies occupying one third of muscle fibres, and absence of type 1 predominance with myosins ATPases techniques (Figure 2). Intermediate
congenital myopathy patients (G2) showed features similar to G1 in P6 and P7, even though the presented higher percentage of rods and type 1 predominance, and well-separated clusters of rods associated with type 1 predominance or uniformity in the other patients. It is noteworthy that P6 and P7 deceased at 5 months, and 2 and a half year, respectively. For this reason these patients could be considered as a ‘Longer survivor severe congenital NM’ subgroup due to their ‘midway’ clinical and morphological features between G1 and G2. Group 3 had a preserved sarcomeric structure with clusters of elongated rods invariantly associated with type 1 predominance/uniformity.

In summary we show a large pathological spectrum ranging from severely damaged sarcomeres with scattered nemaline bodies to globally preserved muscle with clusters of well separated rods occupying the majority of myofibres. Interestingly the degree of sarcomeric disruption directly

Figure 3 Electron microscopy for group G2-intermediate NM. A. P7. Globular ovoid nemaline bodies associated with partial sarcomeric disarray strongly resembling the pathologic picture of G1. B. P9. Presence of elongated nemaline bodies in perinuclear and subsarcolemmal areas. The latter are well separated from well-preserved sarcomeric structure. C. P9. Higher magnification of an elongated nemaline body. The typical periodic net structure composing rods is clearly recognisable. Thin filament spread out of the thinnest edges of rods. Sparse thin filaments are found around the rods intermingled with glycogen granules. Original magnification: A. 11,000x B. 6,400x C. 94,000x.

Figure 4 Electron microscopy for group G3-typical-mild NM. A. P10, G3. Presence of globally preserved sarcomeric structure associated with a cluster of elongated nemaline bodies in the subsarcolemmal areas of muscle fibres. P10. B. P14. Well-delimited clusters of rods surrounded by thin filaments. Original magnification: A. 9,000x B. 8,200x.
related to clinical severity whereas the number of rod invaded fibres seemed to be inversely correlated.

We undertook this study to search for genotype-phenotype correlations. Taken together, the vast majority of NEB mutations are predicted to lead to a degradation and or denaturation of many nebulin isoforms. Markedly reduced amounts of nebulin in muscle samples from patients homozygous for exon 55 deletions have been reported previously [36]. P2 in this study is homozygous for the same exon 55 deletion, resulting in an in-frame deletion of 35 amino acids, and subsequent protein degradation. Nonsense mRNA-mediated decay was demonstrated by RT-PCR in P1, who carries a heterozygous nonsense mutation in exon 45. P1 is also heterozygous for a splice site mutation in intron 122, which was shown to cause exon 122 skipping, resulting in in-frame deletion of 35 amino acids [15]. Splice site mutations are, however, often “leaky”, i.e. some transcripts are spliced correctly, whereas others are incorrectly spliced. Therefore, it seems plausible that P1, as well as the other patients with splice site mutations (P3, P6, P8, P10, P11, P12 and P14) express small amounts of normal nebulin in their muscles. Markedly reduced amounts of nebulin in muscle have been reported in one patient compound heterozygous for a splice site mutation and a frameshift mutation in constitutively expressed exons [37]. The mutations in alternatively spliced exons (exons 174, 175, 176 and 177) only affect nebulin isoforms expressing these exons, leaving other isoforms unaffected. Consequently reduced amounts of nebulin and absence of some isoforms, precluding an appropriate thin filaments assembly, might be responsible for the drastic myofibrillar dissociation revealed in G1 [Figure 4]. Functional studies confirmed that G1 patients’ muscle fibers generate very low force. Although one could argue that age confounded our findings, previous work from our group revealed no major differences in the contractile performance of myofibres isolated from young (age 2–5 years) versus adult (age 20–30) human control biopsies [37]. Note that G1 patients’ muscle fibres expressed only very low levels of neonatal myosin heavy chain isoforms; such low levels are unlikely to account for the major loss of force in this patient. We therefore suggest that nebulin degradation/absence translates into sarcomeric disarray and/or altered actomyosin interaction. This could be responsible for low force generation producing global hypotonia, muscle akinesia, and arthrogryposis. Concordantly, a recent study reported that low levels of nebulin in skeletal muscle are probably responsible for the foetal akinesia and arthrogryposis sequence phenotype [36].

How nebulin deficiency results in nemaline bodies formation is yet to be understood. Some authors suggested that a truncated nebulin would disrupt myofibrillar connectivity leading to Z-disc displacement and, eventually, rods formation [38]. Analysis of nemaline bodies features in our cohort revealed differences in shape across the three groups. These tended to be globular/ovoid in G1, elongated in the other groups. The specificity of this finding is uncertain. What is more striking is that in P8, and P9 from G2 and in all G3 patients, the majority of fibres harboured rod clusters confined to subsarcolemmal and/or perinuclear areas. The myofibrillar structure surrounding them was overall preserved. Some unknown process could try to circumscribe the protein aggregates and avoid a perturbation of muscle contraction. This could explain why muscle fibers in G2 and G3 patients showed a better contractile performance, probably translating into a milder clinical phenotype. It is tempting to speculate that mutations encountered in these groups affect only specific nebulin isoforms, which is certainly true for patients P7, P8, P9, P11, and P14 who all have at least one mutation in an alternatively spliced exon. In this scenario, residual normal isoforms could allow a proper thin filament

### Table 2 Muscle contractility data of permeabilized fibers from nemaline myopathy patients with NEB mutations

|                        | P1  | P7  | P8  | P9  | P10 | P11 | P14 |
|------------------------|-----|-----|-----|-----|-----|-----|-----|
| Maximal active tension (mN/mm²) | 8.1 ± 1.5 | 18.8 ± 2.7 | 76.9 ± 100 | 90.9 ± 105 | 55.5 ± 7.1 | 102.2 ± 21.2 | 61.4 ± 122 |
| Rate of tension redevelopment (k, (s⁻¹)) | 5.1 ± 0.4 | 4.5 ± 0.7 | 5.0 ± 0.5 | 3.4 ± 0.2 | 3.6 ± 0.2 | 4.0 ± 0.6 | 3.8 ± 0.5 |
assembly, while the altered ones might be responsible for protein aggregation/rod formation. If this turned to be true, the presence of rods might be considered unrelated to muscle contractility disturbances. Following this reasoning we could imagine that an additive effect of degraded nebulin isoforms would determine clinical severity.

Type 1 predominance or type uniformity has been reported as a very common feature associated with NM, and many other structural congenital myopathies [39]. It is speculated that this is due to a disturbance of fibre differentiation before phase three of muscle differentiation (35th weeks) [40]. A severe NEB mutated family composed by two brothers has been reported as not having type 1 fibre predominance. However, biopsied muscle and age at muscle biopsy were not specified [41]. In the present study we performed type fibre distribution analysis on ATPase techniques. Surprisingly, all G1 patients failed to show type 1 predominance and constantly showed absence of 2B fibers. Immunostainings for different myosins isoforms (foetal neonatal, fast and slow) revealed a certain degree of myosin isoforms co-expression in numerous fibres suggesting the presence of undifferentiated fibers. This contrasted with the other groups where type 1 predominance/uniformity was present. This finding suggests that G1 NEB-mutated patients were not able to switch towards type 1 predominance due to a possible alteration of muscle maturation. It is tempting to speculate that aberrations in fibre-typing, and absence of type 2B seen with ATPase techniques are due to changes in isoforms imbalance more than related to age at muscle biopsy. Additionally the presence of high percentages of undifferentiated fibres encountered in G1 might turn out to be an important prognostic factor. In fact muscle biopsy analysis of new-borns presenting arthrogryposis and a pathological picture characterised by sarcomeric dissociation, scattered nemaline bodies absence of type 1 predominance and type 2B fibres with ATPases techniques might orientate toward NEB mutations. This is something distinctive from other severe form of congenital myopathies commonly showing type 1 predominance as a prominent feature [42]. In particular we recently demonstrated that MTM1-mutated boys presenting an extremely severe clinical phenotype all had type 1 predominance in their biopsies, regardless of the biopsied muscle, and the gestational age [28].

In conclusion, this study adds on the clinical, morphological and functional characterization of the most recurrent form of NM. We assessed morphological and functional heterogeneity in NEB-mutated NM patients and identified a correlation between disease severity on the one hand, and ultrastructural myofibrillar abnormalities and contractility on the other. We suggest that myofibrillar dissociation and smallness is a primary defect causing the disease while nemaline bodies could be due to a collateral mechanism.

Competing interests
The authors declare that they have no competing interests.

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