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Workshop report

# 1st ENMC European meeting: The EURO-NMD pathology working group *Recommended Standards for Muscle Pathology* Amsterdam, The Netherlands, 7 December 2018

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## Introduction

European Reference Networks, ERNs, were established by the EU commission in December 2016 in order to enhance the access to accurate diagnostics and disease management for patients with rare disorders. EURO-NMD is the dedicated ERN for neuromuscular patients since all the hundreds of different neuromuscular disorders are rare diseases. EURO-NMD has an established structure with 61 centres currently members of the EURO-NMD organisation and with 10 different working groups (WG) on different areas of the clinical aspects of neuromuscular diseases. The 'Neuromuscular Pathology Working Group' is one of these WGs with the explicit task of increasing availability of high quality muscle and nerve pathology diagnostics in Europe for the benefit of the patients. Regular members of the Neuromuscular Pathology WG are: Bjarne Udd chair, Anders Oldfors vice-chair, Montse Olive, Norma Romero, Werner Stenzel, Teresina Evangelista, and the patient representative Massimo Marra.

Based on a survey the WG had performed among the 61 partner centres in 2017, the current methods used in the corresponding pathology laboratories are highly variable. Since all the different current practices on how to perform diagnostic muscle pathology are variable, one of the first tasks of the WG was to produce recommended standards for muscle pathology procedure and analysis. Standardized and harmonized procedures are needed not only for having a secured level of quality among the European centres but also for the exchange of muscle biopsy results of diagnostic dilemmas to be reviewed by the expert panels of EURO-NMD.

From the above it is easy to see why international consensus among the EURO-NMD centers, and beyond, is urgently needed on how to perform muscle biopsies and how to process them technically.

During 2018 the WG members had already worked on and discussed a number of questions of which stainings of muscle biopsy specimens should be included in the recommended standards for all centres of the EURO-NMD. The result of that work was a preliminary version of such recommendations, which was then sent out to 32 muscle pathology experts in different EURO-NMD centers who were listed as being interested to take part in the WG activities. 16 of these pathologists responded with further comments on the draft for recommended standards. In order to continue the final discussion of these responses and to decide on a final version of the Recommended Standards, a one-day ENMC sponsored meeting was approved and scheduled for 7 December 2018, located at the Schiphol airport meeting facilities in Amsterdam, the Netherlands.

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For the comprehensive evaluation and decisions during the meeting the WG was enlarged by the external muscle pathology experts: Caroline Sewry, Hans-Hilmar Goebel, Benno Kusters and Martin Lammens, and a consensus was achieved on recommended standard for muscle pathology.

## EURO-NMD Recommended Standards for Muscle Biopsies

### 1. How to secure best tissue quality

Muscle biopsy samples should be handled very carefully at all stages to avoid artefacts. For cryostat sections, the samples need to be of a size and shape that allows an easy orientation in order to obtain transverse sections.

The samples for cryostat section should be frozen in isopentane cooled in liquid nitrogen; this should be done on site, immediately after collection. The time lapse between the collection of the specimen and freezing should not exceed 2 h, or less than 15 min for biochemical studies of enzyme activities.

Frozen samples should be transported in dry ice or liquid nitrogen and stored at −80 °C. Any thawing during cryosectioning or during other procedures after freezing will damage the sample.

Before storage, specimens fixed in glutaraldehyde should be embedded in the resin for EM.

### 2. Recommended standard lab methods:

### 2.1. Specimen preparation

When processing the biopsy, samples for the following essential techniques should always be available:

- Histology
- Enzyme histochemistry **EHC**
- Immunohistochemistry **IHC**
- Western blotting **WB**
- Semithin sections of resin embedded muscle
- Electron microscopy

**How to proceed:**

- Mounted muscle sample - snap frozen in isopentane *cooled in liquid nitrogen* for 30 sec (longer in the case of a big sample), performing small circular movements.
- Glutaraldehyde-fixed muscle for electron microscopy - fixation best done immediately after removal of the tissue
- (+/− Formalin-fixed, paraffin-embedded muscle)
- Separate frozen muscle (without isopentane) for specific methods such as: Western blot, RNA-cDNA, enzyme biochemistry and +/− secondary paraffin embedding
- Optional: skin biopsy for fibroblast culture, muscle tissue for myoblast culture (specific protocols), for biochemical enzyme assays, and for mitochondrial respiratory chain enzyme activities in pediatric biopsies

**Cryostat Sections (Snap frozen sections)**

- Cryostat at −23 to −25 °C
- For histology, histochemistry and immunohistochemistry 7–10 μm sections

### 2.2. Routine stains for all new biopsies (frozen tissue)

2.2.1. Conventional histology, with at least 2 serial sections from 2 different levels for each of the following staining:
- H&E
- Gömöri Trichrome (modified)
- ORO / Sudan black
- PAS

2.2.2. Enzyme histochemistry
- NADH-TR
- COX-SDH
- Acid phosphatase
- ATPases: Type 1 and Type 2A, 2B and 2C fibres (pH 9.4, 4.6 and 4.3)
- Alternatively, for fiber typing: Myosin heavy chain IHC with antibodies to slow beta and fast IIA with hematoxylin counterstain for Fiber types I, IIA, IIX, and hybrids I+IIA (corresponding to 1–2A-2B-2C with ATPase)

2.2.3. Immunohistochemistry-routine
- Myosin heavy chain neonatal/fetal
- Myosin heavy chain developmental/embryonic
- Myosin heavy chain MyHC fast
- Myosin heavy chain MyHC slow/beta cardiac
- MHC-class1
- p62

2.2.4. Electron microscopy
To be used in samples without diagnostic findings by other methods, to clarify abnormalities observed or not visible on light microscopy. Particularly in: Congenital myopathies, unclear sarcoplasmic abnormalities, dysimmune, toxic, mitochondrial, metabolic myopathies and in neonatal muscle biopsies.

**Optional:** Neuromuscular junction (NMJ) in myasthenic syndromes

### 2.3. Recommended extended methods – context-dependent

Methods that should be available or accessible for all EURO–NMD pathology labs as needed:

2.3.1. Muscular dystrophy, congenital and progressive myopathies

**IHC:** For sarcolemmal protein defects (use Beta Spectrin as the positive control): Dystrophin: N, rod

and C domains, Utrophin, Sarcoglycans $\alpha$,$\beta$,$\gamma$,$\delta$, $\alpha$DG glycosylated (always together with beta dystroglycan), $\alpha$2-Laminin 80&300 kDa, Caveolin-3, Emerin, Telethonin, COL6 (use together with perlecan or COL IV as control). Pediatric biopsies: Laminin $\beta$1, Laminin $\gamma$1, Laminin $\alpha$5
**Optional:** nNOS
**WB:** DYS (at least 2 domains), dysferlin, Calpain3 (2 antibodies), $\alpha$DG (with $\beta$DG)
**Optional:** sarcoglycans

2.3.2. Immune mediated myopathies
**IHC:** p62, CD68, CD8, CD20, C5b-9, CD31
**EHC:** alkaline phosphatase
**Optional:** van Gieson, MHC-class2, CD45/CD3, CD169, CD138, MUM1, MxA, ISG15

2.3.3. Vacuolar and protein aggregate myopathies
**IHC:** p62, TDP-43, Lamp2, Dys1, MHC class1, C5b-9, Desmin, Myotilin, Ubiquitin
**EHC:** Menadione NBT without substrate
**Optional:** LC3, FHL1, Filamin-C, BAG3, HSP70, HSP90, $\alpha$B-Crystallin, CD68, CD45

2.3.4. Congenital myopathies
**IHC:** RYR1, DHPR, SERCA1+2, $\alpha$-actinin 2
**IF:** phalloidin
**Optional:** Sarcomeric and cardiac actin - for actinopathies using high salt for specificity

2.3.5. Mitochondrial myopathies
**EHC:** COX and SDH (both techniques combined and in isolation)
**Optional IHC:** subunits of the mitochondrial respiratory chain complexes I-V

2.3.6. Toxic myopathies
**IHC:** CD45, CD68, LC3

2.3.7. Ion channel myopathies
**Optional:** IHC: CLCN1, RYR1, DHPR, SERCA1, SERCA2

2.3.8. Glycogenoses
**EHC:** Phosphofructokinase, Phosphorylase, LDH, PAS-D
Semithin resin embedded sections: PAS
Biochemical analysis of enzyme defects, which cannot be stained

2.3.9. High CK and exercise intolerance, cramps
**EHC:** Phosphofructokinase, Phosphorylase, LDH, PAS-D
**IHC:** Dystrophin, sarcoglycans, utrophin, beta spectrin, caveolin 3, dysferlin, laminins
**WB:** calpain3 (2 antibodies), dysferlin

2.3.10. Amyloid myopathy
**HC:** Congo red
**IHC:** transthyretin, Immunoglobulin light chains $\kappa$, $\lambda$

2.3.11. Myopathies with affected neuromuscular junctions require special biopsies, i.e. motor point biopsies or entire very short muscles and optional EM.