



# Explorations Multi-omiques dans les maladies neuromusculaires

Ivana Dabaj

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Normandie Université

# THÈSE

Pour obtenir le diplôme de doctorat

Spécialité « SCIENCES DE LA VIE ET DE LA SANTE »

Préparée au sein de « Université de Rouen Normandie »

## Multimic investigations in neuromuscular diseases

Présentée et soutenue par  
**IVANA DABAJ**

Thèse soutenue le 12/01/2023  
devant le jury composé de

|      |            |             |  |                       |
|------|------------|-------------|--|-----------------------|
| M.   | JEROME     | AUSSEIL     | PROFESSEUR DES UNIVERSITES – PRATICIEN<br>HOSPITALIER<br>UNIVERSITE TOULOUSE 3 PAUL SABATIER | Rapporteur du jury    |
| M.   | PATRICK    | VAN BOGAERT | PROFESSEUR DES UNIVERSITES – PRATICIEN<br>HOSPITALIER<br>UNIVERSITE ANGERS                   | Rapporteur du jury    |
| M.   | STEPHANE   | ALLOUCHE    | PROFESSEUR DES UNIVERSITES – PRATICIEN<br>HOSPITALIER<br>UNIVERSITE DE CAEN                  | Membre du jury        |
| Mme. | EMMANUELLE | LAGRUE      | PRATICIEN HOSPITALIER<br>CHU DE TOURS  | Membre du jury        |
| Mme. | SOUMEYA    | BEKRI       | PROFESSEUR DES UNIVERSITES – PRATICIEN<br>HOSPITALIER<br>UNIVERSITE ROUEN NORMANDIE          | Directeur de thèse    |
| M.   | STEPHANE   | MARRET      | PROFESSEUR DES UNIVERSITES – PRATICIEN<br>HOSPITALIER<br>UNIVERSITE ROUEN NORMANDIE          | Co-directeur de thèse |

Thèse dirigée par **SOUMEYA BEKRI (CANCER AND BRAIN GENOMICS)** et **STEPHANE MARRET (CANCER AND BRAIN GENOMICS)**

*On dit qu'avant d'entrer dans la mer,  
une rivière tremble de peur.  
Elle regarde en arrière le chemin  
qu'elle a parcouru, depuis les sommets,  
les montagnes, la longue route sinueuse  
qui traverse des forêts et des villages,  
et voit devant elle un océan si vaste  
qu'y pénétrer ne paraît rien d'autre  
que devoir disparaître à jamais.  
Mais il n'y a pas d'autre moyen.  
La rivière ne peut pas revenir en arrière.  
Personne ne peut revenir en arrière.  
Revenir en arrière est impossible dans l'existence.  
La rivière a besoin de prendre le risque  
et d'entrer dans l'océan.  
Ce n'est qu'en entrant dans l'océan  
que la peur disparaîtra,  
parce que c'est alors seulement  
que la rivière saura qu'il ne s'agit pas  
de disparaître dans l'océan,  
mais de devenir océan.*

**“Le prophète” de Khalil Gibran**

## *Acknowledgments*

I would like to warmly thank Professor Stéphane MARRET, for inviting me to join his medical team in Rouen University Hospital and for believing in my clinical skills and experience. I am thankful for his generous proposition to do the PhD on neuromuscular disorders and for his continuous support as my chief of department and co-director of my PhD. My sincere thanks for the confidence that he constantly shows me. May he be assured of my deepest gratitude.

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## ***Dedication***

*To my daughter,*

*My husband,*

*My parents,*

*My friends*

## Explorations Multi-omiques dans les maladies neuromusculaires

### Résumé

Les maladies neuromusculaires pédiatriques sont relatives à des atteintes du muscle, de la jonction neuromusculaire, du nerf périphérique et de la corne antérieure. La multitude de sites, ainsi que l'hétérogénéité et la rareté de ces troubles, rendent leur diagnostic et leur traitement difficiles. Par conséquent, les efforts conjoints des chercheurs et des cliniciens sont nécessaires pour diagnostiquer et traiter les patients avec plus de précision en utilisant des outils de la biologie des systèmes. Cette approche permet d'analyser différents types de biomolécules et permet, ainsi, d'étudier de manière exhaustive les changements moléculaires en tenant compte des caractéristiques individuelles du patient (contexte clinique, environnement...). Il y a actuellement une recherche très active dans le domaine des maladies neuromusculaires qui a abouti à la mise à disposition de traitements innovants nécessitant une meilleure identification et stratification des patients pour améliorer la prise en charge. Les objectifs de cette thèse sont d'appliquer les techniques omiques afin d'étudier les deux maladies neuromusculaires les plus courantes (l'amyotrophie spinale infantile (SMA) et la myopathie de Duchenne), d'explorer leurs mécanismes physiopathologiques, d'identifier des biomarqueurs de diagnostic potentiels et de mieux stratifier les patients. Le second objectif est d'identifier des biomarqueurs pour suivre la réponse au traitement dans la SMA. Une étude métabolomique sur des biopsies musculaires de patients atteints de la maladie de Duchenne a révélé des changements significatifs dans les métabolismes énergétique et phospholipidique. Par ailleurs, l'étude métabolomique des plasma et LCR de patients SMA a confirmé la présence d'un remodelage métabolique profond chez les patients SMA. De plus, nous avons démontré que ces altérations sont plus prononcées dans le phénotype sévère. L'étude protéomique de ces échantillons est en cours. En conclusion, les outils de biologie des systèmes sont pertinents pour l'étude des maladies neuromusculaires car ils permettent la découverte de biomarqueurs, la compréhension de la physiopathologie et l'identification de nouvelles cibles thérapeutiques.

**Mots clés:** maladies neuromusculaires, omiques, médecine de précision, biologie des systèmes, métabolomique, protéomique, classification, biomarqueurs, amyotrophie spinale infantile, ASI, myopathie de Duchenne, DMD.

## Multiomics in neuromuscular disorders

### Abstract

Pediatric neuromuscular disorders encompass all childhood onset diseases affecting the muscle, the neuromuscular junction, peripheral nerve, and the anterior horn cell. The multitude of sites as well as the heterogeneity and rarity of these disorders, make their diagnosis and treatment challenging. Therefore, joint efforts of researchers and clinicians are mandatory to diagnose and treat patients more accurately using integrative systems biology tools. Such appealing approach uses high-throughput technologies to comprehensively study multilayered molecular changes. It is valuable in the neuromuscular diseases field where innovative treatments are being produced. The objectives of this thesis were to apply omics techniques to study the two most common neuromuscular disorders (Spinal muscular atrophy and Duchenne muscular dystrophy) and explore their pathophysiological mechanisms, identify potential diagnosis biomarkers, and better stratify patients. The second objective was to find biomarkers for response to treatment in SMA. Metabolomics study on muscle biopsies of Duchenne patients revealed significant changes in energy and phospholipid metabolisms. SMA metabolic profiling investigations in plasma and CSF confirmed the presence of profound metabolic remodeling in SMA patients. Moreover, we demonstrated that these alterations are more pronounced in the severe phenotype. SMA proteomics studies are ongoing. In conclusion, systems biology tools are powerful for the study of neuromuscular diseases as they allow the discovery of biomarkers, the understanding of the physiopathology and the identification of new therapeutic targets.

**Key Words:** Neuromuscular disorders, omics, precision medicine, systems biology, metabolomics, proteomics, classification, biomarkers, spinal muscular atrophy, SMA, Duchenne muscular dystrophy, DMD.

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# *Publications & communications*

## **Publications**

This thesis is based on the following papers

1. **Dabaj I**, Tebani A, Marret S, Bekri S. **Neuromuscular disorders in omics era: comprehensive review.** *in preparation*
2. **Dabaj I**, Ferey J, Marguet F, Gilard V, Basset C, Bahri Y, Brehin AC, Vanhulle C, Leturcq F, Marret S, Laquerrière A, Schmitz-Afonso I, Afonso C, Bekri S, Tebani A. **Muscle metabolic remodelling patterns in Duchenne muscular dystrophy revealed by ultra-high-resolution mass spectrometry imaging.** *Sci Rep.* 2021 Jan 21;11(1):1906. doi: 10.1038/s41598-021-81090-1. PMID: 33479270; PMCID: PMC7819988.
3. **Dabaj I**, Lagrue E, Ducatez F, Allouche S, Ausseil J, Seferian A, Benezit A, Plichet T, Pilon C, Vasseur S, Chapart M, Saugier-Verber P, Quijanoroy S, Marret S, Tebani A, Bekri S. **Longitudinal metabolomics profiling of spinal muscular atrophy.** *in preparation.*

Publications associated to the PhD topic (2019-2022)

1. **Dabaj I**, Hassani A, Burglen L, Qebibo L, Guerrot AM, Marret S, Tebani A, Bekri S. **Pontocerebellar Hypoplasia Type 1D: A Case Report and Comprehensive Literature Review.** *J Clin Med.* 2022 Jul 26;11(15):4335. doi: 10.3390/jcm11154335. PMID: 35893425; PMCID: PMC9368788.
2. Cousin MA, Veale EL, Dsouza NR, Tripathi S, Holden RG, Arelin M, Beek G, Bekheirnia MR, Beygo J, Bhambhani V, Bialer M, Bigoni S, Boelman C, Carmichael J, Courtin T, Cogne B, **Dabaj I**, Doummar D, Fazilleau L, Ferlini A, Gavrilova RH, Graham JM Jr, Haack TB, Juusola J, Kant SG, Kayani S, Keren B, Ketteler P, Klöckner C, Koopmann TT, Kruisselbrink TM, Kuechler A, Lambert L, Latypova X, Lebel RR, Leduc MS, Leonardi E, Lewis AM, Liew W, Machol K, Mardini S, McWalter K, Mignot C, McLaughlin J, Murgia A, Narayanan V, Nava C, Neuser S, Nizon M, Ognibene D, Park J, Platzer K, Poirsier C, Radtke M, Ramsey K, Runke CK, Guillen Sacoto MJ, Scaglia F, Shinawi M, Spranger S, Tan ES, Taylor J, Trentesaux AS, Vairo F, Willaert R, Zadeh N, Urrutia R, Babovic-Vuksanovic D, Zimmermann MT, Mathie A, Klee EW. **Gain and loss of TASK3 channel function and its regulation by novel variation cause KCNK9 imprinting syndrome.** *Genome Med.* 2022 Jun 13;14(1):62. doi: 10.1186/s13073-022-01064-4. PMID: 35698242; PMCID: PMC9195326.
3. Quijano-Roy S, Haberlova J, Castiglioni C, Vissing J, Munell F, Rivier F, Stojkovic T, Malfatti E, Gómez García de la Banda M, Tasca G, Costa Comellas L, Benezit A, Amthor H, **Dabaj I**, Gontijo Camelo C, Laforêt P, Rendu J, Romero NB, Cavassa E, Fattori F, Beroud C, Zídková J, Leboucq N, Løkken N, Sanchez-Montañez Á, Ortega X, Kynčl M, Metay C, Gómez-Andrés D, Carlier RY. **Diagnostic interest of whole-body MRI in early- and late-onset LAMA2 muscular dystrophies: a large international cohort.** *J Neurol.* 2022 May;269(5):2414-2429. doi: 10.1007/s00415-021-10806-0. Epub 2021 Sep 24. PMID: 34559299.

## **Oral presentations (2019-2022)**

1. "Histoire naturelle de l'amyotrophie spinale infantile-Nouvelles thérapies. Réunion organisée par le Réseau Périnatalité Eure et Seine-Maritime et le Centre de Compétence des Maladies Neuromusculaires de l'Enfant du CHU de Rouen. Amyotrophie spinale infantile (SMA) : Nouveaux traitements - Nouveaux enjeux du dépistage et de la prise en charge", January 30<sup>th</sup>, 2020, CHU de Rouen, Rouen.
2. "Muscle metabolic remodeling patterns in Duchenne muscular dystrophy revealed by ultra-high-resolution mass spectrometry imaging". Journées de l'école doctorale (JED), May 18<sup>th</sup>, 2021, Rouen.
3. Maladie de Duchenne. Réunion " Nouveautés en Neuropédiatrie", December 2<sup>nd</sup>, 2021, Rouen.
4. "Bilan d'utilisation du Nusinersen pour l'amyotrophie spinale infantile". COMEDIMS, January 18<sup>th</sup>, 2022, CHU de Rouen, Rouen.
5. "Thérapies innovantes dans l'amyotrophie spinale infantile", Séminaire amyotrophie spinale infantile, January 25<sup>th</sup>, 2022, Paris.
6. "Exploration du remodelage métabolique musculaire dans la dystrophie musculaire de Duchenne par imagerie métabolomique". Journée Normande de Recherche Biomédicale, June 10<sup>th</sup>, 2022.
7. "Données de vraie vie dans la prise en charge thérapeutique de la SMA, Agenda Board SMA génération Y" – groupe Ouest– June 21<sup>st</sup>, 2022.
8. "Hypotonie du jeune enfant et urgence à orienter". 10<sup>e</sup> Colloque Ville-Hôpital de Périnatalité. September 24<sup>th</sup>, 2022. Réseau de Périnatalité de Normandie, Caen.
9. "Amyotrophie Spinale Infantile : Des propositions thérapeutiques existent". Association des pédiatres libéraux de la région de Rouen. November 22<sup>nd</sup>, 2022. Clinique Mathilde, Rouen.

## **Training courses:**

1. Courses related to the DIU pédagogie Médicale 2020-2021.
2. Protéger, valoriser et diffuser les résultats et produits de la recherche en Sciences Humaines et sociales, droit, sciences économiques et gestion, November 13<sup>th</sup>, 2020.
3. Mener sa thèse comme un projet, January 27<sup>th</sup> and February 17<sup>th</sup>, 2021.
4. Comprendre la construction du genre et déconstruire les stéréotypes sexistes, February 10<sup>th</sup>, 2021.
5. De la Thèse à l'emploi - Les outils : CV, écrits de motivation, profil réseaux sociaux, April 9<sup>th</sup> and 23<sup>th</sup>, 2021.
6. MOOC Intégrité scientifique, May 2022.

## **Other activities related to the PhD**

I was member of the committee for posters evaluation during the yearly congress organized by the doctoral school, Journée de l'Ecole Doctorale 2021.



## *List of Figures*

Figure 1 : Motor unit [69]

Figure 2 : the Neuromuscular junction architecture and function. We can differentiate three elements in the neuromuscular junction (A, B): pre-synaptic neuron, synaptic, and post-synaptic. The presynaptic neuron consists of the motor nerve terminal (A) while the postsynaptic component is made of the muscle fiber and sarcolemma (A). The action potential pass through the terminal end of the motor neuron and triggers the calcium channels opening. This leads to the flow of calcium inside the motor nerve terminal and activates the release of a neurotransmitter which is acetylcholine (ACh) in the synaptic cleft (B). ACh binds the ACh receptor present in the postsynaptic motor end plate. Action potential reach the motor end plate and propagates through the T-tubules which activates the DHPRs located in the sarcolemma and stimulates the release of calcium by inducing the ryanodine receptors (C). Calcium released from the sarcoplasmic reticulum binds to troponin C and allows the contraction of the muscle and the muscle fiber will shorten [76].

Figure 3: Representation of the cytoskeletal proteins in the striated muscles sarcolemma. Four major linkages between the cytoskeleton and the sarcolemma: (a) cadherin-based linkages: N-cadherin binds to actin, desmocollin and desmoglein anchor desmin filaments at the intercalated disc; (b) integrin-based focal adhesions that links to the extracellular matrix (ECM) via laminin, and to cytoskeleton via desmin and submembraneous  $\gamma$ -actin. Integrins associate with talin,  $\alpha$ -actinin, vinculin and N-RAP to form a strong mechanical link to actin filaments. Integrins could directly interact with  $\alpha$ -actinin and/or other components not depicted here to mediate a connection with actin; (c) dystroglycan complex (made of  $\alpha$ -Dystroglycan,  $\beta$ -dystroglycan and sarcoglycans) links to extracellular components (laminin, neurexin, and agrin), and binds to cytoskeletal proteins (actin, syntrophins and  $\alpha$ -dystrobrevin) and (d) spectrin-based linkage. Spectrin is linked to the membrane through ankyrin and anchors the contractile apparatus to the membrane through an interaction with MLP. All of these linkage complexes can bind to  $\gamma$ -actin which probably helps the interactions between the different proteins [79]

Figure 4: Structure of muscle, muscle fiber and myofibril [80]

Figure 5 : Duchenne de Boulogne (left) and his patient (right) while performing facial focal electric stimulation; photo taken from [242]

Figure 6: Structural representation of dystrophin. Dystrophin is in interaction with actin filaments from one side and binds to the DG complex from the other side. There are neuronal nitric oxide synthase (nNOS) binding sites on dystrophin and  $\alpha$ -dystrobrevin. Dystroglycan (DG) complex made of  $\alpha$ -dystroglycan ( $\alpha$ -DG) ;  $\beta$ -dystroglycan ( $\beta$ -DG) and sarcoglycans is linked to cytoplasmic proteins syntrophins,  $\alpha$ -dystrobrevin and dystrophin [249] .

Figure 7: the different supports of biological information and the corresponding "omic" sciences that allow their exploration [295]

Figure 8: Comparison of frequently working analytical techniques in metabolomics research [347]

Figure 9 : General overview of mass spectrometry imaging workflow. (1) Patient selection; (2) Tissue sampling; (3) Tissue sectioning using a cryomicrotome; (4) Tissue section fixed to the sample probe; (5) Homogenous deposition of matrices using an automatic sprayer; (6) MSI data acquisition using an FT-ICR 12T Solarix Bruker; (7) Mass spectrometry imaging datasets (raw data files) were processed; (8) Raw data were converted into data matrices, including ion mass-to-charge ratios ( $m/z$ ), spatial localization and relative intensity for each ion image, were generated using SCiLS Lab software; (9) Data analysis workflow for comparative analysis to extract the differential metabolic patterns; (10) Annotation of the discriminant features by interrogation mass spectrometry databases; (11) Interpretation of the data according to clinical and biological contexts.

Figure 10: BCAA roles in skeletal muscle

## *Abbreviations*

|   |             |
|---|-------------|
| Acetylcholine   | ACh         |
| Autosomal Dominant                                      | AD          |
| Acide désoxyribonucléique                               | ADN         |
| Autosomal Recessive                                     | AR          |
| Acides ribonucléiques                                   | ARN         |
| Antisense Oligonucleotide                               | ASO         |
| Becker Muscular Dystrophy                               | BMD         |
| Branched Chain Amino Acids                              | BCAA        |
| Congenital disorders of glycosylation                   | CDG         |
| Children's Hospital of Philadelphia Infant Test         | CHOP INTEND |
| Compound Muscle Action Potential                        | CMAP        |
| Congenital muscular dystrophy                           | CMD         |
| Congenital Myasthenic Syndromes                         | CMS         |
| Charcot-Marie-Tooth                                     | CMT         |
| Dystroglycan  | DG          |
| distal Hereditary Motor Neuropathy                      | dHMN        |
| Dihydropyridine Receptor                                | DHPR        |
| Duchenne Muscular Dystrophy                             | DMD         |
| Deoxyribonucleic acid                                   | DNA         |
| Extracellular matrix                                    | ECM         |
| Emery–Dreifuss muscular dystrophy                       | EDMD        |
| European Medicines Agency                               | EMA         |
| European Neuromuscular Center                           | ENMC        |
| Food and Drug Administration                            | FDA         |
| French National Network for Rare Neuromuscular Diseases | FILNEMUS    |
| Facioscapulohumeral Muscular Dystrophy                  | FSHD        |
| Gas Chromatography Mass Spectrometry                    | GC-MS       |
| Glycogen Storage Disease                                | GSD         |
| Genome-Wide Association Study                           | GWAS        |
| Hammersmith Functional Motor Scale Expanded             | HFMSE       |
| Hammersmith Infant Neurologic Examination Section 2     | HINE-2      |
| Hereditary Motor Sensory Neuropathy                     | HMSN        |
| Hereditary Sensory Autonomic Neuropathy                 | HSAN        |
| Hereditary Sensory Autonomic Neuropathy                 | HSAN        |
| Hereditary Spastic Paraplegias                          | HSP         |
| KCNK9 imprinting syndrome                               | KIS         |
| Liquid Chromatography Mass Spectrometry                 | LC-MS       |
| Magnetic Resonance Imaging                              | MRI         |
| Mass Spectrometry                                       | MS          |

|  |               |
|--|---------------|
| Motor Unit Number Estimation             | MUNE          |
| Neurofilament Light chain                | NfL           |
| Next Generation Sequencing               | NGS           |
| Nuclear Magnetic Resonance               | NMR           |
| neuronal Nitric Oxide Synthase           | nNOS          |
| Online Mendelian Inheritance in Man      | OMIM          |
| Pontocerebellar Hypoplasia               | PCH           |
| Polymerase Chain Reaction                | PCR           |
| Phosphorylated Neurofilament Heavy chain | pNfH          |
| Proximal Myotonic Myopathy               | PROMM         |
| Ribonucleic acids                        | RNA           |
| RNA sequencing                           | RNA-seq       |
| Revised Upper Limb Module                | RULM          |
| Spinal Muscular Atrophy                  | SMA           |
| Survival Motor Neuron protein            | SMN protein   |
| Survival Motor Neuron 1                  | SMN1          |
| Sarcoplasmic Reticulum                   | SR            |
| TWIK-related acid-sensitive K channel 3  | TASK3 channel |
| Whole-body Magnetic Resonance Imaging    | WBMRI         |
| Whole Exome Sequencing                   | WES           |
| Whole Genome Sequencing                  | WGS           |

# Synopsis

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*Cette thèse est rédigée en anglais. Le synopsis ci-dessous, résume en langue française les enjeux et les apports de ce travail.*

# ***PARTIE I : INTRODUCTION***

## **CHAPITRE 1 : LES MALADIES NEUROMUSCULAIRES**

Les maladies neuromusculaires pédiatriques englobent toutes les maladies de l'enfance qui affectent le muscle, la jonction neuromusculaire, le nerf périphérique et la corne antérieure [1, 2]. Ces pathologies touchent 1 enfant sur 2000 dans le monde [3]. La multitude de sites susceptibles d'être affectés dans ces pathologies, l'implication du système nerveux central dans certaines formes, ainsi que l'hétérogénéité et la rareté de ces maladies, rendent le diagnostic et le traitement difficiles [4, 5].

Le premier chapitre de cette partie comporte une description anatomique et fonctionnelle du muscle, de la jonction neuromusculaire et du nerf ainsi que la classification des principaux groupes de maladies neuromusculaires héréditaires. L'ataxie héréditaire, les cardiomyopathies héréditaires, l'hyperthermie maligne ont été exclues.

Le chapitre 2 de la partie I porte sur les biomarqueurs dans les maladies neuromusculaires. Les chapitres 3 et 4 détaillent respectivement sur la dystrophie musculaire de Duchenne et l'amyotrophie spinale infantile. Il s'agit des deux pathologies neuromusculaires pédiatriques les plus fréquentes. Ces deux pathologies sont détaillées, car elles ont été étudiées dans le cadre du travail expérimental de cette thèse. Cette partie est clôturée par le chapitre 5 qui décrit les sciences omiques et la médecine de précision.

## **CHAPITRE 2 : BIOMARQUEURS DANS LES MALADIES NEUROMUSCULAIRES**

Dans le cadre de cette thèse, un article de revue est actuellement en préparation et rapporte l'histoire, les classifications utilisées à ce jour, ainsi que les biomarqueurs potentiels (explorations omiques, radiologiques, neurophysiologiques).

**Dabaj I, Tebani A, Marret S, Bekri S. Neuromuscular disorders in omics era: comprehensive review. en préparation**

## CHAPITRE 3 : DYSTROPHIE MUSCULAIRE DE DUCHENNE

### I. Définition, physiopathologie et mode de transmission

Les dystrophinopathies sont des maladies récessives liées à l'X, causées par des variants du gène *DMD*, qui est le plus grand gène connu chez l'homme [6]. L'altération de ce gène conduit à la production d'une protéine dystrophine non fonctionnelle ou absente dans la Dystrophie Musculaire de Duchenne (DMD) et déficiente dans la Myopathie de Becker (BMD). La dystrophine est présente dans les muscles squelettiques et les muscles cardiaques, les neurones corticaux et les neurones cérébelleux [7].

### II. Épidémiologie

La prévalence de la DMD et de la BMD est estimée à 4,8 et 1,6 pour 100 000 personnes respectivement selon une revue systématique portant sur 901 598 055 personnes provenant de 25 articles différents [8]. L'incidence annuelle est d'environ 1/5000 [9].

### III. Diagnostic

Lorsque la myopathie de Duchenne est suspectée, la stratégie diagnostique est maintenant bien établie. Le patient est amené à consulter un médecin lorsque la famille remarque des signes de retard des acquisitions motrices, des chutes fréquentes, des difficultés à courir, sauter ou monter les escaliers, de la fatigue et des douleurs musculaires [10-14]. La plainte principale peut également être un retard cognitif ou des difficultés d'apprentissage. L'âge moyen au diagnostic est de 5 ans et le délai entre les premiers signes et le diagnostic est de 2,2 ans [15].

Le bilan sanguin montre une élévation importante de la créatine kinase (CK >5000 - 10.000 UI/L) et des enzymes hépatiques élevés (ASAT, ALAT). Dans de rares cas, le patient est asymptomatique, mais le diagnostic est suspecté lorsque des bilans sanguins systématiques sont anormaux. Une biopsie musculaire peut être réalisée, et montre une dystrophie à l'examen histologique avec des zones de nécrose et de dégénérescence-régénération. L'absence de dystrophine est le critère de référence pour le diagnostic de la DMD et la protéine quantifiée par une analyse de Western blot. Actuellement, avec la disponibilité de tests génétiques pour diagnostiquer les dystrophinopathies, la biopsie musculaire est un

examen de seconde intention lorsque les phénotypes cliniques et génétiques sont atypiques ou dans un contexte de recherche clinique pour évaluer l'efficacité du traitement traduite par la restauration de la dystrophine [16]. Le diagnostic de DMD est confirmé par le séquençage du gène *DMD* [17]. Les variants sont le plus souvent des délétions ou des duplications d'un ou plusieurs exons, ou plus rarement des variants ponctuels. Un conseil génétique dans la famille du cas index est proposé après confirmation du diagnostic de DMD.

#### IV. Évaluations et prise en charge

Une évaluation initiale est réalisée pour déterminer la gravité de la maladie et les éventuelles complications associées. L'évaluation clinique de l'évolution de la maladie et du pronostic des patients atteints de DMD passe par différents types d'études fonctionnelles qui varient selon le stade de la maladie (dans l'enfance ou âge adulte, avant ou après perte de la marche), des tests de force, amplitude des mouvements, activités de la vie quotidienne et les échelles de fonction motrice [18-24]. Des tests de suivi annuels sont recommandés pour évaluer les fonctions respiratoires et cardiaques.

La prise en charge de la DMD est multidisciplinaire et nécessite la collaboration de différents spécialistes : neuropédiatre, cardiologue, pneumologue, orthopédiste, médecin de Médecine Physique et Réadaptation. Les complications respiratoires et cardiaques sont la principale cause de morbidité et de mortalité, et la prise en charge précoce de ces complications améliore la qualité de vie et prolonge la vie des patients DMD. L'intervention d'autres spécialistes est possible en fonction des besoins du patient. Le patient est vu une à deux fois par an pour un suivi et une prise en charge adaptée. Les psychologues, les kinésithérapeutes, les nutritionnistes jouent un rôle majeur dans la prise en charge de ces patients. Des recommandations internationales ont été publiées afin de standardiser la prise en charge de soins pour les patients atteints de DMD [18-24].

#### V. Thérapies

Actuellement, les glucocorticoïdes sont les seuls traitements dont le bénéfice est prouvé pour ralentir la progression de la maladie [11, 25]. Des thérapies innovantes existent, peu d'entre elles ont reçu un accord réglementaire et aucune n'a montré une efficacité clinique indiscutable.

## CHAPITRE 4 : AMYOTROPHIE SPINALE INFANTILE

### I. Définition, physiopathologie et mode de transmission

L'amyotrophie spinale infantile (SMA) est une maladie neuromusculaire invalidante, autosomique récessive, caractérisée par une dégénérescence progressive des neurones  $\alpha$ -moteurs entraînant une faiblesse musculaire généralisée au niveau des extrémités et du tronc, une atrophie musculaire progressive, une atteinte bulbaire et respiratoire [26]. Il existe un spectre de sévérité de la maladie avec un continuum allant d'une forme prénatale incompatible avec la vie à des formes plus légères et plus bénignes survenant à l'âge adulte [26]. Les délétions homozygotes ou les variants de perte de fonction du gène *SMN1* (Survival Motor Neuron 1) sur le chromosome 5, qui code pour la protéine SMN (survival motor neuron protein) sont la principale cause déterminante de la SMA. Le gène homologue *SMN2* est le principal modificateur de la gravité de la maladie [27-30]. Les deux gènes sont situés sur le chromosome 5q11.1-13.3. Les séquences des gènes *SMN1* et *SMN2* sont similaires, mais ont une différence de 5 nucléotides, l'un d'eux situé dans l'exon 7 du gène *SMN2* ce qui entraîne un épissage alternatif (qui est un événement crucial dans la pathogenèse de la SMA) [31, 32]. Par conséquent, 90 % de la protéine SMN2 exprimée est tronquée et dépourvue de l'exon 7. Cette protéine est instable, et facilement dégradée [31, 32]. Il est à noter que SMN2 est présent au moins à une copie chez les patients, mais le nombre de copies est variable selon les individus. Habituellement, le nombre de copies de SMN2 est inversement corrélé à la sévérité de la maladie SMA [29, 33], mais d'autres facteurs tels que l'hypoxie et l'expression de la Plastine 3 pourraient influencer le phénotype [34].

### II. Épidémiologie

L'incidence mondiale de l'amyotrophie spinale infantile est de 1 : 10 000 [35-37] naissances ou de 7,8-10 sur 100 000 naissances vivantes [38]. L'incidence est similaire chez les hommes et les femmes [39], la fréquence des porteurs varie de 1/40 à 1/60 [36].

### III. Diagnostic

Chez le nourrisson, les premiers signes de la maladie sont : une hypotonie globale "pseudoparalytique" contrastant avec un bon contact et des signes d'éveil associés à une fasciculation de la langue et, dans les cas sévères, des signes de détresse respiratoire [40].



Chez les enfants où la maladie se déclare plus tard dans l'enfance, la présentation clinique peut être une démarche dandinante, une hypo- ou aréflexie sans aucune anomalie des nerfs sensitifs [26]. Les fonctions cognitives sont généralement préservées, même si chez les patients les plus sévèrement atteints, la neurodégénérescence pourrait atteindre non seulement les cellules de la corne antérieure et les ganglions de la racine dorsale et les thalami, mais elle est également répandue dans le cortex cérébral, les ganglions de la base, le tronc cérébral et le cervelet [41].

La maladie est confirmée après avoir effectué un test de diagnostic moléculaire pour le gène *SMN1*. La corrélation entre le phénotype et le génotype n'est pas absolue et le principal déterminant des différents sous-types est lié au nombre de copies du gène *SMN2* [27, 30, 42-44]. Le phénotype est lié au nombre de copies *SMN2* plutôt qu'au niveau d'expression de *SMN2* dans le sang [32].

#### IV. Évaluations et prise en charge

Plusieurs tests fonctionnels sont validés et sont utiles en routine pour l'évaluation des patients SMA selon l'âge, les plus courants sont : Children's Hospital of Philadelphia Infant Test (CHOP INTEND), Hammersmith Infant Neurologic Examination Section 2 (HINE-2), Functional Motor Scale Expanded (HF MSE) et le Revised Upper Limb Module pour SMA (RULM) [39].

La publication de recommandations internationales en 2007 [40] puis une mise à jour faite en 2018 [45, 46], ont permis une reconnaissance précoce de la maladie et un traitement précoce des signes cliniques et par conséquent une amélioration du pronostic. Les standards de soins ont mis en évidence la nécessité d'une prise en charge multidisciplinaire des patients atteints de SMA. La prise en charge respiratoire est primordiale, car les décompensations respiratoires sont la première cause de décès chez ces patients. Cette prise en charge comporte une évaluation initiale de la fonction respiratoire. Lorsque des signes d'insuffisance respiratoire sont détectés, une assistance mécanique est recommandée en utilisant une ventilation invasive ou non invasive. Dans la SMA de type 1 ou dans la forme sévère de la SMA de type 2, une attention particulière doit être accordée à l'atteinte bulbaire. Des protocoles spécifiques sont établis pour la prise en charge aiguë, ambulatoire ou en milieu hospitalier, lors des décompensations respiratoires, déshydratation, hypoglycémie [39].

## V. Thérapies

Ces dernières années, des thérapies innovantes ont été développées pour traiter la SMA. Nusinersen (Spinraza), premier médicament approuvé, est un oligonucléotide synthétique antisens (ASO) qui se lie à l'intron 7 du pré-ARNm *SMN2*, ce qui conduit à la traduction d'une protéine SMN pleinement fonctionnelle [26-28, 44]. Il est administré par voie intrathécale avec une dose de charge au cours des deux premiers mois (jours 1, 15, 29, 64) et des doses d'entretien tous les quatre mois [26]. Le taux de réussite est plus élevé quand le traitement est administré plus tôt [33, 41]. D'autres thérapies sont l'Onasemnogene Apeparvovec (Zolgensma), thérapie génique de remplacement du gène *SMN1*, est administrée par voie intraveineuse ou intrathécale en une seule dose. Le Risdiplam (RG7916), administré par voie orale, est une petite molécule modifiant l'épissage [28, 41]. Les thérapies innovantes actuelles peuvent modifier de manière significative l'évolution de la maladie.

## CHAPITRE 5 : SCIENCES OMQUES ET MEDICINE DE PRECISION

### I. Sciences omiques

Au 20<sup>ème</sup> siècle, des avancées révolutionnaires ont été réalisées avec la découverte de la structure de l'acide désoxyribonucléique (ADN) par Watson et Cricks en 1953 [47]. Cette découverte a été suivie par le développement de la chaîne en réaction par polymérase (PCR) par Mullis en 1986, qui a bouleversé le domaine de la biotechnologie et a ouvert la voie aux sciences omiques [48].

Le suffixe -omique ajouté à un terme moléculaire définit une nouvelle branche de la biologie moléculaire qui utilise des technologies à haut débit pour étudier un ensemble de molécules de manière globale et complète. Il y a une caractérisation et une quantification collectives des molécules provenant de différents échantillons biologiques, ce qui permet de comprendre la structure, la fonction et la dynamique d'un organisme [49, 50]. Il existe des milliers de types de données omiques en biologie, la génomique (l'étude du génome), la transcriptomique (les omiques des acides ribonucléiques (ARN)), la protéomique (l'étude des protéines) et la métabolomique (l'étude des métabolites) seront abordées dans cette thèse.

Les omiques appliquent les théories systémiques aux problèmes. Des efforts sont en cours pour comprendre l'univers entier d'un point de vue biologique en gérant une très grande

quantité de données qui sont inextricablement liées à la bioinformatique et au big data (<http://omics.org/>). La multi-omique intègre différents types de données omiques, ce qui permet de mieux comprendre une grande quantité d'informations recueillies, allant de l'étiologie d'une maladie à ses conséquences [51, 52].

## II. La Médecine de Précision

La médecine de précision est "une approche émergente, pour le traitement et la prévention des maladies, qui prenne en compte la variabilité individuelle des gènes, de l'environnement et du mode de vie de chaque personne" [53-55]. La médecine de précision était auparavant appelée médecine personnalisée, mais l'ancien terme pouvait porter à confusion et laisse sous-tendre que les traitements et les préventions sont uniques pour chaque individu, alors que dans la médecine de précision, le concept est plus large et comprend la découverte de thérapies pour des sous-catégories de maladies, souvent définies par des études omiques [54, 55]. En clinique, les médecins recueillent des informations relatives à la maladie (signes et symptômes), puis, selon les données cliniques et leur expérience, ils proposent un bilan et une prise en charge tout en tenant compte des préférences du patient. Il convient de mentionner que la médecine a toujours été personnalisée et que le traitement était principalement symptomatique plutôt que curatif. Il était donc nécessaire de développer de nouveaux outils pour fournir aux patients les meilleurs soins et découvrir de nouvelles mesures préventives et de nouveaux traitements [54]. Les médecins ont bien délimité les phénotypes qui mènent finalement au diagnostic clinique, mais, avec les progrès des sciences fondamentales et l'utilisation de l'histoire naturelle et des cohortes, les médecins et les scientifiques ont pu déterminer les causes sous-jacentes et les mécanismes sous-jacents (génétique) de nombreuses maladies et ont commencé à rechercher des biomarqueurs pour le suivi, puis ont commencé à débloquent les codes vers le traitement des causes sous-jacentes (traitement ciblé) plutôt que le traitement des symptômes. Ces changements ont conduit à modifier l'histoire naturelle des maladies, ce qui est devenu le nouveau modèle de l'ère de la médecine de précision. Ce nouveau modèle de médecine de précision est né de l'intérêt des entreprises pharmaceutiques pour les maladies rares (en raison de l'augmentation de la portée du développement des médicaments) et de la disponibilité et du caractère abordable des tests génétiques (dont les coûts ont énormément diminué) [54]. Le concept de médecine de précision fondée sur des preuves implique des études multi-omiques en plus des données

cliniques, de laboratoire et d'imagerie afin de mieux comprendre les phénotypes des maladies et de découvrir des biomarqueurs de diagnostic, de pronostic et de développement de traitements [56-59]. En effet, les situations pathologiques sont le plus souvent complexes et multifactorielles et sont déclenchées par l'association de facteurs multiples (génétiques, épigénétiques et environnementaux).

## *OBJECTIFS DE LA THESE*

Les maladies neuromusculaires comprennent un grand groupe de maladies affectant le système nerveux périphérique. Malgré toutes les avancées génétiques, le diagnostic de ces troubles reste difficile. Les cliniciens savent décrire les phénotypes et l'histoire naturelle de la plupart des maladies a été bien retracée. Les traitements (principalement symptomatiques) et la prise en charge multidisciplinaire ont permis d'améliorer la qualité de vie de ces patients. Cependant, la physiopathologie sous-jacente de la plupart de ces troubles reste incomplètement connue.

Nous assistons à une révolution dans le développement des thérapies dans le domaine des maladies neuromusculaires et il existe actuellement sur le marché trois thérapies innovantes approuvées par la FDA (Food and Drug Administration) et l'EMA (European Medicines Agency) pour l'amyotrophie spinale infantile. Cependant, la prédiction des patients répondeurs aux traitements n'est pas possible et il y a réel besoin de trouver des biomarqueurs spécifiques de diagnostic et de suivi. Pour d'autres maladies neuromusculaires, il existe des traitements spécifiques, mais non curatifs. De nombreux processus biologiques doivent être clarifiés pour pouvoir développer une thérapie ciblée. Le développement des sciences omiques ces dernières années a permis l'émergence de la médecine de précision qui vise à traiter les patients en fonction de leurs caractéristiques personnelles et environnementales. Elle utilise les informations obtenues à partir d'études biologiques individuelles et études de population. Les sciences omiques permettent d'extraire des données biologiques d'un système biologique de manière globale avec une approche ciblée ou non ciblée. Ces nouvelles techniques se distinguent des approches conventionnelles qui sont réductionnistes.

L'objectif de cette thèse est l'utilisation des outils omiques pour explorer les deux pathologies neuromusculaires (DMD et SMA) ce qui nous permettra de (i) mieux comprendre les altérations métaboliques des pathologies sous-jacentes, (ii) identifier des biomarqueurs potentiels pour le diagnostic, la stratification des patients et le suivi, et (iii) déterminer de possible cibles thérapeutiques.

La première partie du travail a été appliquée à la dystrophie musculaire de Duchenne. L'étude a porté sur la recherche de profils métaboliques spécifiques de la DMD par des techniques

métabolomiques non ciblées. Mon travail dans cette partie consistait à aider à la préparation d'échantillons pour la spectrométrie de masse à haute résolution, à collecter les données cliniques, à participer à des études métabolomiques sur le tissu musculaire et à analyser et interpréter les résultats.

La deuxième partie de ce travail a été réalisée sur l'amyotrophie spinale infantile en utilisant des explorations métabolomiques et protéomiques avant et après 184 jours de traitement par Nusinersen. L'objectif est de rechercher de nouveaux biomarqueurs spécifiques pour le diagnostic et le suivi de la maladie sous traitement, mais aussi de mieux comprendre les processus biologiques et les voies métaboliques impliqués dans la pathologie. Dans cette thèse les résultats métabolomiques étaient présentés tandis que l'étude protéomique est en cours. Dans ce deuxième projet, mon travail consistait à collecter les échantillons, à les préparer pour la spectrométrie de masse, à recueillir les données cliniques, à participer aux études métabolomiques et à aider à l'analyse et à l'interprétation des résultats.

## ***PARTIE II : PARTIE EXPERIMENTALE***

### **CHAPITRE 1 : LES OMIQUES DANS LA DYSTROPHIE MUSCULAIRE DE DUCHENNE**

#### **I. Contexte**

La dystrophie musculaire de Duchenne est une maladie neuromusculaire récessive liée à l’X causée par des variants du gène *DYS*. Il n'existe actuellement aucun traitement curatif, et le mécanisme physiopathologique sous-jacent reste incomplètement compris. Notre projet consiste à réaliser des études multi-omiques. Nous avons commencé par la métabolomique, car les changements moléculaires peuvent être amplifiés au niveau métabolique, et nous avons utilisé une technique innovante de spectrométrie de masse à ultra-haute résolution pour trouver une signature métabolique sur le tissu musculaire qui aiderait à comprendre les mécanismes moléculaires sous-jacents et à développer des thérapies efficaces.

#### **II. Objectifs**

Le but de cette étude est de réaliser une imagerie métabolomique en utilisant la spectrométrie de masse par résonance cyclotronique ionique à désorption/ionisation laser assistée par matrice, qui est une imagerie basée sur la spectrométrie de masse non ciblée.

#### **III. Collection des données et déroulement de l'étude**

Neuf biopsies musculaires humaines provenant de patients atteints de dystrophie musculaire de Duchenne suivis au CHU de Rouen et neuf biopsies musculaires provenant d'individus témoins. Toutes les biopsies appartiennent à une collection déclarée au ministère français de la santé et située dans le service d’Anatomopathologie du CHU de Rouen. Les dossiers cliniques des patients ont été examinés et des informations cliniques, biologiques et histologiques ont été recueillies. Ces données étaient essentielles pour comprendre la signature métabolique qui varie en fonction de l’environnement, de la génétique, des médicaments.

## IV. Résultats et publication

Cette étude métabolomique non ciblée a révélé des changements significatifs dans 34 métabolites clés. Parmi ces métabolites, 7 étaient diminués dans les biopsies des patients atteints de myopathie de Duchenne par rapport aux biopsies de contrôle, notamment l'adénosine triphosphate et la glycérophosphocholine. Les 27 autres métabolites étaient augmentés dans les biopsies de Duchenne, notamment la sphingomyéline, les phosphatidylcholines, les acides phosphatidiques et les phosphatidylsérines. Les métabolites dérégulés sont impliqués dans le métabolisme énergétique et phospholipidique.

Les détails de ce projet se trouvent dans notre article publié dans Scientific reports :

**Dabaj I, Ferey J, Marguet F, Gilard V, Basset C, Bahri Y, Brehin AC, Vanhulle C, Leturcq F, Marret S, Laquerrière A, Schmitz-Afonso I, Afonso C, Bekri S, Tebani A. Muscle metabolic remodelling patterns in Duchenne muscular dystrophy revealed by ultra-high-resolution mass spectrometry imaging. *Sci Rep.* 2021 Jan 21;11(1):1906. doi: 10.1038/s41598-021-81090-1. PMID: 33479270; PMCID: PMC7819988.**

## V. Conclusion

Cette étude a révélé un remodelage métabolique profond dans les phospholipides et le métabolisme énergétique dans la dystrophie musculaire de Duchenne. Cette étude, qui utilise une approche systémique, a permis d'explorer le métabolisme de la dystrophie musculaire de Duchenne d'une manière holistique et impartiale avec des stratégies sans hypothèse.

# CHAPITRE 2 : LES OMIQUES DANS L'AMYOTROPHIE SPINALE INFANTILE

## I. Contexte

L'amyotrophie spinale (SMA) est une maladie neurodégénérative, autosomale récessive, due à une délétion homozygote du gène *SMN1* ce qui aboutit à la dégénérescence des motoneurones alpha de la corne antérieure de la moelle épinière [60-62]. Jusqu'à récemment, le traitement était uniquement symptomatique et reposait sur une approche multidisciplinaire. Cependant, des thérapies ciblées sont actuellement approuvées et sont bénéfiques pour les patients [29, 33, 41]. Le Nusinersen est le premier médicament approuvé par la FDA et l'EMA pour la SMA. Il s'agit d'un oligonucléotide antisens (ASO) qui se lie à un



site d'inactivation de l'épissage intronique présent dans l'intron 7 de l'ARN pré-messager (pré-ARNm) de *SMN2*, ce qui conduit à une inhibition de l'épissage et à la traduction d'une protéine SMN entièrement fonctionnelle [30, 33]. Malgré toutes les avancées dans le traitement de la SMA, cette maladie n'est toujours pas guérissable et des recherches supplémentaires sont nécessaires pour comprendre la physiopathologie et la réponse au traitement tout en prenant en compte la multitude de facteurs moléculaires et environnementaux. C'est pourquoi les études omiques ont commencé à être appliquées à la SMA, ce qui a permis de mieux comprendre la pathogenèse et d'identifier des biomarqueurs pour le suivi et le développement de thérapies ciblées [63-66].

## II. Objectifs

L'objectif est de réaliser un profilage multi-omique longitudinal de l'amyotrophie spinale infantile ; des études métabolomiques puis protéomiques (incluant NfH et NfL) seront réalisées sur le plasma et le LCR de patients atteints de SMA de type 1, 2 et 3 provenant de centres français, avant et après 6 mois de traitement par Nusinersen. Les résultats sont comparés à ceux de sujets contrôles sans pathologies neurologiques connues.

## III. Collection des données et déroulement de l'étude

Cinquante-trois plasmas de patients atteints de SMA de type 1, 2 et 3 ont été sélectionnés pour l'étude pré-traitement et ont été comparés à 74 plasmas témoins. Vingt-trois plasmas au jour 184 après le début du traitement ont été sélectionnés. Analyse du LCR de 34 patients atteints de SMA avant le traitement, de 52 contrôles, et de 24 patients atteints de SMA au jour 184 après le début du traitement.

Analyse métabolomique ciblée par spectrométrie de masse en tandem permet la quantification de 188 métabolites : acides aminés (21), amines biogènes (21), hexoses (1), acylcarnitines (40), glycérophospholipides (90) et sphingolipides (15). Analyse protéomique ciblée : l'analyse ciblée de 276 protéines (Panels Neurologie, Neuroexploratoire et Réponse immunitaire) sera réalisée à l'aide de la technologie Olink® Biomarker (Olink, Uppsala, Suède). La protéine NF-H du plasma et du LCR sera mesurée à l'aide de tests d'électrochimiluminescence (Meso Scale Discovery [MSD], Rockville, MD, USA). Les données seront acquises à l'aide d'un lecteur de plaques SECTOR S 6000 (Meso Scale Diagnostics, Rockville, MD, USA).

## IV. Résultats

La première partie du projet a consisté à réaliser l'exploration métabolomique. Cette étude a montré un profond remodelage métabolique chez les patients SMA naïfs avec notamment une augmentation de la concentration des acylcarnitines, une diminution de celle des acides aminés et une modulation du métabolisme des glycérophospholipides. Ces résultats sont consistants avec ceux décrits dans la littérature. En effet, il a été rapporté une élévation de la concentration des acylcarnitines chez les patients SMA avec une diminution de la carnitine libre plasmatique. Une dérégulation du métabolisme des acides gras a été caractérisée chez les patients avec une augmentation des acides gras libres plasmatiques et une stéatose hépatique. Ceci peut être dû à la dénervation qui entraîne une perte de fonction de la fibre musculaire et donc une diminution du flux de la beta-oxydation. Par ailleurs, des études ont rapporté une diminution de la concentration de plusieurs acides aminés (alanine, phénylalanine, acides aminés branchés) ainsi que celle de certaines amines biogènes (e.g. hydroxyproline) qui pourraient refléter une diminution du flux du métabolisme protéique. L'étude protéomique est en cours de réalisation. Les résultats qui seront obtenus viendront enrichir les données à intégrer pour obtenir un profil qui reflète le mieux les modifications omiques qui sous-tendent la sévérité de la pathologie et la réponse au traitement.

L'article ci-dessous décrit l'étude et détaille les résultats et leur interprétation.

**Dabaj I**, Lagrue E, Ducatez F, Allouche S, Ausseil J, Seferian A, Benezit A, Plichet T, Pilon C, Vasseur S, Chapart M, Saugier-Weber P, Quijanoroy S, Marret S, Tebani A, Bekri S. Longitudinal metabolomics profiling of spinal muscular atrophy. *en préparation*.

## *CONCLUSION ET PERSPECTIVES*

Les explorations omiques sont en train de révolutionner la recherche dans le domaine de la santé en renforçant le pouvoir de la découverte et en apportant une compréhension plus profonde avec une vision plus holistique. Il s'agit d'un nouveau domaine qui évolue très rapidement ce qui offre une opportunité extraordinaire pour mieux comprendre et explorer la complexité du vivant. Ces avancées technologiques nous ouvrent la porte d'un nouveau monde qui nous éclaire sur de nouvelles notions et nous fait changer notre pratique clinique pour adopter une médecine plus personnalisée qui s'appuie sur des données de grande dimension.

Les patients veulent savoir s'ils vont guérir de leur maladie, si celle-ci va s'aggraver, s'ils peuvent bénéficier d'une thérapie et à quoi s'attendre avec chaque thérapie proposée. Il est extrêmement difficile de répondre à ces questions et les médecins sont confrontés quotidiennement à ce défi permanent.

Les sciences omiques utilisent des approches multiples pour explorer les différents supports de l'information biologique. Cette stratégie permet d'obtenir une vue multidimensionnelle de la biologie humaine afin de comprendre un seul problème. De plus, toutes les métadonnées cliniques, radiologiques, physiologiques, liées à l'environnement peuvent être intégrées pour obtenir une vue globale de l'état du patient à ce moment précis. Des études longitudinales peuvent être aussi menées pour suivre l'état du patient dans le temps (pronostic, suivi de traitement...). Cette approche holistique n'est possible que si des outils mathématiques et informatiques avancés sont déployés pour analyser et visualiser l'information utile à la prise de décision à partir de ces données de grand volume.

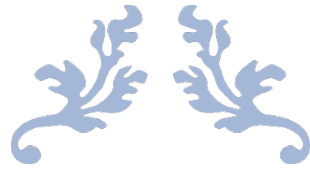
Sur le plan mécanistique, l'adoption de tests multi-omiques permet de comprendre le fonctionnement cellulaire et ses voies métaboliques ainsi que les interactions entre différents types cellulaires. L'utilisation des explorations omiques permet de caractériser de nouvelles voies impliquées dans le processus pathologique et qui pourraient représenter des cibles thérapeutiques. Grâce à ces approches, nous passons de la vision segmentée à une vision globale. Ces études pourraient être d'un grand apport en pratique clinique à l'ère de la médecine de précision. Les explorations omiques sont encore peu utilisées pour l'étude des maladies neuromusculaires. Leur utilisation en clinique pourrait permettre d'accélérer le

diagnostic, de suivre les effets des traitements et communiquer des informations pour choisir le régime thérapeutique le plus adapté à chaque patient. Les études omiques peuvent également contribuer à la conception des essais cliniques : si nous pouvons cibler les patients les plus susceptibles de souffrir des effets indésirables de la maladie, nous pouvons réduire le nombre de patients à impliquer dans l'essai, à réduire la durée et à augmenter la probabilité de succès.

Dans le cadre de cette thèse, des explorations omiques ont été réalisées pour explorer les deux maladies neuromusculaires pédiatriques les plus fréquentes (SMA et DMD).

# Thesis

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## ***PART I: INTRODUCTION***



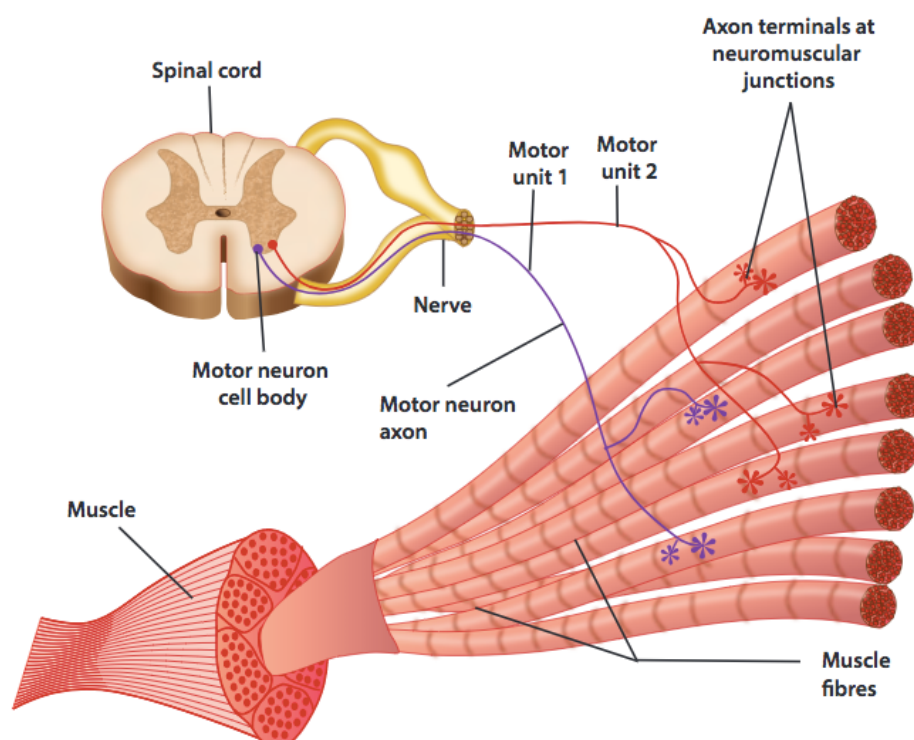
# CHAPTER 1: NEUROMUSCULAR DISORDERS

## I. Neuromuscular anatomy and function

Pediatric neuromuscular disorders encompass all childhood onset diseases affecting the muscle, the neuromuscular junction, peripheral nerve, and the anterior horn cell [1, 2]. These disorders affect 1 in 2000 children worldwide [3]. The multitude of sites that could be affected in these disorders, in addition to the implication of the central nervous system in some forms as well as the heterogeneity and rarity of these disorders, make the diagnosis and treatment challenging [4, 5]. This chapter reviews the basic anatomy and physiology of the muscle and nerve which helps understanding the neuromuscular disorders.

### *Motor unit*

The term motor unit was introduced by Sir Charles Sherrington who detected that there is a force that follows a muscle contraction in stretch [67]. Each motor unit consists of one motor neuron with the innervated muscle fibers [68] (**Figure 1**).



**Figure 1** : Motor unit [69]

### *Motor neurons*

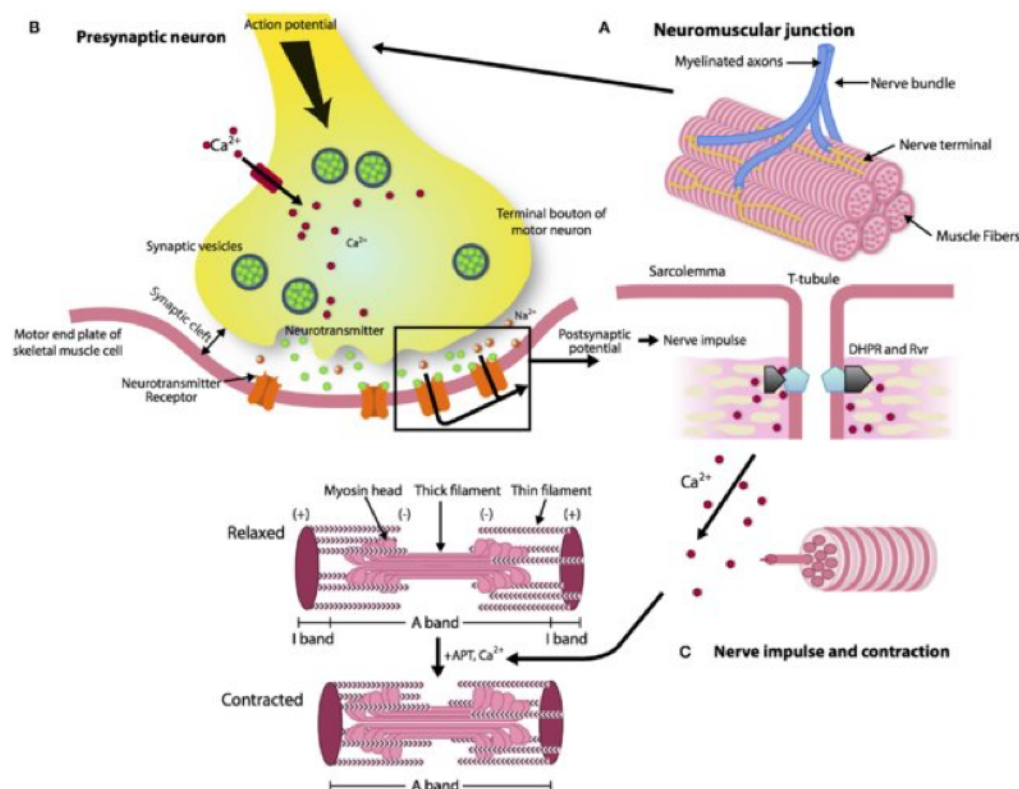
Motor neurons are brain cells with bodies located in the anterior horn of the spinal cord and axons leaving the central nervous system to innervate the muscle. One muscle is innervated by 100 to 200 motor neurons and larger muscles are innervated by more motor neurons [70].

### *Neuromuscular junction and excitation-contraction-relaxation cycle*

Before reaching the target muscle, the myelinated axons get divided into unmyelinated terminal branches that are linked to the muscle by the neuromuscular junction. Each neuromuscular junction consists of a synapse between the motor nerve terminal and the postsynaptic muscle fiber and is adapted to the type and size of the muscle fiber [71]. The nerve impulses (action potentials) that originate in the central nervous system cause the skeletal muscle to contract in an all-or-none fashion (the initiation of the action potential is possible when a threshold value of the membrane is reached) (<https://nba.uth.tmc.edu/neuroscience/m/s1/chapter01.html>). The intensity of the information is coded by the frequency in the nerve cells. The higher the intensity (stretching for skeletal muscles), the greater the number of action potentials triggered. Similarly, the more action potentials there are in a motor neuron, the higher the intensity of contraction of a muscle innervated by that neuron. There is an excitation-contraction-relaxation cycle triggered by the muscle action potential which leads to the contraction and relaxation of the muscle fiber [72]. The term excitation-contraction coupling was first described by Alexander Sandoz in 1952 [73]. The action potential passes through the presynaptic cleft leading to increase in intracellular  $\text{Ca}^{2+}$  which leads to release of the neurotransmitter acetylcholine at the synaptic cleft. Acetylcholine binds to the acetylcholine receptors located at the motor-endplate creating a transmembrane pore for the passage of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ . The flow of the ions yields to the depolarization of the plasma membrane (sarcolemma) and propagation of action potential if the strength of the stimulus reaches the threshold [74]. There are invaginations of the sarcolemma called transverse- tubules or T-tubules that run along the axis of the myofiber, have their lumina communicating with the extracellular fluid space and the sarcoplasmic reticulum surrounding each myofiber [75]. The action potential passes to the T-tubule system then activates the dihydropyridine receptor (DHPR) which is a complex located in the sarcolemma membrane of the T-tubules between the T-tubules and the terminal



cisternae of the sarcoplasmic reticulum (SR). Once the DHRP is activated, it changes conformation and an interaction between DHRP and ryanodine receptors in the SR occurs leading to the release of the intracellular  $\text{Ca}^{2+}$  from the SR and accumulation of  $\text{Ca}^{2+}$  in the myoplasm which give rise to an activation of the contractile apparatus.  $\text{Ca}^{2+}$  buffering is also activated when the  $\text{Ca}^{2+}$  concentration rises in the myoplasm then  $\text{Ca}^{2+}$  reuptake by the SR through the  $\text{Ca}^{2+}$  adenosine triphosphatase. The higher the frequency of nerve action potentials (or in case of pathological depolarization), the greater is the calcium release leading to greater muscle contraction [72, 73] (**Figure 2**).

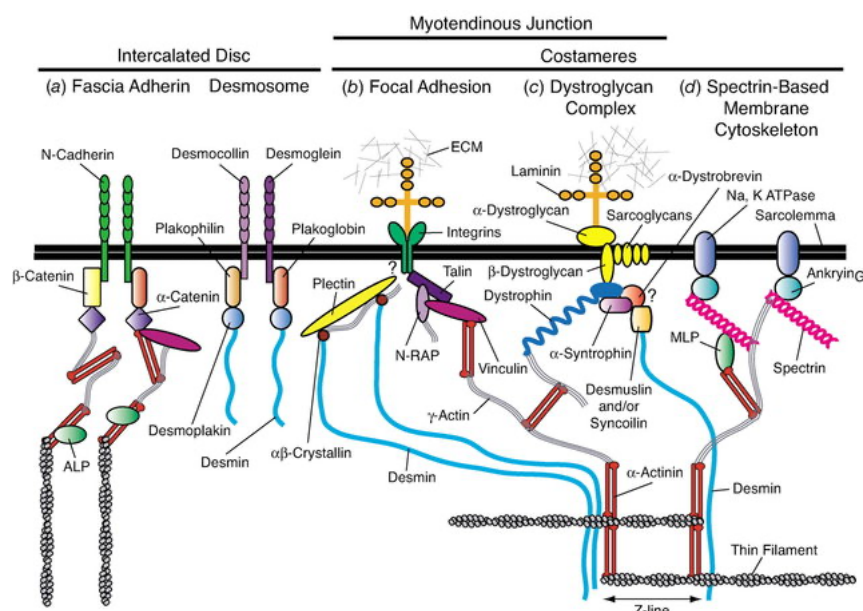


**Figure 2** : the Neuromuscular junction architecture and function. We can differentiate three elements in the neuromuscular junction (A, B): pre-synaptic neuron, synaptic, and post-synaptic. The presynaptic neuron consists of the motor nerve terminal (A) while the postsynaptic component is made of the muscle fiber and sarcolemma (A). The action potential pass through the terminal end of the motor neuron and triggers the calcium channels opening. This leads to the flow of calcium inside the motor nerve terminal and activates the release of a neurotransmitter which is acetylcholine (ACh) in the synaptic cleft (B). ACh binds the ACh receptor present in the postsynaptic motor end plate. Action potential reach the motor end plate and propagates through the T-tubules which activates the DHPRs located in the sarcolemma and stimulates the release of calcium by inducing the ryanodine receptors (C). Calcium released from the sarcoplasmic reticulum binds to troponin C and allows the contraction of the muscle and the muscle fiber will shorten [76].

### *Muscle fibers*

To go into further details of the muscle fiber structure, each muscle fiber has a cytoskeletal network under the sarcolemma and surrounding the myofibrils (group of myofilaments inside

a muscle fiber): the membrane and the cytoskeletal proteins that form costameres composed of focal adhesion, dystroglycan complex, spectrin-based cytoskeleton. The protein complexes of dystrophin, syntrophins, and other molecules bind to F-actin and other cytoskeletal proteins (**Figure 3**). Membrane proteins like sarcoglycan are also linked to the dystroglycan complex and to cytoskeletal proteins which lead to a linkage between the muscle interior and the extracellular matrix. A network of intermediate filaments underly the subsarcolemmal cytoskeleton and is made prominently of desmin, a protein that connects Z-disks, SR, myofibrils, and other organelles to the subsarcolemmal cytoskeleton [77, 78]. Intermediate filaments are also linked to proteins on the surface of lysosomes which are organelles sparse in normal muscle but become abundant in some muscle pathologies [75].

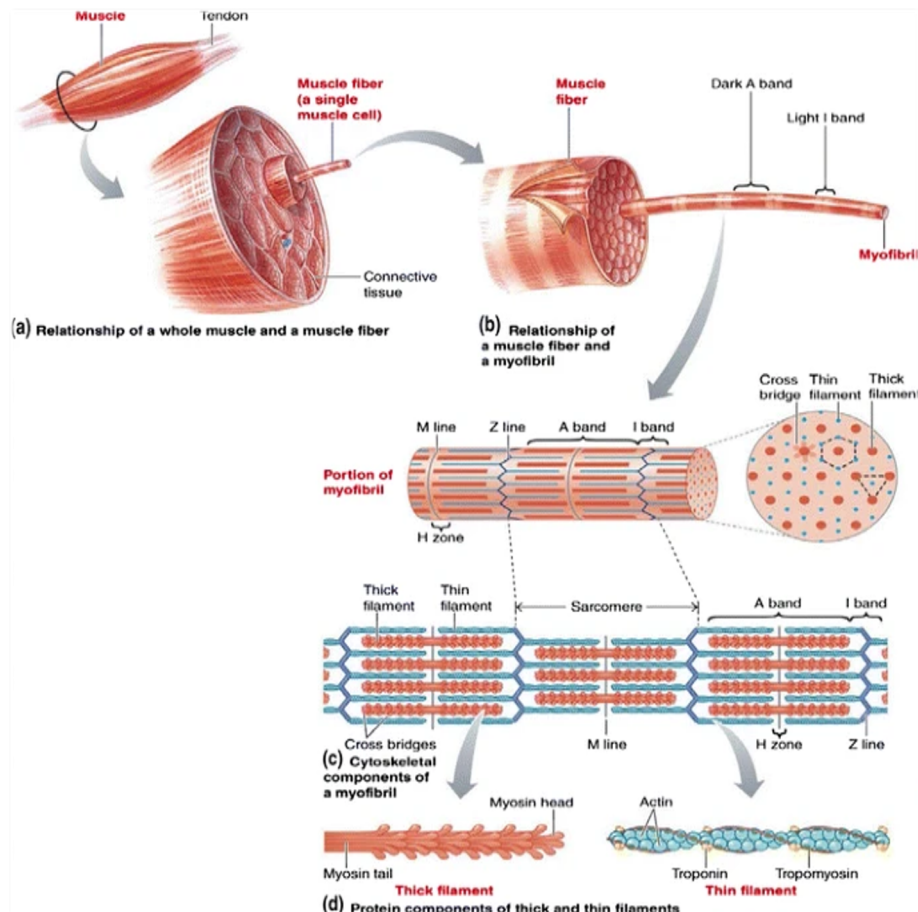


**Figure 3:** Representation of the cytoskeletal proteins in the striated muscles sarcolemma. Four major linkages between the cytoskeleton and the sarcolemma: (a) cadherin-based linkages: N-cadherin binds to actin, desmocollin and desmoglein anchor desmin filaments at the intercalated disc; (b) integrin-based focal adhesions that links to the extracellular matrix (ECM) via laminin, and to cytoskeleton via desmin and submembraneous  $\gamma$ -actin. Integrins associate with talin,  $\alpha$ -actinin, vinculin and N-RAP to form a strong mechanical link to actin filaments. Integrins could directly interact with  $\alpha$ -actinin and/or other components not depicted here to mediate a connection with actin; (c) dystroglycan complex (made of  $\alpha$ -Dystroglycan,  $\beta$ -dystroglycan and sarcoglycans) links to extracellular components (laminin, neurexin, and agrin), and binds to cytoskeletal proteins (actin, syntrophins and  $\alpha$ -dystrobrevin) and (d) spectrin-based linkage. Spectrin is linked to the membrane through ankyrin and anchors the contractile apparatus to the membrane through an interaction with MLP. All of these linkage complexes can bind to  $\gamma$ -actin which probably helps the interactions between the different proteins [79]

### Sarcomere

Inside each muscle fiber there is a contractile unit called the sarcomere consisting of one thick filament and two thin filaments (**Figure 4**). The extremities of the thin filaments of each

sarcomere are linked to the extremities of the filaments of the adjacent sarcomere by the Z disc. The Z disc or Z-line has a structural and signaling role and encloses several proteins such as alpha-actin, titin, nebulin.



**Figure 4:** Structure of muscle, muscle fiber and myofibril [80]

### *Extracellular matrix*

The muscle fiber is surrounded by an extracellular matrix that is composed of the basal lamina, secreted glycoproteins (like laminin), some ligands (like proteoglycans) that binds to the extracellular matrix proteins, and the outermost layer is made of collagen fibers that constitute a connective tissue layer [81]. The basal lamina encompasses extracellular proteins like dystroglycan and integrins and beneath it there are satellite cells and muscle precursors. The extracellular matrix along with the basal lamina play a major role in support and regeneration of muscle fiber.

## Muscles

Efficient contraction of muscles is possible if sarcomeres are activated simultaneously. The activation of sarcomeres is limited by the time needed for an action potential to pass through the muscle fiber and it limits the muscle fibers' length [82, 83]. Muscle fibers are grouped into fascicles that are linked by connective tissue to form a muscle [83]. When stretched, muscles offer resistance that is mostly due to the overlapping between the thick and thin filaments (active contribution to muscle length) but is also influenced by the tendons and internal connective tissue (passive contribution) [84, 85]. Passive contribution to muscle tension is altered in diseased muscle because of an increase in connective tissues and therefore an increase in stiffness and resistance to stretch [86].

Neuromuscular disorders include all diseases caused by the dysfunction of the motor units [87]. They could be acquired or inherited and caused either by genetic defects, immune dysregulation, or iatrogenic [88, 89]. These diseases consist of diseases of the muscle (congenital myopathies and muscular dystrophies), neuromuscular junction (autoimmune myasthenia and congenital myasthenic syndromes), nerves (neuropathies), and anterior horn motor neurons (spinal muscular atrophies or other motor neuron diseases) [90]. Inherited diseases are associated with one of the following defects: 1) sarcolemma and extracellular matrix defects, 2) myonuclear abnormalities, 3) myofibrillar and internal cytoskeletal proteins, 4) ion channel and ion transport defects, 5) complex molecular defects, 6) developmental disorders of skeletal muscle, 7) disorders of catabolic mechanisms, 8) neurotransmission defects, 9) fuel and energy metabolism myopathies [91].

## II. History of neuromuscular disorders

The first neuromuscular nosography was in the early nineteenth century [92] where Aran [93, 94] was the first to describe progressive muscular atrophy in his memoir. Wachsmuth in 1855 [95], and Meryon in 1852 [96, 97] detailed the clinical presentation of patients with progressive atrophy of voluntary muscles and were able to differentiate these cases with primary muscle involvement and familial forms from the group of patients where the peripheral nervous system alteration is secondary rather than inherited [92]. However, it was not until 1868 that neuromuscular disorders were acknowledged, when Duchenne described the first well recognized and clinically devastating disease, currently known as Duchenne

muscular dystrophy [98]. Leyden described in 1876 a familial form with pelvic girdle muscular atrophy and noted the resemblance to the pseudohypertrophic paralysis described by Duchenne. Then, in 1879, Möbius differentiated the diseases with limb girdle muscles involvement including those described by Leyden from other cases with muscular atrophy [99]. At the end of the nineteenth century, Erb described patients with progressive muscular atrophy without central nervous system involvement to illustrate inherited primary muscular disorders with scapulohumeral form [100]. The latter cases (primary muscular disorders with scapulohumeral form) had similar clinical and pathological features to the pseudohypertrophic type described by Duchenne in 1868 [98] and Gowers in 1879 [101] and the atrophy type of Leyden. Later Landouzy and Dejerine in 1884 [102] described in details, for the first time, the fascioscapulohumeral disease. In 1902, Gowers talked for the first time about distal myopathies [103].

Myotonia congenital, another group of diseases involving the neuromuscular junction, was first clearly described by Thomsen in 1876 [104] who was affected by this disease himself as well as other family members. The disability in these patients is due to myotonia (failure of muscle relaxation). Paramyotonia (Eulenburg, 1886) is characterized by myotonia induced by exposure to cold [105]. In 1909, myotonic dystrophies were described by Steinert [106] and Batten and Gibb [107]. In the middle of the 20th century, Maas and Paterson suggested that the three syndromes are due to a single disease process [108].

Regarding the third category of neuromuscular disorders, this involves the peripheral nerves, and the group of diseases is known as inherited neuropathies. It is a diverse group of diseases that could be either syndromic or non-syndromic [109]. The most common non-syndromic condition was originally described in 1886 by Charcot, Marie and Tooth, hence the name of the disease “Charcot-Marie-Tooth” [110]. Prior to reports of Charcot, Marie and Tooth, the disease was reported by Virchow, Eulenburg, Friedreich, Osler, and others [111, 112].

Another major group of inherited neuromuscular disorders is motor neuron diseases. The main disorder of the anterior horn cells in pediatrics is spinal muscular atrophy. The first clinical description of this disease was done by Werdnig in 1891 then Hoffmann in 1893, 1897 and 1900, while late-onset form was described in the 1950s [113]. It took 4 decades to discover the genetic defect in the *SMN1* gene accounting for 95% of the cases [114] and it was until the 2016 that the FDA approved the first specific treatment, Nusinersen. Currently we are witnessing a revolution in the treatment of spinal muscular atrophy with presence of

approved and investigational drugs while diagnosis and prognostic biomarkers are very limited.

### III. Classification of neuromuscular disorders

Classification of neuromuscular disorders is important and could be based on the clinical approach to help the physician putting a framework and categorizing the problem which will yield to the accurate management and possible treatment. It could also be based on the known molecular defect which might be either due to dysfunction in a specific protein, or an organelle or a functional component. Another way to classify is based on the affected gene and the DNA abnormality (stop codon, nucleotide repeat expansion, ... etc.). There is no perfect way to classify and no consensus for one classification including all neuromuscular disorders. We are interested in inherited neuromuscular disorders and currently, these disorders are classified into 16 groups: Muscular dystrophies, congenital muscular dystrophies, congenital myopathies, distal myopathies, other myopathies, myotonic syndromes, ion channel muscle diseases, malignant hyperthermia, metabolic myopathies, hereditary cardiomyopathies, congenital myasthenic syndromes, SMA & motor neuron diseases, hereditary ataxias, hereditary motor and sensory neuropathies, hereditary paraplegias, other neuromuscular disorders [115]. There is a classification for each group based on the correlation between the phenotype and the genotype. Moreover, it is important to note that a disease can be caused by several genes and, on the other hand, in other cases one gene can be responsible for several diseases with different phenotypes [115]. The classification has changed over the years. The first anatomical descriptions of muscle diseases date back to the 16th century while the first clinical reports were in the 19th century [92, 116]. Walton & Nattrass suggested the first comprehensive classification of inherited muscle disorders in 1954, based on the clinical description and mode of inheritance [92]. They excluded myasthenic and unusual congenital myopathies and classified the myopathies into 5 types: Duchenne type, limb-girdle muscular dystrophy, facioscapulohumeral disease, distal, and ocular myopathies [92]. Here, we describe the classification of the major groups. However, we didn't detail hereditary ataxia, hereditary cardiomyopathies, and malignant hyperthermia.

### *Classification of muscular dystrophies*

Muscular dystrophies are a group of muscle disorders due to variants in 66 genes [117], resulting in progressive muscle weakness of the skeletal muscle and dystrophic changes on muscle biopsy [118, 119]. Most diseases were recognized and described long before most of the congenital muscular dystrophies or LGMDs. They include (i) Duchenne and Becker muscular dystrophies, (ii) Emery–Dreifuss muscular dystrophy, and (iii) facioscapulohumeral muscular dystrophy.

Duchenne and Becker muscular dystrophies are a group of diseases caused by variants in the gene *DYS* encoding for the protein dystrophin. DMD is the most common muscular dystrophy in children with an incidence of about 1 in 5000 males per year [9].

Emery–Dreifuss muscular dystrophy (EDMD) is another common muscular dystrophy with different modes of inheritance (AD, AR, X-linked) [120]. The X-linked form distinctive features was first well detailed by Emery and Dreifuss in 1966 , with the following triad of early contractures, slowly progressive muscle wasting and weakness and cardiac abnormalities [121]. However, the first clinical description was reported in 1902 by Cestan [122]. Then in 1955, Becker and Kiener described an X-linked muscular dystrophy with onset later than DMD and a reduced average lifespan and it was until 1979 that the name was officially given as Emery-Dreifuss muscular dystrophy [120]. The first locus for this disease was identified in 1986 by Thomas et al. [123]. It was named *EMD*, and results in EDMD1 subtype which is X-linked recessively inherited [124-126]. The locus for EDMD2 was mapped by Bonne et al. in 1999 [127] *LMNA* was found to be the associated gene. EDMD2 is of autosomal dominant inheritance pattern while also autosomal recessive variants in *LMNA* have been associated with EDMD subtype 3. Forty percent of EDMD cases are due to variants in *LMNA* and *EMD* genes [128, 129]. The other subtypes of EDMD are EDMD4 and EDMD5 (identified in 2007) due to *SYNE1* and *SYNE2* gene alterations respectively [130], in 2009 and 2011 respectively, EDMD6 and EDMD7 were characterized and were linked to *FHL1* [131] and *TMEM43* genes [132, 133]. More genes were identified to be responsible for EDMD phenotype like *TTN*, *SUN1* and *SUN2* genes [134-136].

The third most common muscular dystrophy is facioscapulohumeral muscular dystrophy (FSHD). This clinically heterogeneous disease is due to abnormal expression of the *DUX4* gene in skeletal muscle [137]. Three distinct subtypes are described so far, FSHD type 1, also known



as FSHD1A is the most common type and is caused by contraction of the D4Z4 repeats (1–10 repeat units instead of 11-100 repeats on one 4q chromosome) [138] , type 2 or FSHMD1B is affected by alterations in silencing proteins and is caused by variants in *SMCHD1* gene [139], and the autosomal recessive form FSHD3 due to variants in *LRIF1* gene [140].

To go into further details in the classification of muscular dystrophies, limb girdle muscular dystrophies were classified based on the clinical findings and the age of onset and included a heterogeneous group of disorders [116, 141]. The muscular deficit predominance is in the pelvic and shoulder girdles in all patients, but other clinical findings could be seen such as distal involvement and asymmetry, scapular winging, calf hypertrophy, rippling muscle phenomenon, muscle cramps, cardiac and respiratory complications [142]. With the revolution of genetics since the end of the 20th century, the European Neuromuscular Center (ENMC) suggested a classification in 1995 based on the genetic and molecular criteria [143, 144] the autosomal dominant and recessive loci were designated as LGMD type 1 and 2 respectively. A progressive alphabetic designation for genetic mapping was used, however, all alphabetic letters were occupied, and the classification system used in the Online Mendelian Inheritance in Man (OMIM) included other myopathies with normal CK levels and therefore was not used by the clinicians. This led to the organization of the 2nd ENMC workshop in 2017 and a new classification was suggested, naming the autosomal dominant LGMDs as D and numbering from 1 to 5, the recessive ones as R with numbers from 1 to 23 [145] and this classification was complemented by a new one adding the protein involved and the genetic defect [116]. Patients to be considered having LGMD they should have elevated CK, abnormal muscle imaging with evidence of degeneration, and dystrophic pattern on muscle biopsy. The condition must be described at least in two unrelated families with affected individuals that achieved walking independently [145]. Of note, the same gene can be responsible for LGMD but also for other muscular diseases for example variants in *TTN* and *DYSF* genes are linked to distal myopathies [141], variants in *LMNA* gene give LGMD type 1B and other phenotypes like Emery-Dreifuss myopathy, lipodystrophy, cardiomyopathy, Charcot-Marie-Tooth type 2B1 [146, 147]. This leads to further complications in the classification, and this will affect the feasibility in clinical trials when patients are to be included. The classification of these disorders is still problematic, and a new classification is needed with the evolution of genetics and the discovery of new genes using whole exome



sequencing and next generation sequencing and new informatics tools [116, 148, 149]. Limb girdle muscular dystrophies are part of a larger group of disorders, congenital muscular dystrophies which encompasses 53 diseases, 56 genes according to the muscle gene table 2022 [117].

### *Classification of congenital muscular dystrophies*

Congenital muscular dystrophies (CMDs) are one of the inherited muscular disorders with weakness presenting soon after birth and during infancy [150]. Classification in this group of disorders, like in congenital myopathies, is based on muscle biopsy finding and genetic evaluation. In 1989, Leyten et al. [151] suggested two types of CMDs with CNS involvement Fukuyama and Muscle-eye-brain syndrome. In 1990, Topaloglu et al. proposed the addition of intermediate forms with normal intelligence and without neurological abnormalities [152]. Since the mid-nineties, the classification has been continuously updated. Patano et al. suggested four types: 1) classical CMD (without mental retardation), 2) CMD with forebrain malformations-mental retardation, 3) CMD with mental retardation not otherwise classified, 4) atypical CMDs [153, 154]. Then in 2004 Voit et Tomè [155] distinguished 4 groups 1) deficit of laminin alpha 2 – MCD1A, 2) defects of alpha dystroglycan, 3) disorders with prominent contractures, rigid spine, and Ulrich CMD, 4) primary or secondary alpha 7 integrin deficiency. Bonneman et al. [156] reported the CMD entities: a) laminin alpha 2 related CMD (primary laminin 2/merosin deficiency); b) alpha-dystroglycan related dystrophies; c) congenital disorders of glycosylation (CDG) with abnormal alphadystroglycan glycosylation; d) collagen VI and integrin related CMD forms; e) intracellular and nuclear CMD forms. The major categories are based on the affected proteins: merosin-related myopathies (CMD1A due to variants in *LAMA2* gene) [157-159], Collagen type VI related myopathy (Ullrich congenital muscular dystrophy -AR; Bethlem myopathy- AD) [160, 161], CMD with abnormal glycosylation of alpha dystroglycan (Walker Warburg syndrome, Muscle-eye-brain, Fukuyama, CMD1C, and CMD1D) [162] and CMD with integrin deficiency. Other forms in this group of disorders are CMD1B, CMD with lamin A/C deficiency, rigid spine syndrome [163, 164].

### *Classification of congenital myopathies*

This group comprises 68 diseases, 48 genes [117]. This term was first used by Magee and Shy in 1956 [165]. Clinically patients present with hypotonia and weakness that could occur from birth with static or poorly progressive deterioration [166]. The spectrum represents a range of severity comprising fetal akinesia to adult-onset mild phenotype [167, 168]. The congenital myopathies were historically classified according to the specific morphologic features on muscle biopsy [166, 167] such as cores in central core disease [165, 169] and multiminicore disease [170], rods in nemaline myopathy [171], central nuclei in centronuclear/myotubular myopathy [172], selective hypotrophy of type 1 fibres in congenital fibre type disproportion [173]. However, overlapping morphological abnormalities is common, therefore making the diagnosis more challenging. In addition, the genetic basis of the different forms of congenital myopathies is complex because one disease can be caused by variants in more than one gene [166], one gene can be responsible of different diseases [174-179] and the same gene variant can lead to different pathological features in the same family or even in the same individual at different ages [166, 180]. Lately, some congenital myopathies were reported without any hallmarks on muscle pathology but with specific molecular defects [181]. Nevertheless, genetic revolution did not help in resolving some cases where few congenital myopathies remain without any molecular defect [181]. The later information adds more complexity to the classification of this group which is currently classified by using both the histological and molecular findings. In 2018, Mathis et al. [182] suggested a three-step nomenclature: step 1 for phenotype starting by the I) generic term (hereditary myopathy), then II) the clinical phenotype (distal, proximal,...etc.) or III) the pathological phenotype (core, centronuclear,...etc.), followed by step 2 for inheritance (AD, AR,...) and step 3 for genotype (gene, or unknown).

### *Classification of distal myopathies*

The distal myopathies were initially classified by eponyms. It started in 1951 when Welander [183] described an autosomal dominant form (Welander distal myopathy) in his publication of a series of 249 individuals, the recessive Myoshi myopathy [184, 185], the recessive distal myopathy with rimmed vacuoles, the dominant tibial muscular dystrophy (also known as Udd myopathy) [186]. It was until 1995 that the genetic era started with the mapping of the autosomal dominant distal myopathy published by Laing et al. [187]. Progressively, more

genes have been identified to be causative and we can differentiate three main categories: 1) late-onset autosomal dominant forms, 2) adult-onset autosomal dominant forms, 3) early-onset autosomal dominant forms [188]. Currently there are 28 disease phenotypes based on the clinical-molecular basis [117].

#### *Classification of myotonic syndromes*

The most common myotonic dystrophies, are myotonic dystrophy 1 (Steinert) which is of childhood onset, caused by expansion of CTG repeat in the *DMPK* gene on chromosome 19 [189-193] and the myotonic dystrophy type 2 (proximal myotonic myopathy also known as PROMM) which is of adult onset, predominant proximal weakness, absent abnormalities in CTG repeat and different histological pattern [194, 195]. Both these diseases are dominantly inherited. More disorders belong to this group and are less common: 1) chloride channel 1 skeletal muscle channelopathy, could be dominantly inherited (Thomsen disease) [196] or recessive (Becker disease) [197], 2) rippling muscle disease with dominant and recessive inheritance [198, 199], 4) Schwartz-Jampel syndrome [200], 5) Brody disease [201].

#### *Classification of ion channel muscle diseases*

Ion channel muscle diseases also known as skeletal-muscle channelopathies are a group of disorders with defects related to ion-channel dysfunction (sodium, potassium, chloride, calcium) [202]. This group currently includes Thomsen and Becker myotonia due to chloride channel dysfunction, hypokalemic periodic paralysis type 2 and hyperkalemic periodic paralysis, paramyotonia congenita, potassium aggravated myotonia due to sodium channel dysfunction; hypokalemic PP type 1, Acetazolamide responsive hereditary paroxysmal cerebellar ataxia, episodic ataxia type-2 due to calcium channel dysfunction; hypokalemic periodic paralysis. Episodic ataxia/myokymia syndrome, thyrotoxic hypokalemic periodic paralysis, Andersen's syndrome and long QT syndrome due to potassium channel [115]. Clinically, patients present with periodic paralysis with episodic attacks of weakness or nondystrophic myotonias with electrical or clinical myotonia (impairment of relaxation following a sudden voluntary contraction or external stimulation) [202, 203]. The diagnosis is based on the clinical suspicion, electromyography with specific patterns (I-V) directing the molecular diagnosis and confirmed by molecular testing [204].

### *Classification of metabolic myopathies*

Metabolic myopathies, a clinically heterogeneous group of disorders with abnormalities in glycogen storage, the glycolytic pathway, and the lipid metabolism [117]. In 1951, McArdle described the disease as “characterized by a gross failure of the breakdown of glycogen to lactic acid” and the disease was named by its eponym rather than the biochemical defect which is myophosphorylase deficiency [205]. There are different glycogen storage diseases (GSD) associated with exercise intolerance, cramps and myoglobinuria (type V, VII, VIII also called IXd, IX, X, XI, XIII) [206]. The disorders of energy supply to muscle could result in exercise intolerance or in chronic weakness. The latter sign is seen for example in GSD type II. Currently and according to the latest gene table of neuromuscular diseases [117], there are 30 gene alterations responsible for 30 disease phenotypes in metabolic myopathies group, most of them with autosomal recessive inheritance except GSD type IXd [207], phosphoglycerate kinase 1 deficiency [208, 209], GSD of heart/lethal congenital [210], GSD type XIII [211], and myopathy due to lactate transporter defect [212] that are of X-linked recessive inheritance. Muscular glycogenoses are divided into type 0, II, IIIa, IV, V, VII, IXd, XIV, XV, GSD of heart, polyglucosan storage myopathy, polyglucosan body myopathy 2. Diseases of glycolytic pathway are phosphoglycerate kinase 1 deficiency, GSD type X, GSD type XI, GSD type XIII and myopathy due to lactate transporter defect. Disorders of lipid metabolism are carnitine palmitoyl-transferase II deficiency, primary systemic carnitine deficiency, carnitine translocase deficiency, multiple acyl-coA dehydrogenase deficiency, VLCAD deficiency, Chanarin-Dorfmann syndrome, mitochondrial complex I deficiency due to ACAD9 deficiency, neutral lipid storage disease with myopathy without ichthyosis, acute recurrent myoglobinuria, mitochondrial myopathy with lactic acidosis, lipid storage myopathy due to Flavin Adenine Dinucleotide Synthetase deficiency. The correct diagnosis became more important with the availability of specific therapy in some of these disorders i.e., enzyme replacement therapy in Pompe disease [213].

### *Classification of myasthenic syndromes*

Congenital myasthenic syndromes (CMS) are rare inherited neuromuscular disorders first described in 1948 [214] and the first genetic defects were reported in 1995 [215]. This group of diseases is due to alteration in the neuromuscular junction and presents clinically with fatigable weakness but the specific phenotypic manifestations as well as the response to

treatment are variable between the different types of CMS [216]. As in the other neuromuscular disorders, with the advent of molecular diagnostic studies, the number of genetic defects reported to be causative of this group of disorders has increased considerably. Several workshops hosted by the European Neuromuscular Centre were organized to update the classification [217-220] as well as some review publications [216, 221-223]. The CMS were classified based on the anatomic location of the disease protein into presynaptic, synaptic, and postsynaptic and defects in glycosylation. Then, Engel et al. suggested to add a new group with defects of endplate development and maintenance as well as a group to include other syndromes [224].

### *Classification of hereditary motor and sensory neuropathies*

The first classification of the inherited neuropathies was done by Dyck and Lambert in 1968 [225]. They differentiated type 1 for demyelinating neuropathies and type 2 for axonal neuropathies.

Further classification was using the alphabet for subtypes of neuropathies with different genetic causes. This classification is confusing since there are more genes discovered than the 26 letters of the alphabet and the same gene can be linked to different modes of inheritance [226]. The same gene can be related to different phenotypes. For example, variants in *GARS1* gene can lead to Charcot-Marie-Tooth (CMT) disease type 2D, distal hereditary motor neuropathy (dHMN) type V, and infantile spinal muscular atrophy [227]. Furthermore, things are even more complicated since the same gene can have different mode of inheritance. *MFN2* variants are a frequent cause of either dominant or recessive forms of Charcot-Marie-Tooth disease type 2, with variable degree of severity [228].

The heterogeneity of these disorders makes it difficult and challenging to classify these disorders. Mathis et al. [116] suggested to start the denomination by the name of clinical phenotype (CMT, dHMN,...etc) followed by the mode of inheritance (AD for autosomal dominant, AR for autosomal recessive, XL for X-linked, Spo for sporadique, Mit for mitochondrial) and replace the numbers by 'de' for demyelinating, 'ax' for axonal and 'in' for intermediate forms. Instead of using the letters that follow the numbers in the old classification they proposed to add the name of the gene followed by 'dup' for duplication and 'del' for deletion or 'dig' for digenism when two genes are implicated. If the gene is unknown in this case, they put 'unknown'. Therefore, an example would be AD-CMTde-

PMP22dup instead of CMT1A. The same group conducted a survey asking the opinion of more than 300 physicians and scientists that were attending the Peripheral Nerve Society meeting. 65% of the 107 specialists who answered the survey agreed with the new classification [229]. In 2022, the French National Network for Rare Neuromuscular Diseases (FILNEMUS) consortium suggested a genetic classification of peripheral neuropathies based on next generation sequencing [230]. Three subgroups are distinguished: Hereditary Motor Sensory Neuropathy (HMSN), distal Hereditary Motor Neuropathy (dHMN), Hereditary Sensory Autonomic Neuropathy (HSAN) with overlap between the different subgroups. Despite the different suggested classifications, we still face a problem in the nosography of the inherited neuropathies and the proposed nomenclatures are not yet fully validated internationally.

#### *Classification of motor neuron disorders*

The most common motor neuron disorders and the second most frequent neuromuscular disease is spinal muscular atrophy (SMA). SMA is an autosomal recessive neuromuscular disorder caused by pathogenic variants in the Survival Motor Neuron 1 (*SMN1*) gene, which encodes the SMN protein (survival motor neuron protein). This disease is characterized by progressive degeneration of the motor neurons resulting in generalized muscular weakness and secondary moderate to severe impairment, with an incidence of 1:8400 births in Europe and 1:11000 births in the USA [27-30, 66]. Clinically, there is a broad spectrum of age onset, disease course and severity. The current classification differentiates five subtypes based on age of onset, severity, maximal motor milestone reached: type 0 is the most severe with death shortly after birth, type 1 non-sitters, type 2 sitters, type 3 walkers, type 4 adult onset [28, 33, 44]. In the past few years, innovative therapies have been developed to treat SMA and there is an urgent need to identify biomarkers.

#### *Classification of hereditary paraplegias*

Hereditary spastic paraplegias (HSP) are a group of neurodegenerative disorders presenting with progressive spastic gait disturbance [231]. These disorders could be of autosomal dominant, autosomal recessive, X-linked recessive, or mitochondrial mode of inheritance [90, 232, 233]. There are different classifications. Harding's classification based on signs and symptoms differentiate between pure/uncomplicated and complicated HSP. Another

Harding's classification is based on age of onset of spasticity: type I (early onset) and type II classical (onset after 35 years of age). This group of disorders could also be classified based on the mode of inheritance (AD, AR, XR, mitochondrial). The last type of classification is based on intracellular pathophysiological mechanisms: membrane trafficking and organelle trafficking, axonal transport, mitochondrial dysfunction, lipid metabolism disturbances, myelination abnormalities [234].

The upcoming chapter (chapter 2) focuses on biomarkers in neuromuscular diseases. Chapters 3 and 4 detail the most common childhood neuromuscular disorders: Duchenne muscular dystrophy (chapter 3) and spinal muscular atrophy (chapter 4). A special interest was accorded to these two pathologies since they were studied in the experimental work of this thesis. At the end of Part 1, chapter 5 focuses on omics sciences and precision medicine.

## CHAPTER 2: BIOMARKERS IN NEUROMUSCULAR DISEASES

Biomarkers play a central part in most parts of biomedical disclosure and translational medicine. A biomarker is defined as an objectively measured specific entity that is used as an indicator for normal or pathologic processes, or for response to therapy or to other health care interventions [48]. Even though the term biomarker became popular in the 1980s, biomarkers existed a long time before. One example is in ancient Hindus who discovered that “honey urine” is a marker for diabetes [235].

We can differentiate different types of biomarkers, some are circulating, others are instrumental/invasive [236]. Furthermore, biomarkers could be surrogate endpoints for clinical trials in an era where treatments are emerging and there is an urgent need for accurate diagnostic and prognostic tools. In the past decades, extensive research efforts were devoted to developing biomarkers but, surprisingly, few biomarkers were adopted clinically. An estimate in 2018, only 100 biomarkers are in clinical use which is a very small fraction of the potential biomarkers in the literature [237] only 8 qualified biomarkers that have achieved regulatory acceptance as of July 2021 according to the FDA biomarker qualification program [238]. The qualification of a biomarker by the FDA program means that it has been proven useful in a specific context of use and has met the specific analytical and measurement criteria mentioned by the FDA. However, this qualification is not mandatory to be able to use a biomarker in drug development [239].

In this chapter, we designed a comprehensive review that is based on the most recent literature about biomarkers, including multi-omics strategies, in inherited neuromuscular disorders in the past twenty years. A historical review of the identification of neuromuscular disorders as well as the challenges in diagnosis and treatment will be followed by a description of the current potential biomarkers. Very few biomarkers are used in clinical practice and in drug development related to these disorders. Our article will be submitted soon:

**Dabaj I, Tebani A, Marret S, Bekri S. Neuromuscular disorders in omics era: comprehensive review. *in preparation.***



# **Neuromuscular disorders in omics era: comprehensive review**

Dabaj Ivana<sup>1</sup>, Tebani Abdellah<sup>2</sup>, Marret Stephane<sup>1</sup>, Bekri Soumeiya<sup>2</sup>

## **Abstract**

Neuromuscular disorders includes diseases where the primary lesion is in the peripheral nervous (anterior horn cell, peripheral nerve muscle, the neuromuscular junction, and the muscle). In pediatrics these disorders are mainly of genetic origin. Despite the revolution in the modern diagnostic tools and in genetics the diagnosis remains challenging due to the variability of clinical phenotype, the heterogeneity, and the rarity of diseases. These disorders were first described in the 19<sup>th</sup> century and historically they were considered without treatment. This myth has changed progressively and in the past years we witnessed the emergence of innovative therapy and a change in the natural history in some diseases where targeted therapy has been developed. This work provides a historical perspective on pediatric neuromuscular disorders as well as the current potential biomarkers described that could be useful for the pediatric population with inherited neuromuscular disorders.

## **Author affiliations:**

1 Normandie University, UNIROUEN, CHU Rouen, INSERM U1245, Department of Neonatology, Pediatric Intensive Care, and Neuropediatrics, 76000 Rouen, France

2 Normandie University, UNIROUEN, INSERM U1245, CHU Rouen, Department of Metabolic Biochemistry, 76000 Rouen, France

Correspondence to: Ivana Dabaj, MD, Department of Neonatology, Pediatric Intensive Care, and Neuropediatrics, Normandie University, UNIROUEN, CHU Rouen, INSERM U1245, 1, Rue de Germont, 76000 Rouen, France E-mail: [ivana.dabaj@chu-rouen.fr](mailto:ivana.dabaj@chu-rouen.fr)

**Running title:** Neuromuscular disorders in omics era

**Keywords:** neuromuscular disorders, biomarkers, omics sciences, Duchenne muscular dystrophy, spinal muscular atrophy, classification of neuromuscular disorders, history of neuromuscular disorders, precision medicine, omics

## Introduction

Pediatric neuromuscular disorders encompass all childhood onset diseases affecting the muscle, the neuromuscular junction, peripheral nerve, and the anterior horn cell [1, 2]. These disorders affect 1 in 2000 children worldwide [3]. The multitude of sites that could be affected in these disorders, in addition to the implication of the central nervous system in some forms as well as the heterogeneity and rarity of these disorders, make the diagnosis and treatment challenging [4, 5]. Until the end of the 20<sup>th</sup> century, the diagnosis used to rely mainly on clinical expertise, creatine kinase blood levels, electromyography, and muscle biopsy. Afterward, the characterization of the genes involved in these pathologies has allowed improving and accelerating the diagnosis [4]. In pediatrics, an inherited or a de novo pathogenic variant in a single gene is identified as causal to a variety of neuromuscular disorders, and clinicians now routinely access next generation sequencing techniques leading to rapid diagnosis [3]. The advances in diagnostic tools added to the complexity of a challenging clinical field and despite the enormous progress, the genetic cause is unsolved in 50% of patients with rare neurological diseases [6]. The clinical heterogeneity and difficulty in diagnosis prompted scientists worldwide to put considerable efforts to search for diagnostic, prognostic, and therapeutic biomarkers [5]. A biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention” as defined by the National Institute of Health in 2001 [7]. We can differentiate different types of biomarkers, some are circulating, others are instrumental/invasive [8]. Furthermore, biomarkers could be surrogate endpoints for clinical trials in an era where treatments are emerging and there is an urgent need for accurate diagnostic and prognostic tools.

This review is based on the most recent literature about biomarkers, including multi-omics strategies, in inherited neuromuscular disorders in the past twenty years. A historical review of the identification of neuromuscular disorders as well as the challenges in diagnosis and

treatment will be followed by a description of the current biomarkers used in these disorders in the pediatric population.

## **Anatomy and function of motor units and muscle fibers**

The motor neurons are nerve cells with bodies located in the anterior horn of the spinal cord and axons leaving the central nervous system to innervate the muscle. One muscle is innervated by 100 to 200 motor neurons and larger muscles are innervated by more motor neurons [9]. Each motor unit consists of one motor neuron with the innervated muscle fibers [10]. Before reaching the target muscle, the myelinated axons get divided into unmyelinated terminal branches that are linked to the muscle by the neuromuscular junction. Each neuromuscular junction consists of a synapse between the motor nerve terminal and the postsynaptic muscle fiber and is adapted to the type and size of the muscle fiber [11]. There is an excitation-contraction-relaxation cycle triggered by the muscle action potential which leads to the contraction and relaxation of the muscle fiber [12].

To go into further details of the muscle fiber structure, each muscle fiber has a cytoskeletal network under the sarcolemma (plasma membrane) and surrounding the myofibrils (group of myofilaments inside a muscle fiber). There are membrane and cytoskeletal proteins. Membrane proteins like sarcoglycan are also linked to the dystroglycan complex and to cytoskeletal proteins which lead to a linkage between the muscle interior and the extracellular matrix.

Neuromuscular disorders include all diseases caused by the dysfunction of the motor units [13]. They could be acquired or inherited and caused either by genetic defects, immune dysregulation, or iatrogenic [14, 15]. These diseases consist of diseases of the muscle (congenital myopathies and muscular dystrophies), neuromuscular junction (auto-immune myasthenia and congenital myasthenic syndromes), nerves (neuropathies), and anterior horn motor neurons (spinal muscular atrophies or other motor neuron diseases) [16]. Inherited diseases are associated with one of the defects: 1) sarcolemma and extracellular matrix defects, 2) myonuclear abnormalities, 3) myofibrillar and internal cytoskeletal proteins, 4) ion channel and ion transport defects, 5) complex molecular defects, 6) developmental disorders of skeletal muscle, 7) disorders of catabolic mechanisms, 8) neurotransmission defects, 9) fuel and energy metabolism myopathies [17].

## History of neuromuscular disorders identification

The first neuromuscular nosography was in the early nineteenth century [18] where Aran [19, 20] was the first to describe progressive muscular atrophy in his memoir. Wachsmuth in 1855 [21], and Meryon in 1852 [22, 23] detailed the clinical presentation of patients with progressive atrophy of voluntary muscles and were able to differentiate these cases with primary muscle involvement and familial forms from the group of patients where the peripheral nervous system alteration is secondary rather than inherited [18]. However, it was not until 1868 that neuromuscular disorders were acknowledged, when Duchenne described the first well recognized and clinically devastating disease, currently known as Duchenne muscular dystrophy [24]. Leyden described in 1876 a familial form with pelvic girdle muscular atrophy and noted the resemblance to the pseudohypertrophic paralysis described by Duchenne. Then, in 1879, Möbius differentiated the diseases with limb girdle muscles involvement including those described by Leyden from other cases with muscular atrophy [25]. At the end of the nineteenth century, Erb described cases with progressive muscular atrophy without central nervous system involvement to illustrate inherited primary muscular disorders with scapulohumeral form [26]. The latter cases had similar clinical and pathological features to the pseudohypertrophic type of Duchenne in 1868 [24] and Gowers in 1879 [27] and the atrophy type of Leyden, and later Landouzy and Dejerine in 1884 [28] described in detail for the first time the fascioscapulohumeral disease. In 1902, Gowers talked for the first time about distal myopathies [29].

Myotonia congenita, another group of diseases involving the neuromuscular junction, was first clearly described by Thomsen in 1876 [30] who was affected by this disease himself as well as other family members. The disability in these patients is due to myotonia (failure of muscle relaxation). Paramyotonia (Eulenburg, 1886) is characterized by myotonia induced by exposure to cold [31]. In 1909, myotonic dystrophies were described by Steinert [32] and Batten and Gibb [33]. In the middle of the 20<sup>th</sup> century, Maas and Paterson suggested and the three syndromes are due to a single disease process [34].

We gave a brief history of diseases of the muscle and neuromuscular junction. Now concerning the third category of neuromuscular disorders, this involves the peripheral nerves, and the group of diseases is known as inherited neuropathies. It is a diverse group of diseases that could be either syndromic or non-syndromic [35]. The most common non-syndromic condition was originally described in 1886 by Charcot, Marie and Tooth, hence the name of the disease

“Charcot-Marie-Tooth” [36]. Prior to reports of Charcot, Marie and Tooth, the disease was reported by Virchow, Eulenburg, Friedreich, Osler, and others [37, 38].

Another major group of inherited neuromuscular disorders is motor neuron diseases. The main disorder of the anterior horn cells in pediatrics is spinal muscular atrophy. The first clinical description of this disease was done by Werdnig in 1891 then Hoffmann in 1893, 1897 and 1900, while late-onset form was described in the 1950s [39]. It took 4 decades to discover the genetic defect in the *SMN1* gene accounting for 95% of the cases [40] and it was until the 2016 that the FDA approved the first specific treatment, Nusinersen. Currently we are witnessing a revolution in the treatment of spinal muscular atrophy with presence of approved and investigational drugs while diagnosis and prognostic biomarkers are very limited.

## **Classification of neuromuscular disorders**

Classification of neuromuscular disorders is important and could be based on the clinical approach to help the physician putting a framework and categorizing the problem which will yield to the accurate management and possible treatment. It could also be based on the known molecular defect which might be either due to dysfunction in a specific protein, or an organelle or a functional component. Another way to classify is based on the affected gene and the DNA abnormality (stop codon, nucleotide repeat expansion, ... etc.). There is no perfect way to classify these disorders and no consensus for one classification that includes all neuromuscular disorders.

We are interested in inherited neuromuscular disorders and currently, these disorders are classified into 16 groups: muscular dystrophies, congenital muscular dystrophies, congenital myopathies, distal myopathies, other myopathies, myotonic syndromes, ion channel muscle diseases, malignant hyperthermia, metabolic myopathies, hereditary cardiomyopathies, congenital myasthenic syndromes, SMA & motor neuron diseases, hereditary ataxia, hereditary motor and sensory neuropathies, hereditary paraplegia, other neuromuscular disorders [41]. There is a classification for each group based on the correlation between the phenotype and the genotype. Moreover, it is important to note that a disease can be caused by several genes and on the other hand, in other cases one gene can be responsible for several diseases with different phenotypes [41]. The classification has changed over the years. The first anatomical descriptions of muscle diseases date back to the 16th century while the first clinical reports

were in the 19<sup>th</sup> century [18, 42]. Walton and Nattrass suggested the first comprehensive classification of inherited muscle disorders in 1954, based on the clinical description and mode of inheritance [18]. They excluded myasthenic and unusual congenital myopathies and classified the myopathies into 5 types: Duchenne type, limb-girdle muscular dystrophy, facioscapulohumeral disease, distal, and ocular myopathies [18]. In our review we describe the classification of the major groups (hereditary ataxia, hereditary cardiomyopathy, and malignant hyperthermia were not detailed).

## **Classification of muscular dystrophies**

Muscular dystrophies are a group of muscle disorders due to variants in 66 genes [43], resulting in progressive muscle weakness of the skeletal muscle and dystrophic changes on muscle biopsy [44, 45]. Most diseases were recognized and described long before most of the congenital muscular dystrophies or LGMDs and include Duchenne and Becker muscular dystrophies, Emery–Dreifuss muscular dystrophy, and Facioscapulohumeral muscular dystrophy.

Duchenne and Becker muscular dystrophies are a group of diseases caused by variants in the gene *DYS* encoding for the protein dystrophin. DMD is the most common muscular dystrophy in children with an incidence of about 1 in 5000 males per year [46].

Emery–Dreifuss muscular dystrophy (EDMD) is another common muscular dystrophy with different modes of inheritance (AD, AR, X-linked) [47]. The X-linked form distinctive features was first well detailed by Emery and Dreifuss in 1966, with the following triad of early contractures, slowly progressive muscle wasting and weakness and cardiac abnormalities [48]. However, the first clinical description was in 1902 by Cestan [49]. Then in 1955, Becker and Kiener described an X-linked muscular dystrophy with onset later than DMD and a reduced average lifespan and it was until 1979 that the name was officially given as Emery-Dreifuss muscular dystrophy [47]. The first locus for this disease was identified in 1986 by Thomas et al. [50]. It was named *EMD*, and results in EDMD1 subtype which is X-linked recessively inherited [51-53]. The locus for EDMD2 was mapped by Bonne et al. in 1999 [54] *LMNA* was found to be the associated gene. EDMD2 is of autosomal dominant inheritance pattern while also autosomal recessive variants in *LMNA* have been associated with EDMD subtype 3. 40% of EDMD cases are due to variants in *LMNA* and *EMD* genes [55, 56]. The other subtypes of EDMD are EDMD4 and EDMD5 due to *SYNE1* and *SYNE2* genes discovered in 2007 [57], EDMD6 due to variants in *FHL1* gene discovered in 2009 [58], and EDMD7 due to variants in

*TMEM43* gene in 2011 [59, 60]. More genes are also discovered to be responsible of EDMD phenotype like *TTN*, *SUN1* and *SUN2* genes [61-63].

The third most common muscular dystrophy is facioscapulohumeral muscular dystrophy (FSHD). This disease with clinical heterogeneity, is due to abnormal expression of the *DUX4* gene in skeletal muscle [64]. Three distinct subtypes are described so far, FSHD type 1, also known as FSHD1A which is caused by contraction of the D4Z4 repeats [65], type 2 or FSHMD1B is affected by alterations in silencing proteins and is caused by variants in *SMCHD1* gene [66], and the autosomal recessive form FSHD3 due to variants in *LRIF1* gene [67].

To go into further details in the classification of muscular dystrophies, limb girdle muscular dystrophies were classified based on the clinical findings and the age of onset and included a heterogeneous group of disorders [42, 68]. The muscular deficit predominance is in the pelvic and shoulder girdles in all patients, but other clinical findings could be seen such as distal involvement and asymmetry, scapular winging, calf hypertrophy, rippling muscle phenomenon, muscle cramps, cardiac and respiratory complications [69]. With the revolution of genetics since the end of the 20<sup>th</sup> century, the European Neuromuscular Center (ENMC) suggested a classification in 1995 based on the genetic and molecular criteria, [70, 71] the autosomal dominant and recessive loci were designated as LGMD type 1 and 2 respectively. A progressive alphabetic designation for genetic mapping was used, however, all alphabetic letters were occupied, and the classification system used in the Online Mendelian Inheritance in Man (OMIM) included other myopathies with normal CK levels and therefore was not used by the clinicians. This led to the organization of the 2<sup>nd</sup> ENMC workshop in 2017 and a new classification was suggested, naming the autosomal dominant LGMDs as D and numbering from 1 to 5, the recessive ones as R with numbers from 1 to 23 [72] and this classification was complemented by a new one adding the protein involved and the genetic defect [42]. Patients to be considered having LGMD they should have elevated CK, abnormal muscle imaging with evidence of degeneration, and dystrophic pattern on muscle biopsy. The condition must be described at least in two unrelated families with affected individuals that achieved walking independently [72]. Important to note that the same gene can be responsible of LGMD but also of other muscular diseases for example variants in *TTN* and *DYSF* genes are linked to distal myopathies [68], variants in *LMNA* gene gives LGMD type 1B and other phenotypes like Emery-dreifuss myopathy, lipodystrophy, cardiomyopathy, Charcot-Marie-Tooth type 2B1

[73, 74]. This leads to further complications in the classification, and this will affect the feasibility in clinical trials when patients are to be included. The classification of these disorders is still problematic, and a new classification is needed with the evolution of genetics and the discovery of new genes using whole exome sequencing and next generation sequencing and new informatics tools [42, 75, 76]. Limb girdle muscular dystrophies are part of a larger group of disorders, congenital muscular dystrophies which encompasses 53 diseases, 56 genes according to the muscle gene table 2022 [43].

## **Classification of congenital muscular dystrophies**

Congenital muscular dystrophies (CMDs) are one of the inherited muscular disorders with weakness presenting soon after birth and during infancy [77]. Classification in this group of disorders, like in congenital myopathies, is based on muscle biopsy finding and genetic evaluation. In 1989, Leyten et al. [78] suggested two types of CMDs with CNS involvement Fukuyama and Muscle-eye-brain syndrome. In 1990, Topaloglu et al. proposed the addition of intermediate forms with normal intelligence and without neurological abnormalities [79]. Since the mid-nineties, the classification has been continuously updated. Patano et al. suggested four types: 1) classical CMD (without mental retardation), 2) CMD with forebrain malformations-mental retardation, 3) CMD with mental retardation not otherwise classified, 4) atypical CMDs [80, 81]. Then, in 2004, Voit et Tomè [82] distinguished 4 groups 1) deficit of laminin alpha 2 – MCD1A, 2) defects of alpha dystroglycan, 3) disorders with prominent contractures, rigid spine, and Ulrich CMD, 4) primary or secondary alpha 7 integrin deficiency. Bonneman et al. [83] reported the CMD entities: a) laminin alpha 2 related CMD (primary laminin 2/merosin deficiency); b) alpha-dystroglycan related dystrophies; c) congenital disorders of glycosylation (CDG) with abnormal alphasdystroglycan glycosylation; d) collagen VI and integrin related CMD forms; e) intracellular and nuclear CMD forms. The major categories are based on the proteins affected : merosin-related myopathies (CMD1A due to variants in *LAMA2* gene) [84-86], Collagen type VI related myopathy (Ullrich congenital muscular dystrophy -AR; Bethlem myopathy- AD) [87, 88], CMD with abnormal glycosylation of alpha dystroglycan (Walker Warburg syndrome, Muscle-eye-brain, Fukuyama, CMD1C, and CMD1D) [89] and CMD with integrin deficiency. Other forms in this group of disorders are CMD1B, CMD with lamin A/C deficiency, rigid spine syndrome [90, 91].



## **Classification of congenital myopathies**

This group comprises 68 diseases, 48 genes [43]. The term was first used by Magee and Shy in 1956 [92]. Clinically patients present with hypotonia and weakness that could occur from birth with static or poorly progressive deterioration [93]. The spectrum represents a range of severity comprising fetal akinesia to adult-onset mild phenotype [94, 95]. Concerning the classification, the congenital myopathies were historically classified according to the specific morphologic features on muscle biopsy [93, 94] such as cores in central core disease [92, 96] and multiminicore disease [97], rods in nemaline myopathy [98], central nuclei in centronuclear/myotubular myopathy [99], selective hypotrophy of type 1 fibres in congenital fibre type disproportion [100]. However, overlapping morphological abnormalities is common thus making diagnosis more challenging. In addition, the genetic basis of the different forms of congenital myopathies is complex because of one disease can be caused by variants in more than one gene [93], one gene can be responsible of different diseases [101-106] and the same gene variant can lead to different pathological features in the same family or even in the same individual at different ages [93, 107]. Lately, some congenital myopathies were reported without any hallmarks on muscle pathology but with molecular defects identified [108]. Nevertheless, genetic revolution didn't help in resolving some cases where few congenital myopathies remain without any molecular defect [108]. The later information adds more complexity to the classification of this group which is currently classified by using both the histological and molecular findings. In 2018, Mathis et al. [109] suggested a three-step nomenclature: step 1 for phenotype starting by the I) generic term (hereditary myopathy), then II) the clinical phenotype (distal, proximal,...etc.) or III) the pathological phenotype (core, centronuclear,...etc.), followed by step 2 for inheritance (AD, AR,...) and step 3 for genotype (gene, or unknown).

## **Classification of distal myopathies**

The distal myopathies were initially classified by eponyms. It started in 1951 when Welander [110] described an autosomal dominant form (Welander distal myopathy) in his publication of a series of 249 individuals, the recessive Myoshi myopathy [111, 112], the recessive distal myopathy with rimmed vacuoles, the dominant tibial muscular dystrophy (also known as Udd myopathy) [113]. It was until 1995 that the genetic era started with the mapping of the autosomal dominant distal myopathy published by Laing [114]. Progressively more genes have been identified to be causative and we can differentiate three main categories: 1) late-onset

autosomal dominant forms, 2) adult-onset autosomal dominant forms, 3) early-onset autosomal dominant forms [115]. Currently there are 28 disease phenotypes based on the clinical-molecular basis [43].

## **Classification of myotonic syndromes**

The most common myotonic dystrophies, are myotonic dystrophy 1 (Steinert) which is of childhood-onset, caused by expansion of CTG repeat in the *DMPK* gene on chromosome 19 [116-120] and the myotonic dystrophy type 2 (proximal myotonic myopathy PROMM) which is of adult-onset, predominant proximal weakness, absent abnormalities in CTG repeat and different histological pattern [121, 122]. Both these diseases are dominantly inherited. More disorders belong to this group and are less common: 1) chloride channel 1 skeletal muscle channelopathy, could be dominantly inherited (Thomsen disease)[123] or recessive (Becker disease)[124], 2) rippling muscle disease with dominant and recessive inheritance[125, 126], 4) Schwartz-Jampel syndrome [127], 7) Brody disease [128].

## **Classification of ion channel muscle diseases**

Ion channel muscle diseases also known as skeletal-muscle channelopathies are a group of disorders with defects related to ion-channel dysfunction (sodium, potassium, chloride, calcium) [129]. This group includes currently in Thomsen and Becker myotonia due to chloride channel dysfunction, hypokalemic periodic paralysis type 2 and hyperkalemic periodic paralysis, paramyotonia congenita, potassium aggravated myotonia due to sodium channel dysfunction; hypokalemic PP type 1, Acetazolamide responsive hereditary paroxysmal cerebellar ataxia, episodic ataxia type-2 due to calcium channel dysfunction; hypokalemic PP Episodic ataxia/myokymia syndrome, thyrotoxic hypokalemic periodic paralysis, Andersen's syndrome and long QT syndrome due to potassium channel [41]. Clinically patients present with periodic paralysis with episodic attacks of weakness or nondystrophic myotonias with electrical or clinical myotonia (impairment of relaxation following a sudden voluntary contraction or external stimulation) [129, 130]. The diagnosis is based on the clinical suspicion, electromyography with specific patterns (I-V) directing the molecular diagnosis and confirmed by molecular testing [131].

## **Classification of metabolic myopathies**

Metabolic myopathies, a clinically heterogeneous group of disorders with abnormalities in glycogen storage, the glycolytic pathway, and the lipid metabolism[43]. In 1951 McArdle described the disease as "characterized by a gross failure of the breakdown of glycogen to lactic acid" and the disease was named by its eponym rather than the biochemical defect which is myophosphorylase deficiency [132]. There are different glycogen storage diseases (GSD) associated with exercise intolerance, cramps and myoglobinuria (type V, VII, VIII also called IXd, IX, X, XI, XIII) [133]. The disorders of energy supply to muscle could result in exercise intolerance or in chronic weakness. The later sign is seen for example in GSD type II. Currently and according to the latest gene table of neuromuscular diseases [43], there are 30 gene variants responsible for 30 disease phenotypes in metabolic myopathies group, most of them with autosomal recessive inheritance except GSD type IXd [134] and phosphoglycerate kinase 1 deficiency [135, 136] that are X-linked recessively inherited and GSD of heart/lethal congenital [137], GSD type XIII [138], and myopathy due to lactate transporter defect [139] that are X-linked recessive inheritance. Muscular glycogenoses are divided into type 0, II, IIIa, IV, V, VII, IXd, XIV, XV, GSD of heart, polyglucosan storage myopathy, polyglucosan body myopathy 2. Disease of glycolytic pathway are phosphoglycerate kinase 1 deficiency, GSD type X, GSD type XI, GSD type XIII and myopathy due to lactate transporter defect. Disorders of lipid metabolism are Carnitine palmitoyl transferase II deficiency, primary systemic carnitine deficiency, carnitine translocase deficiency, glutaric aciduria type IIA, B and C, VLCAD deficiency, Chanarin-Dorfmann syndrome, mitochondrial complex I deficiency due to ACAD9 deficiency, Neutral lipid storage disease with myopathy without ichthyosis, acute recurrent myoglobinuria, mitochondrial myopathy with lactic acidosis, lipid storage myopathy due to Flavin Adenine Dinucleotide Synthetase deficiency. The correct diagnosis became more important with the availability of specific therapy in some of these disorders i.e., enzyme replacement therapy in Pompe disease [140].

## **Classification of myasthenic syndromes**

Congenital myasthenic syndromes (CMS) are rare inherited neuromuscular disorders first described in 1948 [141] and the first genetic defects were reported in 1995 [142]. This group of diseases is due to alteration in the neuromuscular junction and presents clinically with fatigable weakness but the specific phenotypic manifestations as well as the response to treatment are variable between the different types of CMS [143]. As in the other neuromuscular

disorders, with the advent of molecular diagnostic studies, the number of genetic defects reported being causative of this group of disorders has increased considerably. Several workshops hosted by the European Neuromuscular Centre were organized to update the classification [144-147] as well as some review publications [143, 148-150]. The CMS were classified based on the anatomic location of the disease protein into presynaptic, synaptic and postsynaptic and defects in glycosylation then Engel et al. suggested to add a new group with defects of endplate development and maintenance as well as a group to include other syndromes [151].

## **Classification of hereditary motor and sensory neuropathies**

Concerning the inherited neuropathies, the first classification was done by Dyck and Lambert in 1968 [152]. They differentiated type 1 for demyelinating neuropathies [153] and type 2 for axonal neuropathies while taking into consideration the mode of inheritance, neurophysiologic and pathologic characteristics. Further classification was using the alphabet for subtypes of neuropathies with different genetic causes. The latter classification is confusing since there are more genes discovered than the 26 letters of the alphabet and the same gene can be linked to different modes of inheritance [154]. The same gene can be related to different phenotypes. For example, variants in *GARS1* gene can lead to Charcot-Marie-Tooth disease type 2D, distal hereditary motor neuropathy type V, and infantile spinal muscular atrophy [155]. Furthermore, things are even more complicated since the same gene can have different mode of inheritance. *MFN2* variants are a frequent cause of either dominant or recessive forms of Charcot-Marie-Tooth disease type 2, with variable degree of severity [156].

The heterogeneity of these disorders makes it difficult and challenging to classify these disorders. Mathis et al.[42] suggested to start the denomination by the name of clinical phenotype (CMT, dHMN, or HSAN) followed by the mode of inheritance (AD for autosomal dominant, AR for autosomal recessive, XL for X-linked, Spo for sporadic, Mit for mitochondrial) and replace the numbers by 'de' for demyelinating, 'ax' for axonal and 'in' for intermediate forms. Instead of using the letters that follow the numbers in the old classification they proposed to add the name of the gene followed by 'dup' for duplication and 'del' for deletion or 'dig' for digenism when two genes are implicated. If the gene is unknown in this case, they put 'unknown'. Therefore, an example would be AD-CMTde-PMP22dup instead of CMT1A. The same group conducted a survey asking the opinion of more than 300 physicians

and scientists that were attending the Peripheral Nerve Society meeting. 65% of the 107 specialists who answered the survey agreed with the new classification [157].

In 2022, the French National Network for Rare Neuromuscular Diseases (FILNEMUS) consortium suggested a genetic classification of peripheral neuropathies based on next generation sequencing [158]. Three subgroups are distinguished: Hereditary Motor Sensory Neuropathy (HMSN), distal Hereditary Motor Neuropathy (dHMN), Hereditary Sensory Autonomic Neuropathy (HSAN) with overlap between the different subgroups.

Despite the different suggested classifications, we still face a problem in the nosography of the inherited neuropathies and the proposed nomenclatures are not yet fully validated internationally.

## **Classification of motor neuron disorders**

The most common motor neuron disorders and the second most frequent neuromuscular disease is spinal muscular atrophy. Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by pathogenic variants in the Survival Motor Neuron 1 (*SMN1*) gene, which encodes the SMN protein (survival motor neuron protein). This disease is characterized by progressive degeneration of the motor neurons resulting in generalized muscular weakness and secondary moderate to severe impairment, with an incidence of 1:8400 births in Europe and 1:11000 births in the USA [159-163]. Clinically, there is a broad spectrum of age onset, disease course and severity. The current classification differentiates five subtypes based on age of onset, severity, maximal motor milestone reached: type 0 is the most severe with death shortly after birth, type 1 non-sitters, type 2 sitters, type 3 walkers, type 4 adult onset [161, 164, 165]. In the past few years, innovative therapies have been developed to treat SMA and there is an urgent need to identify biomarkers.

## **Classification of hereditary paraplegias**

Hereditary spastic paraplegias (HSP) are a group of neurodegenerative disorders presenting with progressive spastic gait disturbance[166]. These disorders could be of autosomal dominant, autosomal recessive, X-linked recessive, or mitochondrial mode of inheritance [16, 167, 168]. There are different classifications. Harding's classification based on signs and symptoms differentiate between pure/uncomplicated and complicated HSP. Another Harding's classification is based on age of onset of spasticity: type I (early onset) and type II classical (onset after 35 years of age). This group of disorders could also be classified based

on the mode of inheritance (AD, AR, XR, mitochondrial). The last type of classification is based on intracellular pathophysiological mechanisms: Membrane trafficking and organelle trafficking, axonal transport, mitochondrial dysfunction, lipid metabolism disturbances, myelination abnormalities [169].

## **Omics analyses**

Omics is a field in the biological sciences used to identify, characterize and quantify the biological molecules that are present in living organisms such as DNA, RNA, metabolites, and proteins [170]. These studies are potent to obtain an integrated and comprehensive view of complex biological processes, the evolution of diseases, or the efficacy of the treatment [171]. Multi-omics approaches have been used in different neuromuscular disorders like Duchenne muscular dystrophy, spinal muscular atrophy, and myopathies. In this review, we pay particular attention to the role of omics-based approaches in biomarker discovery and in informing therapeutic target identification and guiding future treatment strategies.

## **Materials and Methods**

### **Literature Analysis**

In this study, we performed a programmatic literature search for a more efficient and reproducible review process using the Adjutant R package[41]. We searched for articles related to omics-based investigation in neuromuscular diseases that were published between January 1999 and February 2022. We used two queries ((neuromuscular diseases) AND (\*omic)). The resulting document corpus included PubMed IDs, year of publication, authors, article titles, article abstract and any associated Medical Subject Heading (MeSH) terms. Titles and abstracts were decomposed into single terms, stemmed, and filtered by Adjutant package. The term frequency inversed document frequency metrics for each term and created a sparse Document Term Matrix (DTM) for further analysis. t-Distributed Stochastic Neighbor Embedding (t-SNE) [172] and hdbscan [173] algorithms were used to perform unsupervised clustering using DTM data. The coordinates generated by t-SNE were used in hdbscan algorithm to derive the

topic clusters. Each cluster was then assigned a topic by using the five most frequent terms within the cluster.

## **Manual Curation: Inclusion and Exclusion Criteria**

Following the topic clustering step, we validated our clusters using external manual curation assessing the correspondence between articles and cluster topics. Each sampled article was examined and either considered acceptable for further analysis or rejected. Inclusion criteria were topic relevance assessed by a pediatric neurologist, article in English; human data; original research or clinical trial. We further refined corpus and cluster naming. Supplementary Table S1 contains a list of all the articles along with their corresponding cluster.

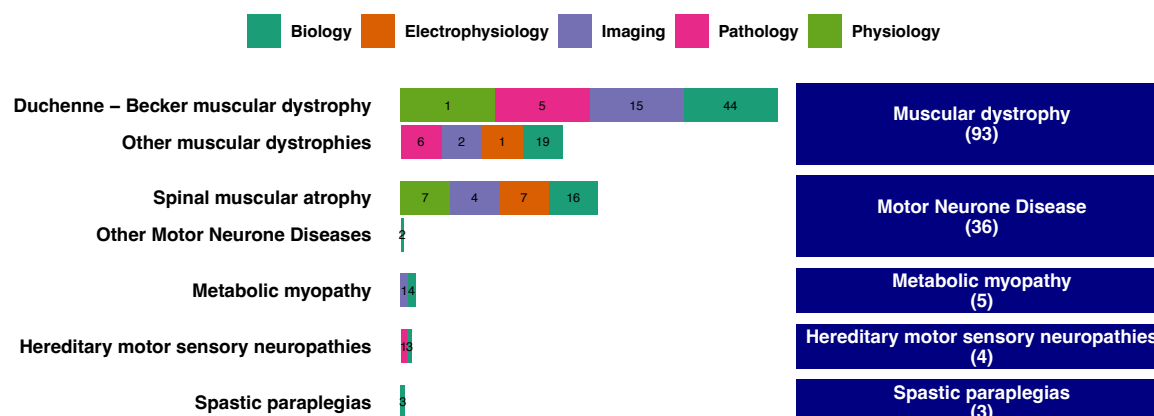
## **Data Analysis**

All the data analysis and visualization have been done using the R software [174].

## **Results**

### **Literature Mining and Topic Clusters**

The first-round analysis generated a document corpus of 3102 articles related to inherited neuromuscular diseases biomarkers published over the past 20 years (**Supplementary Table S1**). We chose journal articles, multi-center, randomized control trials. Using article titles and abstracts, we derived topic clusters in an unsupervised manner, and classified articles according to their clustering status. Articles that never formed part of a cluster were removed from further analysis, leaving 502 documents that formed 23 clusters. Cluster topics were assigned using the five most frequent terms within the cluster along with a manual curation of the included articles. We chose only articles related to pediatric neuromuscular disorders and were left with 101 articles. Further data mining was done manually to keep interesting articles and the final analysis was performed on 141 articles. (**Figure 1**). The full list of articles and related clusters are presented in **Supplementary Table S1**.



**Figure 1: Biomarkers in neuromuscular disorders.** These were classified into 5 categories: biology, electrophysiology, imaging, pathology, physiology. On the left, the list of neuromuscular diseases, Duchenne/Becker muscular dystrophy being the disease with the most frequent number of biomarkers detected, followed by spinal muscular atrophy, and other muscular dystrophies. Very few biomarkers described for metabolic myopathies, hereditary motor sensory neuropathies, other motor neurones or spastic paraplegias. On the right, the number of articles per disease classification: ninety-three articles talking about biomarkers in muscular dystrophies, 36 in motor neuron diseases, 5 articles in metabolic myopathy, 4 in hereditary motor sensory neuropathies, and 3 in spastic paraplegias.

## Muscular dystrophy

The disease group that was thoroughly studied in last two decades is muscular dystrophy and most precisely Duchenne muscular dystrophy. 52 articles treated dystrophinopathies (DMD/BMD) among the 70 articles treating biomarkers in muscular dystrophies. Forty-nine potential biomarkers were described in 41 articles. In 6 articles omics analyses were performed and identified 808 analytes in proteomic studies and 41 analytes in metabolomics [175-180].

## Circulating biomarkers

Blood biomarkers were mainly circulating proteins detected in whole blood, serum, or plasma. This type of biomarkers is becoming important since it is non-invasive yet helpful in monitoring DMD progression and disease milestones [180, 181].

Skeletal muscle derived protein, creatinine kinase (CK) is known to be elevated early in the disease but does not correlate well with the disease severity [176, 178, 180, 182]. However, it could be affected by the disease progression as it is lower in non-ambulant DMD patients compared to ambulant [183]. CK could be a marker for treatment efficacy as it declined in



patients treated with Drisapersen (exon skipping 51) versus placebo [184] but, on the other hand no changes in CK levels were seen in patients on steroid treatment [183]. Matrix metalloproteinase 9 (MMP9) levels were found significantly increased in DMD patients compared to control, but this marker is not helpful in the treatment follow-up [181, 185, 186].

Other circulating potential biomarkers of disease progression that are significantly increased in DMD and are inversely correlated to muscle strength, function and quality of life are miR-1, miR-133, miR -206 detected using reverse-time PCR [187].

A potential marker that could be used in clinical trials is Dynamic Thiol/disulphide homeostasis [188].

Since in DMD the main cause of death is cardiac insufficiency, most research about biomarkers was mainly focused on searching for biomarkers for prediction and for prognosis of cardiomyopathy. In heart failure, BNP, tenascin C, thrombospondin-4, Interleukin 1 receptor-like 1 protein (ST2) and galectin-3 (Gal3) are proven biomarkers [189]. In DMD, both Brain Natriuretic Peptide (BNP) and N-terminal pro-brain natriuretic peptide (NT-pro-BNP) could predict poor prognosis when there is an underlying cardiomyopathy. BNP [190-192] is highly sensitive to systolic dysfunction and is a marker of poor prognosis. It is not helpful in early detection of cardiac dysfunction. NT-pro-BNP [193, 194] is a predictor of poor prognosis and death when disease is advanced but it is not a predictive biomarker to follow the response to cardiac treatment if its level was low before treatment with combination therapy Metoprolol and Enalapril compared to placebo, as its level stayed low after 19 months of treatment in adolescents with DMD. On the other hand, Cardiac Troponin T and cardiac Troponin I are possible cardiac biomarkers of early detection of cardiomyopathy in patients with DMD [195]. Cardiac MRI performed in DMD patients that suffered from chest pain and transient elevation of cardiac troponin I, showed early cardiac degeneration which suggests that cardiac Troponin I could be predictive for cardiac involvement. Troponin I is not correlated to muscle enzyme CK while cardiac troponin T could be increased and related to skeletal muscle abnormality [175, 176, 180, 196, 197]. miR-222 is also a potential circulating biomarker reflecting structural but not functional cardiac alteration in DMD/BMD [198].

ST2 is found to be significantly increased in the serum of patients with DMD affected with cardiomyopathy compared to normal controls and therefore ST2 could be used as a biomarker for detection of cardiac involvement [181].

## **Biomarkers on muscle biopsy**

Since DMD/BMD is a muscular disorder, some researchers were interested in looking for biomarkers in muscle. Muscle dystrophin quantitation could be used for the diagnosis of DMD/BMD [199]. Besides, the instability of dystrophin mRNA could be a potential biomarker of response to antisense oligonucleotide mediated exon skipping [200].

## **Urine**

Very few studies were performed to search for urinary biomarkers. Urinary ferritin [201] and 8-hydroxy-2'-deoxyguanosine [202] concentrations are higher in DMD compared to other neuromuscular disorders. Urinary prostaglandin PDG2 could help in monitoring disease progression [203].

## **Imaging**

Bone mineral density (Dexascan) is correlated to bone health in DMD and is found to be low especially in non-ambulant DMD [204, 205]. MRI/MRS (Magnetic Resonance Imaging – Magnetic Resonance Spectroscopy) biomarkers are reflective of disease progression especially when performed early stages of the disease <12 years of age [206-209]. MRI/MRS could also be useful in monitoring response to treatment as it detects decrease in inflammation after corticosteroids administration [210]. Cardiac ultrasound is also helpful to monitor the disease progression as cardiac involvement is a marker of severity. [192, 207, 211, 212]. Conventional ultrasound can be informative, but quantitative ultrasound is a more sensitive tool [213]. Left ventricular tonic contraction using conventional ultrasound and left ventricular strain using speckle-tracking echocardiographic imaging are a promising tools of early detection of cardiomyopathy in DMD and thus could help prevent cardiomyopathy [214-216].

## **Genetics**

Diagnosis of DMD/BMD is confirmed by genetic testing. Several variant types have been identified in *DYS* gene such as copy number variants (CNV - deletion, duplication), single nucleotide variant (SNV), small insertion [217-219]. Preimplantation genetic diagnosis is indicated in DMD [220]. The identification of carrier females is important for future pregnancies and for the follow-up of these women. Germinal mosaicism should be considered when assessing risk of recurrence of dystrophinopathies [221]. Prediction of cardiac

involvement could also be possible using genetic testing as some variants seemed to be related to cardiomyopathy while others protect against cardiac alteration [222].

## **Motor function testing**

As for motor function tests the 6-minutes walking test and time function test are used in DMD as biomarkers for follow-up [175, 223].

## **Other muscular dystrophies**

In other muscular dystrophies (FSHD, LGMD, EDMD, congenital muscular dystrophy, myotonic dystrophy), 21 articles [175, 192, 202, 217, 224-239] identified 41 potential biomarkers. They are mainly circulating cardiac, or muscle related. The most relevant are cardiac troponin T [175, 227, 240], cardiac troponin I [226, 240], BNP, microRNAs (miR-133a, miR-193b, miR-191, miR-140-3p, miR-454, miR-574, miR-885-5p, miR-886-3p, miR-27b, miR-1, miR-27b, miR-133a, miR-133b, miR-206, miR-140-3p, miR-454, miR-574) or genetic variants [224, 225, 229, 230, 241]. Muscle biopsy could help orienting the diagnosis using paraffin wax embedded muscle [228].

## **Motor Neuron Disease**

With the therapeutic revolution in spinal muscular atrophy, several studies were conducted to find therapeutic follow-up and diagnostic biomarkers.

## **Blood, cerebral spinal fluid, and urine biomarkers**

Most biomarkers are either circulating plasma proteins, metabolites and transcripts identified using proteomics, metabolomics, and transcriptomic studies [242, 243]. (Omics are detailed in the next section). Molecular biomarkers frequently used are *SMN2* copy number, *SMN* transcript level, SMN protein, *SMN* genetic testing [244]. Neurofilaments are potential biomarkers in blood [245]. In CSF, malondialdehyde, superoxide dismutase, catalase are potential biomarkers [246]. Metabolomic study using urine samples enabled to identify a large number of differentially expressed analytes [243].

## **Motor function testing**

Motor functional studies and strength pulmonary studies were more regularly used in SMA studies [247]. The motor function tests include Motor Function Measure (MFM) [248], wrist angular velocity [248], wrist acceleration velocity [248], Brooke upper limb score [248], dominant side and non-dominant side [248], predicted functional reaching volume [248], Hammersmith Functional Motor Scale (HFMSE) [249], Alberta Infant Motor Score (AIMS) [247], Children's Hospital of Philadelphia Infant Test for Neuromuscular Disorders (CHOP INTEND) [247], Test of Infant Motor Performance Screening Items (TIMPSI) [247], Microsoft Kinect [250]. As for the strength studies, respiratory function tests useful as biomarkers of follow-up and disease progression are Forced Vital Capacity (FVC), Maximum Expiratory Pressure (MEP), Maximum Inspiratory Pressure (MIP), Peak Cough Flow (PCF) [248].

## **Electrophysiology**

Electromyography is a diagnostic tool but also a disease biomarker in SMA. Motor Unit Number Index (MUNIX) is a biomarker in adults with SMA but could also be used in children [251, 252]. Compound muscle action potential (CMAP) is more frequently used in children with SMA and is a biomarker of disease severity and used for the treatment follow-up [247, 248, 253]. Electrical impedance is another myography technique that could be used to classify SMA subtypes [254].

## **Imaging**

Different imaging tools could be used for SMA investigation such as MRI [248], Dexascan [253] and ultrasound [254].

## **Metabolic Myopathy**

Pompe disease is a metabolic myopathy for which an enzyme replacement therapy is available. Serum microRNAs (miR-1-3p, miR-133a-3p, miR-206) have been identified as potential biomarkers [255], while myostatin and IGF1 were identified as therapeutic biomarkers [256]. Serum Troponin T is a prognosis biomarker in metabolic myopathies [197].

Regarding imaging, a study about cardiac phenotypes in mitochondrial myopathies showed that cardiac MRI features could be a biomarker in patients with different phenotypes and genotypes [257]. Some genetic variants could be linked to a given phenotype e.g. R631C variant is a biomarker of severity in muscular form of carnitine palmitoyl-transferase II deficiency [256].

## **Hereditary Motor Sensory Neuropathies**

In this group of disorders, very few biomarkers have been characterized. Neurite density on skin biopsy, most specifically PGP9.5-immunolabeled skin biopsies showed absent epidermal innervation distally in the lower limb with relative sparing in the thigh. Neurite density was highly correlated with disease severity and electrophysiologic evaluation (median motor amplitude) [258].

When suspected hereditary axonal neuropathy, the first-line analyses should include *MFN2* gene sequencing irrespective of the mode of inheritance since this entity is clinically heterogeneous [197]. In Japanese adults, *MME* gene sequencing to characterize autosomal recessive axonal neuropathy could help in early diagnosis [259].

## **Spastic Paraplegia**

Few blood biomarkers were found in hereditary spastic paraplegias: variants in *atlastin* (*ATLN-1*), *spastin* (*SPAST*), *REEP1* genes account for half of the autosomal dominant HSP [260]. In hereditary spastic paraplegias due to the *CYP7B1* gene alteration, a decreased *CYP7B1* mRNA levels were indicative of the possible loss of function [261].

## **Omics in neuromuscular disorders**

### **Omics in DMD/BMD**

Large scale proteomics explorations on serum of patients with DMD/BMD compared to controls in cross-sectional study showed 10 potential biomarkers of diagnosis of dystrophinopathies, 285 proteins separate DMD from healthy, 121 proteins differentiated BMD from DMD, and 13 proteins separated BMD from healthy individuals in longitudinal studies [176]. 427 proteins were correlated to disease progression muscle loss and fatty replacement in muscles as well cardiac involvement [176]. Combined results of MS- and ELISA-based

quantifications were not sufficient to provide protein signature but revealed various myofibrillar proteins, muscle-derived titin, myosin, and carbonic anhydrase I as well as Vitamin D binding protein, Fibulin-1, Gelsolin as potential biomarkers [262]. Another proteomics study using SOMAscan assays (Slow Off-rate Modified Aptamers) detected 44 proteins that could be potential biomarkers (24 increased, 20 decreased) differentiating DMD patients from healthy controls [180]. In this latter exploration the analytes distinguishing DMD, were mostly of muscle origin and were very high at young age (4-10 years) then decline : creatine kinase M-type (CK-M), fatty acid binding protein 3 (FABP3), myoglobin (MB), carbonic anhydrase III (CA3), malate dehydrogenase (MDH1), lactate dehydrogenase B (LDHB), glucose phosphate isomerase (GPI), Hsp70 (HSPA1A), troponin I, fast skeletal muscle (TNNT2), troponin I, cardiac muscle (TNNT3), mitogen-activated protein kinase 12 (MAPK12), and calcium-calmodulin-dependent protein kinase II $\alpha$  (CAMK2A). Proteins associated with connective tissue remodeling are lower in DMD to note prolyl endopeptidase FAP (FAP), protein jagged-1 (JAG1), bone sialoprotein 2 (IBSP), ADAM metalloproteinase domain 9 (ADAM9), cadherin-5 (CDH5), neural cell adhesion molecule L1-like protein (CHL1), osteomodulin (OMD), and contactin-5 (CNTN5). Several other proteins were detected in that study and are associated with inflammation and innate immune response [180].

Metabolic signatures could be potential marker to monitor DMD disease progression and response to treatment. Fourteen metabolites were significantly altered in serum of DMD compared to healthy controls (arginine, creatine, and unknown compounds at m/z of 357 and 312 while decreased metabolites included creatinine, androgen derivatives and other unknown) in serum metabolomics using LC-MS [178].

Protein studies on muscle biopsy (Western blot and LC-MS/MS methods) identified an increase in skeletal troponin T rather than cardiac troponin T in DMD patients while proteomics on serum studies showed increase in cardiac troponin T as possible biomarker for diagnosis of neuromuscular diseases [175].

Other -omics studies on muscle biopsies of DMD were metabolomics analysis using untargeted mass spectrometry imaging (matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry) revealed 34 metabolites that are significantly altered compared to control and these metabolites are involved in energy and phospholipid metabolism [263]. However, verification and validation of MS-discovered serum biomarkers remain challenging [264].

## Omics in SMA

Non-targeted CSF proteomics studies using mass spectrometry were done in 10 adults with SMA type 2 and 3 before and after 10 months of treatment. Interestingly, two groups of SMA patients with different age and expression of nerve degeneration and regeneration proteins were identified [165, 265]. Transcriptomics assessed the perineural net elements' function and showed that HAPLN1 plays a fundamental role in clinical variability among SMA patients with the same phenotype [266]. Proteomics studies identified some proteins that are either genetic modifier like PLS3 and NCALD, SMN modifiers Elavl4 (HuD), Copa, Uba1, Mapk10 (Jnk3), Nrnx2 and Tmem41b, and some proteins that are downregulated while others are upregulated [266]. Genomics approach has been used alone or in a multi-omics approach along with transcriptomics in clinical trials in SMA [267]. Moreover, metabolic abnormalities (alteration of fatty acid metabolism, glucose tolerance impairment, and mitochondrial defects) were reported in SMA animals and patients and there is a possible impact of SMN deficiency on metabolic abnormalities therefore metabolomics studies could be helpful in enlightening the underlying mechanisms of the disease and in finding potential biomarkers [268].

Relatively few potential biomarkers have been studied such as functional outcome measures to assess the response to treatment. NatHis-SMA study showed that Motor Function measure (MFM), pulmonary function testing, strength, electroneuromyography, muscle MRI and workspace volume discriminate between SMA types 1, 2, 3 and 4. MyoSet devices and Active-seated discriminate type 2 and 3 [248]. Body composition was also evaluated as a potential biomarker but the correlation with motor function was weak in SMA type 2 and moderate in SMA type 1 [269]. Concerning electrophysiological biomarkers, NeuroNEXT study showed that compound muscle action potential (CMAP) is a potential biomarker [247]. In addition to CMAP, Motor unit number estimation (MUNE) has been studied and amplitude of both declined with age in SMA 1 and 2. These measurements delineate motor function and may reveal abnormalities before symptoms appear. The stability of these two electrophysiological biomarkers is an indication of capacity for prolonged motor neuron survival. While CMAP may remain stable over time, MUNE has been shown to increase [163, 270, 271]. Circulatory biomarkers have gained significant attention for therapeutic monitoring. The first one is SMN protein that can be measured in biological samples and could be useful to monitor treatment that increase SMN levels [271]. Czech et al. study showed that the expression of SMN mRNA and protein in blood greatly overlap between different disease types and thus cannot be used as a marker for clinical severity [272]. The second potential biomarkers are neurofilaments

which are intermediate filaments of the neuronal axons. Phosphorylated neurofilament heavy chain (pNfH) and neurofilament light chain (NfL) have been associated with axonal degeneration and their levels tend to increase in cerebrospinal fluid, plasma/serum of patients with motor neuronopathy [159, 165, 271]. There is a positive correlation between CSF NfL levels and age of treatment as well as duration of disease, but no correlation found with CSF pNfH. To note that there is a slight but significant decrease in both neurofilaments type before and after 6 months of treatment with Nusinersen [159]. Although plasma neurofilaments seem to be reliable biomarkers in infants that is correlated to motor function improvement [245], contradicting results concerning adult patients' response to treatment [165]. Besides, the protein Tau is considered as a cortical neuronal marker. Its concentration is high in CSF of SMA types 1 and 2 children and decreases with Nusinersen treatment, but it is not altered in SMA type 3 patients [165]. Serum creatinine could be an interesting biomarker of muscle deterioration; however, the range of variability is very wide. The combination of increased tendency of creatinine concentration with the decrease in creatine kinase activity during Nusinersen treatment suggests decreased muscle mass wasting and an improvement in the muscular energy metabolism. However, prospective studies are needed [273, 274]. Some potential imaging biomarkers have been studied: muscle MRI and ultrasound, electrical impedance myography, MR neurography, imaging flow cytometry combined with spot analysis, magnetization transfer ratio [275-277]. Splicing regulators like miRNAs could be interesting as biomarkers and therapeutic targets for neurodegenerative diseases like SMA [278]. MyomiR serological levels decrease after Nusinersen treatment in infantile SMA [279]. Another regulator is SMN2 methylation that could change the disease phenotype by modulating its transcription. Differences in methylation patterns in certain genes through genome-wide methylation studies could be involved in pathogenesis of SMA [271].

## **Perspectives and conclusion**

Pediatric neuromuscular disorders are heterogenous, rare neurological diseases. The diagnosis of these disorders is challenging despite the advances in genetics. These challenges encouraged scientists to join efforts to search for biomarkers for diagnosis, for (i) prevention of the disease or its complications, (ii) understanding the pathophysiology, (iii) designing trials more efficiently with smaller groups and more efficient outcome measures, and (iv) developing targeted therapy. In the past years, the neuromuscular field marked a real breakthrough in healthcare where the real benefit was seen after early intervention and using targeted treatment.



The best example is SMA where targeted therapy changed the natural history. However, monitoring these patients is still challenging and more research is needed to find biomarkers. One way to find biomarkers is using omics approaches. Omics sciences are revolutionizing research in the health field by enhancing the power of discovery and providing deeper understanding with a more holistic view. As we notice in this review, there are few biomarkers identified that could be useful in clinical practice and few omics studies performed. More precisely, there are very few metabolites identified as potential biomarkers as few metabolomics studies were performed. Metabolomics studies are sensitive to subtle functional changes in the genome and proteome as these changes can be amplified at the metabolic level. We expect that metabolomics studies would reflect the wide spectrum of clinical phenotype by the detection of a broad scope of biochemical signature which therefore would offer more possibilities for treatment development. Metabolomics alone are not sufficient and multi-omics explorations which uses multiple approaches by layering various omics on top of each other for a multidimensional view of human biology would help understanding a single problem. With these sciences we go from large scale to depths. The concept of evidence-based precision medicine involves multi-omics studies in addition to the clinical, laboratory and imaging data to better understand the disease phenotypes and to discover biomarkers of diagnosis, prognosis, and to develop treatments [280-283].

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## **Supplementary material**

‘Supplementary material is available at *Brain* online’.

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## CHAPTER 3: DUCHENNE MUSCULAR DYSTROPHY

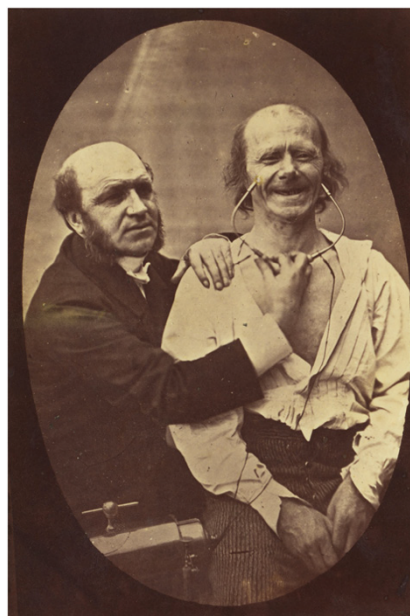
### I. Duchenne de Boulogne (1806-1875)

Guillaume Benjamin Amand Duchenne (**Figure 5**) was the son of Jean Duchenne, a sea captain during Napoleonic wars, and was destined to have a career at sea, but he did not follow his father's will and decided to move to Paris in 1827 to complete his medical studies after graduating from a local college at Douai. He had renowned teachers like René-Théophile-Hyacinthe Laënnec (1781–1826), Baron Guillaume Dupuytren (1777–1835), François Magendie (1783–1855) and Léon Cruveilhier (1791–1874) and obtain his MD degree in 1831 in Paris. He returned to his town of origin Boulogne-sur-Mer where he worked in private practice but soon after returned to Paris in 1842 after he was devastated by the death of his young wife during childbirth [240]. He was criticized for his provincial accent and his coarse behavior and was named Duchenne de Boulogne referring to his city of origin in order not to confuse him with the renowned society physician Edouard Adolphe Duchesne (1804-1869). Duchenne worked in private practice in several charity clinics and hospitals and never applied and was never offered to work in a university hospital in Paris. However, he was known to be rigorous, determined, and meticulous in recording clinical histories and in completing investigations which helped him obtain outstanding research material [241]. His introduction to the academic world was through the publication of his innovative technique in electrophysiology, localized electrization, consisting of stimulation of craniofacial groups using fine electrodes [241, 242]. Using the simple electrical stimulator, he performed detailed analyses and described various neuromuscular disorders particularly pseudohypertrophic muscular dystrophy known today by his eponym as Duchenne muscular dystrophy (DMD). Other disorders described by Duchenne were facio-scapulo-humeral muscular dystrophy, progressive bulbar paralysis, poliomyelitis, progressive locomotor ataxia, syringomyelia and lead poisoning [242, 243].

At the end of his life, he became friend with Jean-Marie Charcot who called Duchenne “The Master” due to his exceptional clinical abilities. Charcot was one of the founders of modern neurology, an internationally renowned professor of neurology that described several neurological disorders like multiple sclerosis, amyotrophic lateral sclerosis, inherited neuropathies...etc. He invited Duchenne to work at *La Salpêtrière*. Duchenne became famous internationally at the end of his career. He held dinner parties monthly for his colleagues



(Charcot, Pierre Paul Broca, Auguste Nélaton and Edmé Félix Alfred Vulpian) and discussed histological slides as first attempts for muscle pathology. Duchenne was the first to construct the needle muscle biopsy done percutaneously without anesthesia (which he called histological punch) in order to obtain tissue from living patients and to perform histopathological diagnosis [244]. His idea to perform biopsy on living patients was a consequence of his scant access to post-mortem biopsy even to the patients that he took care of for years as he didn't work in hospitals in Paris [245]. His contribution to the neuromuscular field was not only in diagnosis (electrophysiology, biopsy, and clinical description) but also in management where he designed several orthoses [242, 243]. Duchenne was an expert in documentation, and he published the first illustration of photography of living patients in *Album de photographies pathologiques* in 1862 as a complement to the second edition of his book "*De l'électrisation localisée*" [245]. He also used photography in his neuroanatomical and neuropathological studies where he presented the first illustrations of brain neurons and nuclei. He also contributed to arts through his book "*Mécanisme de la physionomie* " [241] where he showed various forms of emotional physiognomies obtained by electric stimulation of distinct facial muscles.



**Figure 5 :** Duchenne de Boulogne (left) and his patient (right) while performing facial focal electric stimulation; photo taken from [242]

## II. Duchenne and Becker Muscular Dystrophies

### *Definition*

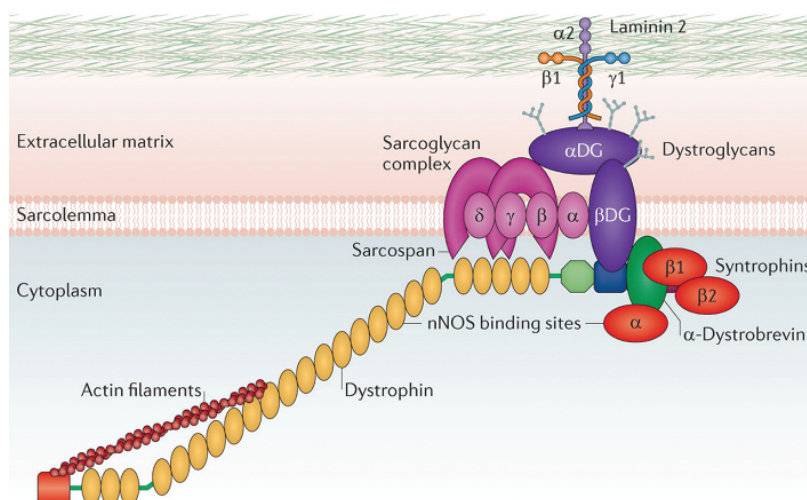
Muscular dystrophy is a group of hereditary disorders presenting with progressive alteration of the muscle due to abnormal function or absence of proteins in the muscle cell plasma membrane [246]. DMD is the most common childhood muscular dystrophy characterized by absence of dystrophin while the protein is deficient in Becker muscular dystrophy (BMD) [247].

### *Epidemiology*

The worldwide prevalence of DMD and BMD is estimated 4,8 and 1,6 per 100.000 people respectively according to a systematic review involving 901.598.055 people from 25 articles [8]. The annual incidence is around one in 5000 live males [9]. Early diagnosis of DMD is crucial in the era of disease-modifying treatments. Average age at diagnosis is 5 years and the time between first signs and diagnosis is 2,2 years [15].

### *Mode of inheritance and pathophysiology*

DMD and BMD are X-linked recessive disorders, caused by variant of *DMD* gene, which is the largest gene known in humans [6]. This leads to production of a non-functional dystrophin protein that is absent in DMD and deficient in BMD. Dystrophin is present in skeletal and cardiac muscles, cortical neurons and cerebellar neurons [7]. It is a membrane protein that links the ECM to the internal cytoskeleton via glycoproteins [6]. It has a major role in stability of the muscle fiber as it anchors the actin cytoskeleton to the basement membrane with a membrane glycoprotein complex and it provides a shock-absorbing connection between the cytoskeleton and ECM [10, 248] (**Figure 6**).



**Figure 6:** Structural representation of dystrophin. Dystrophin is in interaction with actin filaments from one side and binds to the DG complex from the other side. There are neuronal nitric oxide synthase (nNOS) binding sites on dystrophin and  $\alpha$ -dystrobrevin. Dystroglycan (DG) complex made of  $\alpha$ -dystroglycan ( $\alpha$ -DG) ;  $\beta$ -dystroglycan ( $\beta$ -DG) and sarcoglycans is linked to cytoplasmic proteins syntrophins,  $\alpha$ -dystrobrevin and dystrophin [249] .

Dystrophin absence or alteration leads to destruction of dystroglycan and weakens the membrane therefore destroy the muscle cells [246, 250]. The contractile unit of actin/myosin shortens and has altered function thus leading to muscle weakness and lesions in cell membrane. The damaged muscle cells suddenly leak the creatinine kinase which reaches abnormally high levels in plasma. This leak is responsible for an abnormal inflammatory response with scar tissue formation inside the muscle thus giving the pseudohypertrophic appearance in the calf muscles [251, 252]. Altered muscle fibers are replaced by fat and connective tissue in skeletal, cardiac, and gastrointestinal smooth muscles[253, 254] . In the brain the defect in dystrophin manifests structural deformity [253].

### *Clinical presentation*

Clinically, first symptoms appear after the first year of life with delayed gross motor milestones and failure to achieve the ability to walk fast, run, jump and climb stairs, inability to get up from the floor without pushing in the knees and getting the help with hands (Gower's maneuver) [10-14]. In addition to proximal muscle weakness, calf pseudohypertrophy [11], the muscular dystrophy is stable the first years until around 7 years then rapid progressive degradation and loss of ambulation occur at 12 years (mean age of 9.5 years), scoliosis, stiffness of distal muscle, upper limb weakness, respiratory obstructive and

restrictive insufficiency, cardiomyopathy, gastrointestinal complications (constipation, dysphagia, feeding problems, obesity or anorexia) [255], death in the late teens. This natural history changed with the use of corticosteroids and optimization of standards of care (respiratory, cardiac, orthopedic) [11, 25, 256]. The mean age of loss of ambulation became 13-14 years old, the risk of scoliosis is decreased, cardiac health and long-term survival improved [7, 257]. Dystrophin is also present in the brain in multiple isoforms, Dp140 and Dp71 [258], therefore its alteration leads to variable brain involvement that is non-progressive in DMD patients [259, 260]. Furthermore, brain MRI study of DMD patients compared to healthy subjects revealed smaller volume of grey matter mainly in the subgroup of patients lacking the brain dystrophin isoform Dp140. Patients also have smaller total brain size, lower white matter fractional anisotropy, and higher white matter mean and radial diffusivity [261]. The severity of a patient's cognitive delay variability is correlated to the variability in the location of the dystrophin gene variant and a consequence alteration of brain isoforms [262-266]. Mental retardation, speech delay, autism spectrum disorder, attention deficit hyperactivity disorder, learning difficulties, anxiety could be observed in DMD patients [258, 264, 267, 268]. Cognitive delay affects the ability of walking independently that was found to be altered in DMD patients with variant affecting different brain dystrophin isoforms [263].

### *Diagnosis*

When DMD is suspected, the diagnostic strategy is now well established. Early diagnosis is important in order not to delay the implementation of an adapted management and for genetic counseling. Usually, the diagnosis is made around the age of 3 to 4 years.

The patient is brought to medical attention when the caregivers notice clinical symptoms of motor delay, frequent falls, difficulty in running, jumping, or climbing stairs, fatigue, and muscle pain. The chief complaint could also be cognitive delay or learning difficulties. In rare cases, the patient is asymptomatic, but diagnosis is suspected when abnormal laboratory workup is seen in a suggestive family background.

Blood workup shows markedly increased levels of creatine kinase concentrations (>5000 – 10.000 IU/L) and elevated liver function tests (SGOT, SGPT). A muscle biopsy is performed, and dystrophy is seen on histology with areas of necrosis and degeneration-regeneration. The absence of dystrophin is gold standard for diagnosis of DMD and is quantified by western blot

analysis. Currently, with the availability of genetic testing to diagnose dystrophinopathies, the muscle biopsy is useful when clinical and genetic phenotypes are not suitable or in research to evaluate treatment efficacy translated by dystrophin restoration [16]. The diagnosis of DMD is confirmed by genetic sequencing of *DMD* gene (OMIM\*300377) [17]. Variants are most often deletions or duplications of one or more exons, or more rarely point variants. Genetic counseling in the family of the index case is suggested after confirmation of the diagnosis of DMD.

## *Evaluation*

### *1. Primary evaluation*

An initial evaluation is performed to determine the disease severity and the possible associated complications. This includes an evaluation of motor function and strength as well as determination of orthopedic complications (retractions, scoliosis, etc.). Concerning the associated complications, the primary evaluations of respiratory function (vital capacity, SNIFF nasal inspiratory pressure) and cardiac function (electrocardiogram, cardiac ultrasound, ...etc.) are performed initially and on the follow-up. Cognitive function tests are performed if the clinical presentation is suggestive.

### *2. Evaluation at follow-up*

Clinical evaluation of disease progression and prognosis of patients with DMD is through different types of functional studies that varies with the stage of disease. These studies include timed function tests: 6-minute walk test [18-20, 22-24] is the most popular outcome measure in ambulant patients, time from supine to standing position, time to climb four-step stairs, and hand-to-mouth function [21]. Other studies to evaluate DMD are strength testing, range of motion, activities of daily living and motor function scales [17]. Yearly follow-up tests are recommended to evaluate the respiratory and cardiac function.

## *Management*

Management of DMD is multidisciplinary and requires the collaboration between different specialists: pediatric neurologist, cardiologist, pulmonologist, orthopedist, rehabilitation therapist. Respiratory and cardiac complications are the major cause of morbidity and mortality, and the early management of these complications improved the quality of life and

prolonged the life of DMD patients. The intervention of other medical specialists is possible depending on the patients' needs. The patient is seen once to twice per year for follow-up and appropriate management. Psychologists, physical therapists, nutritionists play a major role in the management of these patients. International guidelines have been published to provide standard of care for patients with DMD [11, 17, 25, 256].

### *Therapies*

Currently glucocorticoids are the only treatment with proven benefit in slowing disease progression [11, 25, 256]. Emerging therapies exist, few of them received regulatory approval and none has shown undebatable clinical efficacy. Three intravenous gene therapy drugs are on trial PF-0639926 and SGT-001, which are AAV9-based, and SRP-9001, which is AAVrh74-based and one intramuscular gene therapy rAAVrh74.MCK.micro-dystrophin has been completed [269]. The trial of the first IV gene therapy mentioned PF-0639926 was on hold after the death of one patient in 2021 by trials are now resuming [270].

Other therapies to mention are stop codon readthrough (Ataluren (PTC-124)), approved exon skipping therapies target exon 45, 51, and 53. Exon skipping 51 (Eteplirsen (AVI-4658), SRP-5051)[271-273], Exon 45 skipping Casimersen (SRP-4045) is approved, [274] while DS-5141b is under study (no peer review publication). Approved Exon 53 skipping is Golodirsen (SRP-4053) [275-277], while Vitolarsen (NS-065, NCNP-01) and WVE-N531 are under trial. Exon 2 skipping under development (scAAV9.U7.ACCA)[270]. There are also stem cells and cell-based therapy and upregulation of dystrophin surrogate proteins [270].

### *Newborn screening*

In 1976, a myologist (Dr. Dubowitz), proposed dosing creatine kinase levels to screen for DMD and by this approach he had initiated the concept of newborn screening for neuromuscular disorders [278]. In 1977, a screening program for DMD in Germany was developed by a researcher (Dr. Scheuerbrandt) at a time where ethics committee or review board were not mandatory for such scientific investigations. This program aimed to screen for high creatine kinase to identify those with DMD and BMD during 34-year period [279]. Creatine kinase does not reflect the genetic defect and could be falsely positive or falsely negative. Mendell et al. developed a 2-tier system of analysis to measure CK activity using a fluorometric assay based upon the enzymatic transphosphorylation of adenosine diphosphate to adenosine

triphosphate, followed by variant analysis using genomic DNA extracted from the dried blood spot then they did whole genome amplification using multiplex ligation-dependent probe amplification to detect deletions/duplications in the *DMD* gene [280]. Despite these advances, one should consider that approved therapy is limited to a small group of DMD patients and targeted therapy using gene therapy is still not approved and not yet administered in newborns, therefore a screening program targeting deletions responsive to exon-skipping therapy (exon 45, 51 and 53 skipping) [281]. Targeted screening could help in optimizing the management and treatment and probably might help introducing pre-symptomatic care. To date, screening programs in DMD are limited worldwide probably due to ethical considerations and limitations of treatment.

## CHAPTER 4: SPINAL MUSCULAR ATROPHY

### I. Aran-Duchenne

In 1850, François-Amilcar Aran described patients with “progressive spinal muscular atrophy” presenting with slowly progressive disease, muscular atrophy sparing some muscles, muscle fasciculations, claw hands, without alteration in sensitivity in order to refer to the first clinically described patients with spinal muscular atrophy (SMA) [94, 282]. Aran was a French scientist and doctor that was born in Bordeaux, and he completed his medical studies in Paris. He was very active in publishing and even published his first paper in 1843 before obtaining the medical doctor degree [283]. Duchenne de Boulogne helped Aran in the studying these first cases of SMA in 1848 and 1850 and he performed the innovative localized electrical stimulation that permitted the differentiation from paralysis and the identification of different types of SMA [38, 244]. Duchenne asserted that he described the first cases of SMA before Aran’s first publication during a weekly meeting of the French Academy of Science in 1849 (that was intended to be used as evidence but never left a record of its existence) [284]. The disease was named previously after both scientists by the eponym “Aran-Duchenne hand” while Charcot used the term “Duchenne-Aran atrophy” and nowadays the disease is known as spinal muscular atrophy [38, 244].

### II. Spinal Muscular Atrophy

#### *Definition*

Spinal muscular atrophy is a debilitating neuromuscular disease characterized by progressive muscular atrophy and weakness leading to irreversible paralysis. There is a spectrum of disease severity with a continuum going from prenatal form that is incompatible with life to milder and more benign forms with adulthood onset [285] .

#### *Epidemiology*

Spinal muscular atrophy worldwide incidence is 1:10,000 [35-37] births or 7,8-10 in 100,000 live births [286], similar incidence between male and female [39], carrier frequency range between 1 in 40 to 1 in 60 [36].



### *Inheritance and pathophysiology*

The disease inheritance is autosomal recessive. Homozygous deletions of the *SMN1* gene or loss of function variants are the main SMA-determining cause while its homologous gene *SMN2* is the major modifier of the disease severity by pathogenic variants in the Survival Motor Neuron 1 (*SMN1*) gene on chromosome 5 which encodes the SMN protein (survival motor neuron protein) [27-30]. Both genes are located on chromosome 5q11.1-13.3. *SMN1* and *SMN2* sequences are very similar, only changes in 5 nucleotides occur, one of them located in exon 7 of the *SMN2* gene results in alternative splicing (which is a crucial event in SMA pathogenesis) [287, 288]. As a result, 90 % of the expressed *SMN2* protein is truncated lacking the exon 7. This protein is unstable, and easily degraded [31, 32]. Of note, *SMN2* is present at least at one copy in patients but the number of copies is variable among individuals. Usually, the *SMN2* copy number is inversely correlated with SMA disease severity [29, 33] but other factors such as hypoxia and Plastin 3 expression could influence the phenotype [34].

### *Classification*

There is a broad spectrum of age onset, disease course and severity. The historical classification differentiates five subtypes based on age of onset, severity, maximal motor milestone reached: type 0 is the most severe with death shortly after birth, type 1 non-sitters, type 2 sitters, type 3 walkers, type 4 adult onset [28, 33, 44]. This classification does not take into consideration the change in disease phenotype due to treatment with the new innovative approved therapies (Nusinersen, Onasemnogene abeparvovec and Risdiplam) [40]. For example, treated patients with SMA type 1 can achieve sitting position and develop a posture that wasn't detected before giving these medications [45]. Moreover, pre-symptomatic newborns with SMA that currently received therapy, have normal development in many cases and there are ways to monitor their response to treatment that need to be discovered [46].

### *Diagnosis*

#### *1. Clinical presentation*

It is characterized by progressive degeneration of the  $\alpha$ -motor neurons resulting in generalized muscular weakness of limbs and trunk and secondary moderate to severe impairment, progressive muscle atrophy, bulbar and respiratory muscles involvement [40]. When disease onset in infants and toddlers, they present with tongue fasciculation and in

severe cases with signs of respiratory distress (use of accessory muscles) [26]. In children where disease onset is later in childhood, the clinical presentation could be waddling gait, hypo- or areflexia without any signs of sensory nerve abnormalities [26].

Cognitive functions are usually preserved, even if in the most severely affected patients, neurodegeneration could reach not only the anterior horn cells and dorsal root ganglia and thalami, but is also widespread in cerebral cortex, basal ganglia, brainstem, and cerebellum [41].

## *2. Electromyography (EMG)*

When the disease is suspected an EMG could be beneficial and shows signs of axonal motor neuropathy (motor conduction velocities are normal or slightly decreased, needle study is abnormal with signs of neuropathic alterations)

## *3. Genetic testing*

The disease is confirmed after performing molecular diagnostic testing for *SMN1* gene. The correlation between the phenotype and the genotype is not absolute and the main determinant of the different subtypes relates to the number of copies of the gene *SMN2* [27, 30, 42-44]. The phenotype is related to the *SMN2* copy number rather than the expression levels of *SMN2* in the blood [42].

## *4. Other diagnostic tools*

Creatine kinase is slightly elevated which leads to suspicion of muscular dystrophy, therefore an EMG could help in differentiating motor neuron disease from muscular dystrophy. There is no role for muscle biopsy in the diagnosis of SMA.

## *5. Management and follow-up*

The establishment of international guidelines published in 2007 [289] and updated in 2018 [290, 291], has allowed the early recognition of the disease and early treatment of clinical signs and consequently improved the prognosis. Standard-of-care highlighted the necessity of multidisciplinary management for SMA patients. Different specialists are directly and regularly involved in the management of these patients: pediatric neurologist, physical therapist and rehabilitation physician, orthopedist, pulmonologist, and other specialists depending on the situation. Orthopedic and rehabilitation specialists monitor and work on

preventing deterioration of scoliosis/contractures/weakness and on stabilizing or improving the muscle function and range of motion. Several functional tests are validated and are helpful in routine use: Children's Hospital of Philadelphia Infant Test (CHOP INTEND), Hammersmith Infant Neurologic Examination Section 2 (HINE-2), Functional Motor Scale Expanded (HFMSE) and the Revised Upper Limb Module for SMA (RULM) [40].

Respiratory care includes an initial evaluation for respiratory function (forced vital capacity, oxygen and CO<sub>2</sub> levels sleep study). When signs of respiratory distress are detected, mechanical support is recommended by using invasive or non-invasive ventilation.

In SMA type 1 or in the severe form of SMA type 2, special attention should be given to bulbar involvement. This includes regular evaluation and management by a nutritionist and speech therapist. Specific protocols are established to monitor acute care remotely or in hospital setting (respiratory decompensation, dehydration, hypoglycemia) [40].

#### *6. Innovative therapies*

In the past few years, innovative therapies have been developed to treat SMA. Nusinersen (Spinraza), the first drug approved, is an antisense synthetic oligonucleotide (ASO) that binds to intron 7 of the *SMN2* pre-mRNA, which leads to the translation of a fully functional SMN protein [27, 28, 44, 292]. It is administered intrathecally with a loading dose in the first two months (day 1, 15, 29, 64) and maintenance doses every fourth months [292]. The earlier the drug is given, the higher the success rate [33, 293]. Other therapies are Onasemnogene Apeparvovec (Zolgensma), a one-time *SMN1* gene replacement therapy given intravenously or intrathecally and Risdiplam (RG7916), a small molecule splicing modifier given orally [28, 293]. The current innovative therapy can significantly alter the disease course.

#### *7. Newborn screening*

Concerning early diagnosis, newborn screening programs have been developed in several countries: the USA, Germany, Belgium, and Australia [28]. The aim of these programs is to allow presymptomatic initiation of therapy [33]. These tools are important to detect affected children, but some patients show very mild disease course with late onset of symptoms. Therefore, predictive measures of the disease severity were developed to help clinicians in their decision to treat. In France a pilot study for SMA newborn screening will start in Grand

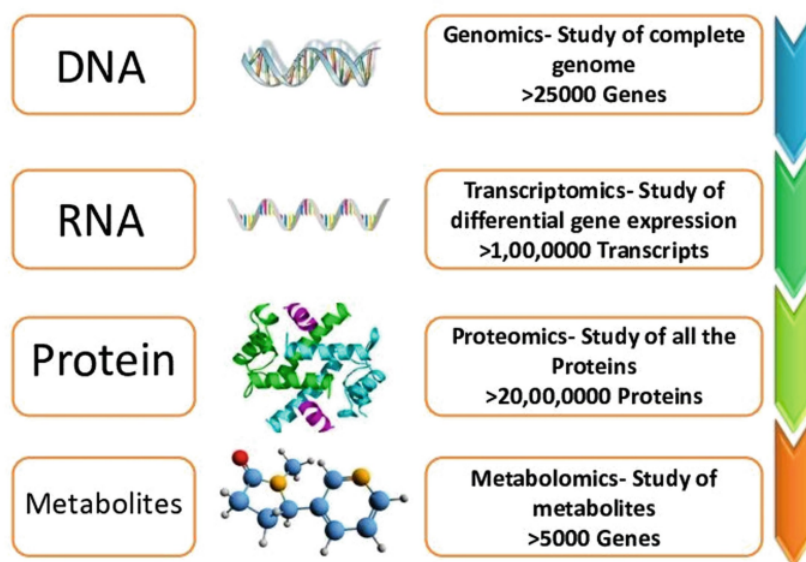
Est and Nouvelle-Aquitaine and will be for 2 years to test feasibility on the screening program before extending all over France.

## CHAPTER 5: OMICS SCIENCES AND PRECISION MEDICINE

### I. Introduction

In the 20th century, revolutionary advances have been made with the discovery of deoxyribonucleic acid (DNA) structure by Watson and Cricks in 1953 [47]. This was followed by the development of Polymerase Chain Reaction (PCR) by Mullis in 1986, which revolutionized the field of biotechnology and paved the way for the omics sciences [48].

The suffix -omics added to a molecular term define a new branch of systems biology that uses high-throughput technologies to study a set of molecules in a comprehensive and global way. There is a collective characterization and quantification of molecules from different biological samples therefore allowing the structure, function, and dynamics of an organism to be understood [49, 294]. There are thousands of omics data types in biology, the first one appears to be genomics, studying the genome. Genomics in addition to other omics studies like transcriptomics (the omics of ribonucleic acids (RNA), proteomics (proteins) and metabolomics (metabolites) will be discussed in this chapter (**Figure 7**).



**Figure 7:** the different supports of biological information and the corresponding "omic" sciences that allow their exploration [295]

Omics apply systems theories to the problems and efforts are ongoing to understand the whole universe from biological perspective by managing a very large amount of data that are

inextricably linked to bioinformatics and big data (<http://omics.org/>). Multi-omics integrate different types of omics data together which allows greater understanding of a large amount of information gathered, going from the etiology of a disease to the consequences [52, 296].

## II. Genomics

The genome is the complete set of DNA present in a cell or an organism that is necessary for its development and functioning [297]. The word "genome" is a conjunction of the words "gene" and "chromosome". In every cell of the body there is a complete copy of the approximately 3 billion DNA base pairs to constitute the genome ([www.genome.gov](http://www.genome.gov)). DNA is found mainly in the nuclei of cells (cellular DNA) but is also present in the mitochondria (mitochondrial DNA). The sequence of DNA in a species varies little over time (except for variants and chromosomal rearrangements) [298, 299]. Genomics focuses on identifying the role of genes in determining the disease traits in a complex setting whereas different factors other than genetics (example environmental) could modify or influence these traits. These latter factors establish the epigenetic phenomena which could be modulated by the environment in which the patient lives. These changes may modify the phenotype and can be transmitted to future generations [300]. An important tool used to identify thousands of genetic variants that are statistically linked with complex diseases or traits in multiple human populations is the genome-wide association study (GWAS). It is a research approach that help understanding complex phenotypes (GWAS catalog [www.genome.gov/gwastudies](http://www.genome.gov/gwastudies)). In such studies, the genomes of thousands of individuals are sequenced to look for genomic variants that occur between cases with a specific disease or trait compared to controls. Other linked technologies used in genetics include targeted analysis of several genes of interest by sequencing a panel of genes using new generation sequencing (NGS) [301], genotype arrays [302-304], the study of all exonic sequences and intron-exon junctions is performed by a Whole Exome Sequencing (WES) [305] and the analysis of the whole genome is implemented by Whole Genome Sequencing (WGS) [306].

Pediatric genomics is rapidly evolving by incorporating NGS, WES, WGS into clinical practice and research. Since the majority of rare diseases in children are of genetic etiology, genomic studies have resulted in increased discovery rate of causative genes leading to a better understanding of the genetic pathophysiology, better diagnosis, better management,

accurate prognosis, increase in the emergence of new therapies and a better support for the patient and his family [307].

### III. Transcriptomics

The transcriptome is the complete set of RNA produced by the genome in a cell or tissue at a given time, or under specific circumstances. The RNA varies and has different expressions of the genes according to the cell type, the environment, and the biological processes in progress [308]. We can distinguish several types of RNA, to note ribosomal RNA, messenger RNA, transfer RNA, micro-RNA, and other non-coding RNA. Transcriptomics is used to study the amount of gene expression by identifying the amount of RNA transcripts, the structure of the transcript, and by quantifying the different levels of expression of the transcripts in space and time and under varying physiological conditions [309]. It can be used to compare RNA between two populations (e.g., patients versus healthy controls), to evaluate the response of a cell to its environment (e.g., response to treatment), to evaluate the evolution of transcription during development (e.g., during embryogenesis), or to compare RNA in different cell types. Transcriptomics is the study of the transcriptome using high-throughput methods, such as microarray analysis and RNA sequencing [310, 311].

Microarray technology is an important tool, it provides a quick analysis of the transcriptome and has been useful for drug development and clinical research by analyzing thousands of genes from multiple samples. The main drawback of this technique is that it analyzes only known sequences and therefore does not allow the detection of new transcripts [312].

The latest technology in transcriptome analysis is RNA sequencing (RNA-seq) [313], based on deep sequencing technology [314]. It allows to study all RNA (coding and non-coding) present in a cell, or a biological fluid. It is therefore appropriate for revealing RNA processing variants and has been successfully useful in rare diseases especially in neuromuscular diseases [315, 316] and is an effective diagnostic tool for cases of cortical malformation without identified variant [317]. It analyzes differential gene expression and differential splicing of mRNAs. It sequences the cDNAs and compares them to a reference genome or transcriptome. An advanced form of RNA sequencing is RNA-seq in situ that analyses a cell in a fixed tissue.

Moreover, information about the genes expressed by a microbial community is called metatranscriptomics [318]. Metatranscriptomics provides a functional profile of the microbiome under varying physiological conditions [318]. The data generated are useful for

enrichment analysis and phylogenetic analysis of microbes. Several bioinformatics pipelines are currently designed or under development for the analysis of the metatranscriptome datasets [319].

#### IV. Proteomics

The proteome is made up of all the proteins in a genome, cell or tissue at a given time [320]. This concept was first introduced in 1994 first by Wilkins [321]. From the same genome, a significant number of different proteins is generated. The proteome is dynamic and varies according to the physiological and pathological conditions, to the cell type, and the role assigned to the protein and considers the co and post-translational modifications of the protein during the translation process [322]. The aim of proteomics is to characterize the expression, structure, function, and interactions of proteins present in a biological sample [323]. A better knowledge of proteins helps to understand the molecular mechanisms of biological processes. Proteomics can also be used to search for biomarkers specific to a disease [324, 325]. The analysis and quantification of proteins have been revolutionized by mass spectrometry (MS) to study macromolecules, which earned John Fenn and Koichi Tanaka the Nobel Prize in Chemistry in 2002. MS methods have been modified to perform high-throughput analyses of thousands of proteins in cells or body fluids [326-328]. Before the arrival of MS, the analysis of proteins was laborious, and the biologist had to purify a large quantity of proteins in a sample before beginning their study.

Different methods of proteomics studies have been developed and could be divided into targeted approach and non-targeted approach, some are gel-dependent, and others gel independent [329-332]. In the non-targeted approach studies, all the proteins are contained in a biological sample in an exhaustive way. Nuclear magnetic resonance (NMR) and high-resolution MS are used for this non-targeted approach. On the other hand, targeted analyses allow the study of a preselected group of proteins in order to deliver more precise, quantitative, sensitive data [333]. This latter analysis is possible by MS and immunoassays [334, 335].

In this work, we used two targeted proteomics approaches: (i) *Olink technology* is a high multiplex, high throughput protein analysis using Proximity Extension Assay (for each protein, a matched pair of antibodies linked to unique oligonucleotides binds to the protein target. The oligonucleotides hybridize to each other, and this sequence can be detected and



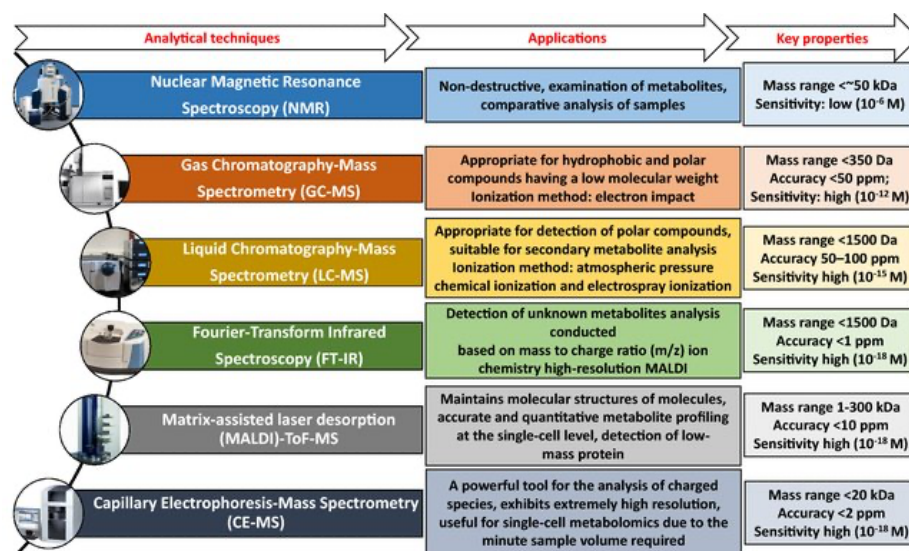
quantified by quantitative real-time PCR or NGS). This powerful technology enables to assess more than 3000 proteins. (ii) Meso Scale Discovery (*MSD*) *Technology* is an immunoassay based on electrochemiluminescence and multi-array approach. This novel sandwich approach uses an electrochemical stimulation and light emission to provide precise quantification of multiple proteins in a single sample.

## V. Metabolomics

The term "metabolome" was first used in 1998 by Oliver and colleagues during their work on yeast metabolism [336]. It is defined as the complete set of metabolites which are small organic molecules resulting from or at the origin of an enzymatic chemical reaction present in a biological fluid, tissue or cell at a given time [337]. Metabolomics refer to the analysis of the metabolome [338]. The metabolome is characterized by a great variability establishing the expression of the genome and is more dynamic than the proteome and genome. The expression of metabolites varies in response to the surrounding environment, the genetic, drug or dietary variables and other factors [339-342]. This omics science has found application in different fields of biology and in disease understanding, drug development, and treatment follow-up process [337, 343-345]. It is sensitive to subtle functional changes in the genome and proteome as these changes can be amplified at the metabolic level which makes it easier to be detected by metabolomics studies.

Currently two research strategies are available to conduct metabolomics studies: the untargeted and the targeted approaches [337]. The untargeted approach analyzes all the molecules present in a fluid or biological tissue in an exhaustive way. It is typically used to compare samples with significant phenotypic differences [346]. It reveals the correlation between metabolites and pathophysiological changes. Its objective is to obtain as much data as possible in an unbiased way to generate hypotheses that can be used for a subsequent targeted analysis. The metabolic differences between groups are obtained by statistical analysis and combined with bioinformatics tools to reveal the mechanisms of metabolic changes in biological systems. Targeted metabolomics is used to analyze the specific content of selected metabolites in a biological system. It completes the non-targeted analysis and validates the differences found between the metabolites in a highly specific, highly sensitive and accurate quantification. It could also be used to directly quantify certain metabolites using quantitative analyses. Targeted metabolomics requires prior knowledge of the

molecules of interest. Nuclear magnetic resonance, liquid chromatography mass spectrometry (LC-MS) and Gas chromatography mass spectrometry (GC-MS) are used as metabolome assay technologies. The choice of the platform is done based on substance nature, sample type, experimental purposes, and funding (**Figure 8**).



**Figure 8:** Comparison of frequently working analytical techniques in metabolomics research [347]

Metabolomics affords quantifiable data of a patient's current biochemical status as compared to the genetic background and gene expression which do not always track the health status. That is why metabolomics will continue to be an essential part of precision medicine's toolbox. The more metabolomics enters the field of clinical testing, the more data will be obtained, the broader the scope of biochemical signatures of diseases reflecting a wider spectrum of the clinical phenotype therefore offering more possibilities for treatment development.

The full emergence of clinical metabolomics is currently limited by some challenges, like method standardization, data analysis, data interpretation, quality control, and analytical validation [348]. Despite the challenges previously noted, we cannot neglect the importance of clinical metabolomics and with its emergence it is expected to play an essential key role in clinical diagnostics and decision-making [349].

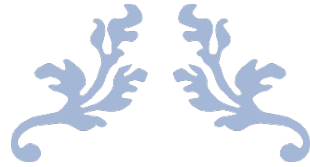
## VI. Precision medicine

According to the Precision Medicine Initiative, precision medicine is “an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person” [53-55]. Precision medicine was previously called personalized medicine, but the old term could lead to misinterpretation to infer that treatments and prevention are unique for each single individual while in precision medicine the concept is broader and would include discovering therapies to subcategories of disease, often defined by omics studies [54, 350]. In clinical practice physicians collect information related to the disease (signs and symptoms) then based on evidence and their experience they suggest the workup and management while checking the patient’s preferences. This is to mention that medicine in practice has always been personalized and treatment was mainly symptomatic rather than curative therefore new tools were needed to be developed to provide patients with the best care and to discover new preventive measures and treatment [54]. Physicians have been good in delineating phenotypes which ultimately lead to clinical diagnosis, but, with the advances of basic sciences and the use of natural history and cohorts, physicians and scientists were able to determine underlying causes and the underlying mechanisms (genetics) of many diseases and started looking for biomarkers for follow-up then started unlocking the codes towards treatment of the underlying causes (targeted treatment) rather than treatment of symptoms. These changes have led to change in the natural history of the diseases, and this became the new model in the precision medicine era. This new model of precision medicine emerged from the interest of pharmaceutical companies in rare disease (due to increase expenses in drug development) and the availability and affordability of the genetic testing (which costs decreased tremendously) [54]. The concept of evidence-based precision medicine involves multi-omics studies in addition to the clinical, laboratory and imaging data to better understand the disease phenotypes and to discover biomarkers of diagnosis, prognosis, and to develop treatments [56-59].

In childhood neurology, precision medicine gained new insights in some neurological diseases like autoimmune encephalopathy [351], cerebral palsy [352], ceroid lipofuscinosis type 2 [353, 354] and other neurometabolic diseases (San Filippo, Aromatic L-aminoacid decarboxylase deficiency, Alexander, Niemann pick type C, ...etc.) where the discovery of the

underlying etiology has led to better and earlier intervention in management and to the development of targeted therapy. In epilepsy, the treatment is usually symptomatic, and a lot of antiepileptic drugs have been developed in the past years. Many of these drugs have been found by accident to have an anti-convulsant effect and epilepsy itself is a symptom of many diseases rather than a disease by itself. Surgery in epilepsy [355], vitamins in responsive epilepsies [356, 357], ketogenic diet in GLUT1 deficiency [358], mTOR inhibitor therapy (Everolimus [359] and metformin [360]) for patients with tuberous sclerosis can be considered a targeted approach that are effective in epilepsy. A lot of research is ongoing to develop targeted therapies in epilepsy such as ASO [361] and dead CAS9 mediated *SCN1A* gene activation [362] in Dravet syndrome and gene therapy in focal epilepsy [363].

The real breakthrough came in neuromuscular diseases where the real benefit was seen after early intervention and using targeted treatment. Natural history is changing using targeted therapy in neuromuscular diseases for example in SMA type 1, the prognosis is devastating without treatment with poor prognosis (median time to death or permanent ventilation 6.1 [364] to 10.5 months [365] and progressive loss in motor function) while with advent of innovative treatment antisense oligonucleotides and/or viral gene therapy natural history is completely different (more details in chapter 3 : Spinal muscular atrophy).



## *THESIS OBJECTIVES*



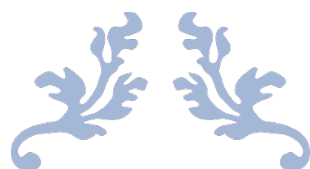
Neuromuscular disorders include a wide range of diseases affecting the peripheral nervous system. Despite all the advances in molecular testing, the diagnosis of these disorders remains challenging. Clinicians are good in describing the phenotypes and natural history in most of the diseases have been well traced. Treatment was mainly symptomatic and multidisciplinary management has improved the quality of life of these patients. However, the underlying pathophysiology of most of these disorders remains incompletely understood.

We are witnessing a revolution in therapy development in these disorders and currently three innovative FDA and EMA approved drugs exist in the market for spinal muscular atrophy. However, the prediction of responders is not possible and the follow-up using specific biomarkers is still insufficient. In other neuromuscular diseases, there are some specific but not curative treatments available. Many biological processes need to be clarified to be able to develop targeted therapy. The development of omics sciences in recent years has enabled the emergence of precision medicine which aims to treat patients according to their personal and environmental characteristics. It uses information obtained from individual and population-based biological studies. The omics sciences make it possible to extract biological data from a biological system in a global way with a targeted or non-targeted approach. These new techniques are different from conventional approaches which are reductionist and sequential.

The objective of this thesis is to use omics tools to interrogate the complexity of two NMD (DMD and SMA) enabling thus (i) a better understanding of the metabolic alterations underlying the pathology, (ii) the identification of potential biomarkers for the diagnosis, the patient's stratification, and the follow-up and (iii) the determination of possible therapeutic targets.

The first part of the work was applied to Duchenne muscular dystrophy. The study focused on the search for specific metabolic profiles of DMD using non-targeted metabolomics techniques on muscle biopsies. My work in this part consisted in assisting in preparation of samples for high resolution mass spectrometry, collection of clinical data, participating to metabolomics studies on muscle tissue and analyzing and interpreting the results.

The second part of this work was carried out on spinal muscular atrophy using metabolomics and proteomics approaches before and after 184 days of Nusinersen treatment. The objective was to search for specific new biomarkers for the diagnosis and follow-up of the disease under treatment but also to better understand the biological processes and metabolic pathways involved in the pathology. In this thesis the results of metabolomic study were presented while the proteomics study is ongoing. In this second project, my work comprised collecting the samples, their preparation for mass spectrometry, collection of clinical data, participating in metabolomics studies and assisting in analysis and interpretation of the results.



## ***PART II: EXPERIMENTAL SECTION***





# CHAPTER 1: OMICS IN DUCHENNE MUSCULAR DYSTROPHY

## I. Background

Duchenne muscular dystrophy, a common neuromuscular disorder, characterized by alteration or absent dystrophin. It is an X-linked disorder that is caused by variants in the *DYS* gene. Currently there is no curative treatment, and the underlying pathophysiological mechanism remain incompletely understood. Therefore, our project was to undergo multi-omics studies. We started by metabolomics as the molecular changes can be amplified at the metabolic level, and we used an innovative technique of ultra-high-resolution mass spectrometry to find a metabolic signature on muscle tissue which would help understand the underlying molecular mechanisms and would help develop effective therapies.

## II. Objectives

The aim of this study is to perform metabolomic imaging using matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry which is an untargeted mass spectrometry-based imaging.

## III. Data collection and study workflow

Nine human muscle biopsies from Duchenne muscular dystrophy patients followed at Rouen University medical center and nine muscle biopsies from control individuals. All biopsies belong to a collection declared to the French Ministry of Health located in the Pathology Department of Rouen University Hospital. Clinical charts of patients were reviewed, and clinical, biological, and histological information were collected: genetic testing, motor skills, symptoms at onset, cognitive involvement, signs of disease severity (motor, respiratory, cardiac), medication or clinical trial, laboratory workup, multidisciplinary management. These data were essential to understand the metabolite signature that varies in response to the surrounding environment, the genetic, drug factors.



**Figure 9 :** General overview of mass spectrometry imaging workflow. (1) Patient selection; (2) Tissue sampling; (3) Tissue sectioning using a cryomicrotome; (4) Tissue section fixed to the sample probe; (5) Homogenous deposition of matrices using an automatic sprayer; (6) MSI data acquisition using an FT-ICR 12T Solarix Bruker; (7) Mass spectrometry imaging datasets (raw data files) were processed; (8) Raw data were converted into data matrices, including ion mass-to-charge ratios ( $m/z$ ), spatial localization and relative intensity for each ion image, were generated using SCiLs Lab software; (9) Data analysis workflow for comparative analysis to extract the differential metabolic patterns; (10) Annotation of the discriminant features by interrogation mass spectrometry databases; (11) Interpretation of the data according to clinical and biological contexts.

#### IV. Ethics

The study protocols have been approved by ethics committee of NORD WEST1 – Rouen University Hospital (collection number DC-2015–2468, accession number AC-2015–2467).

#### V. Collaboration

This project benefited from the expertise of the Department of Metabolic Biochemistry Department, the Department of Neonatal Pediatrics, Intensive Care-Neuropediatrics, Department of Genetics and Reference Center for Developmental Disorders, Normandy Center for Genomic and Personalized Medicine and the Department of Pathology at Rouen University Hospital and the research unit Inserm U1245. The Ultra-high resolution mass

spectrometry imaging was performed at COBRA laboratory UMR 6014 Et FR 3038 Univ Rouen-IRCOF.

## VI. Results and publication

This untargeted metabolomics study revealed significant changes in 34 keys metabolites. Among these metabolites, 7 were decreased in the Duchenne biopsies compared to control biopsies including adenosine triphosphate, and glycerophosphocholine. The other 27 metabolites were increased in the Duchenne biopsies, including sphingomyelin, phosphatidylcholines, phosphatidic acids and phosphatidylserines. Most of these dysregulated metabolites are tightly related to energy and phospholipid metabolism.

Details of this project are found in our article published in Scientific Reports:

**Dabaj I**, Ferey J, Marguet F, Gilard V, Basset C, Bahri Y, Brehin AC, Vanhulle C, Leturcq F, Marret S, Laquerrière A, Schmitz-Afonso I, Afonso C, Bekri S, Tebani A. **Muscle metabolic remodelling patterns in Duchenne muscular dystrophy revealed by ultra-high-resolution mass spectrometry imaging.** *Sci Rep.* 2021 Jan 21;11(1):1906. doi: 10.1038/s41598-021-81090-1. PMID: 33479270; PMCID: PMC7819988.

## VII. Conclusion

This study revealed a deep metabolic remodeling in phospholipids and energy metabolism in Duchenne muscular dystrophy. This study using a systems-based approach enabled exploring the metabolism in DMD in an unprecedented holistic and unbiased manner with hypothesis-free strategies.



OPEN

## Muscle metabolic remodelling patterns in Duchenne muscular dystrophy revealed by ultra-high-resolution mass spectrometry imaging

Ivana Dabaj<sup>1,2,9</sup>, Justine Ferey<sup>3,9</sup>, Florent Marguet<sup>2,4</sup>, Vianney Gilard<sup>3,5</sup>, Carole Basset<sup>4</sup>, Youssef Bahri<sup>6</sup>, Anne-Claire Brehin<sup>7</sup>, Catherine Vanhulle<sup>1</sup>, France Leturcq<sup>8</sup>, Stéphane Marret<sup>1,2</sup>, Annie Laquerrière<sup>2,4</sup>, Isabelle Schmitz-Afonso<sup>6</sup>, Carlos Afonso<sup>6</sup>, Soumeia Bekri<sup>2,3</sup>✉ & Abdellah Tebani<sup>3</sup>

Duchenne muscular dystrophy (DMD) is a common and severe X-linked myopathy, characterized by muscle degeneration due to altered or absent dystrophin. DMD has no effective cure, and the underlying molecular mechanisms remain incompletely understood. The aim of this study is to investigate the metabolic changes in DMD using mass spectrometry-based imaging. Nine human muscle biopsies from DMD patients and nine muscle biopsies from control individuals were subjected to untargeted MSI using matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry. Both univariate and pattern recognition techniques have been used for data analysis. This study revealed significant changes in 34 keys metabolites. Seven metabolites were decreased in the Duchenne biopsies compared to control biopsies including adenosine triphosphate, and glycerophosphocholine. The other 27 metabolites were increased in the Duchenne biopsies, including sphingomyelin, phosphatidylcholines, phosphatidic acids and phosphatidylserines. Most of these dysregulated metabolites are tightly related to energy and phospholipid metabolism. This study revealed a deep metabolic remodelling in phospholipids and energy metabolism in DMD. This systems-based approach enabled exploring the metabolism in DMD in an unprecedented holistic and unbiased manner with hypothesis-free strategies.

Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy in children, with an incidence of 1 in 5000 live-born boys annually and an estimated prevalence of 15.9 per 100,000 boys in the USA and 19.5 per 100,000 boys in the UK<sup>1–3</sup>. This lethal X-linked recessive neuromuscular disorder is caused by mutations in the *DMD* gene resulting in absent or reduced functional dystrophin (DYS). As in many genetic diseases, DMD exhibits a continuum of disease severity, which may be correlated to the presence or absence of a functional protein. Becker muscular dystrophy is a milder form of dystrophinopathy in which *DYS* levels are reduced, but a residual partially functional protein remains<sup>3</sup>. The dystrophin-associated protein complex underlies the link between the extracellular matrix and the cytoskeleton, thus ensuring the skeletal muscle strength. Dystrophin dysfunction results in severe architectural muscle changes with subsequent degeneration and impaired regeneration<sup>4,5</sup>.

<sup>1</sup>Department of Neonatal Pediatrics, Intensive Care and Neuropediatrics, Rouen University Hospital, 76031 Rouen, France. <sup>2</sup>Normandie Univ, UNIROUEN, CHU Rouen, INSERM U1245, 76000 Rouen, France. <sup>3</sup>Department of Metabolic Biochemistry, Rouen University Hospital, 76031 Rouen, Cedex, France. <sup>4</sup>Department of Pathology, Rouen University Hospital, Rouen, France. <sup>5</sup>Department of Neurosurgery, Rouen University Hospital, Rouen, France. <sup>6</sup>Normandie Univ, COBRA UMR 6014 Et FR 3038 Univ Rouen; INSA Rouen; CNRS IRCOF, 1 Rue Tesnière, 76821 Mont-Saint-Aignan Cedex, France. <sup>7</sup>Department of Genetics and Reference Center for Developmental Disorders, Normandy Center for Genomic and Personalized Medicine, Normandie Univ, UNIROUEN, Inserm U1245 and Rouen University Hospital, 76000 Rouen, France. <sup>8</sup>APHP, Laboratoire de Génétique Et Biologie Moléculaire, HUPC Cochin, Paris, France. <sup>9</sup>These authors contributed equally: Ivana Dabaj and Justine Ferey. ✉email: soumeia.bekri@chu-rouen.fr

Dystrophin has been shown to be temporo-spatially expressed by discrete neuronal populations, mainly in the pyramidal cells of the hippocampus, in the amygdala, in Purkinje cells and granular neurons of the cerebellum. Thus, DYS is involved in several cognitive functions and human brain development, although the precise mechanisms remain unclear<sup>6</sup>. Muscle weakness and motor delay are usually early symptoms. In the absence of steroid treatment, the ambulation loss occurs at about 10 years of age and near paralysis by age 20, with scoliosis, loss of upper limb function, cardiac involvement, and respiratory insufficiency. Brain dysfunction also occurs at the start of various neurobehavioral developmental disorders, such as intellectual disability, or more specific cognitive disorders, delayed speech and coordination acquisition, executive dysfunction, attention deficit disorder and autism spectrum disorders<sup>1–3,7</sup>. To date, there is no cure for DMD, but the life expectancy of DMD patients has improved owing to steroid treatments and better care. DMD patients lose the ability to walk around 13–14 years of age and eventually die in their 30 s due to cardiopulmonary complications<sup>1,3</sup>. However, numerous clinical trials and experimental approaches are ongoing, such as exon skipping, gene therapy, myostatin inhibitors, trophin modulation, CRISPR/Cas9 suppression of stop codons and stem cell therapy<sup>8</sup>. Validated outcome measures are required to assess the potential benefits of these treatments. Currently, several functional tests are used such as the 6 min walk test, the North Star ambulatory assessment, and the performance of upper limb test or time items in addition to muscular imaging. However, these tests are often ineffective, and their results do not necessarily correlate with the patient's condition<sup>9</sup>. Owing to wide phenotypic variability, reliable outcome measurements that would enable assessing treatment efficacy remain tentative, and new biomarkers must be identified to better understand the DMD pathophysiology and more accurately evaluate patient prognosis and follow-up<sup>1</sup>. Metabolic impairments have been associated with DMD since the 1970s<sup>10–13</sup>. However, the advent of new omics technologies allows exploring the metabolism and its components in an unprecedented holistic and unbiased manner with hypothesis-free strategies<sup>14</sup>. Metabolites are small organic molecules involved in enzymatic reactions to form the metabolism. The “metabolome” refers to all metabolites present in a given biological system, fluid, cell, or tissue. Metabolomics is the omics technology that enables exploring the metabolome by comprehensively measuring metabolite levels in a given biological sample<sup>15</sup>. Its main goal is to parse the biochemical changes in a biological system by probing the metabolite variations related to genetic, environmental, drug, dietary and other factors or interventions. Mass spectrometry is a widely used metabolomics technology because of its high detection sensitivity, metabolome coverage and rapid data acquisition turnover<sup>16</sup>. The spatial distribution of metabolites can be tracked holistically and systematically, which is highly valuable for gaining a mechanistic understanding of biological processes. Mass spectrometry imaging (MSI) achieves this by simultaneously revealing the spatial distribution of multiple molecules in a single experiment from various biological samples, particularly tissue Sects.<sup>17</sup>. Thus, this technology shows promising translational potential in the biomedical field<sup>18,19</sup>. MSI has been applied to study DMD muscle biopsies using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) imaging and TOF secondary ion mass spectrometry. Previous investigations highlighted lipid compositional changes<sup>20,21</sup> or Krebs cycle intermediates<sup>22</sup> in muscles from DMD mouse models and DMD patients<sup>23</sup>. However, the relatively low mass resolution of the TOF-MS instruments makes peak annotation very challenging. Thus, powerful new mass spectrometry instruments based on Fourier-transform ion cyclotron resonance (FTICR) offer better analytical performance in terms of resolution, sensitivity and specificity<sup>24–26</sup>. Thus, this technology offers unprecedented exhaustive metabolome coverage and non-ambiguous molecular formula assignments<sup>26</sup>.

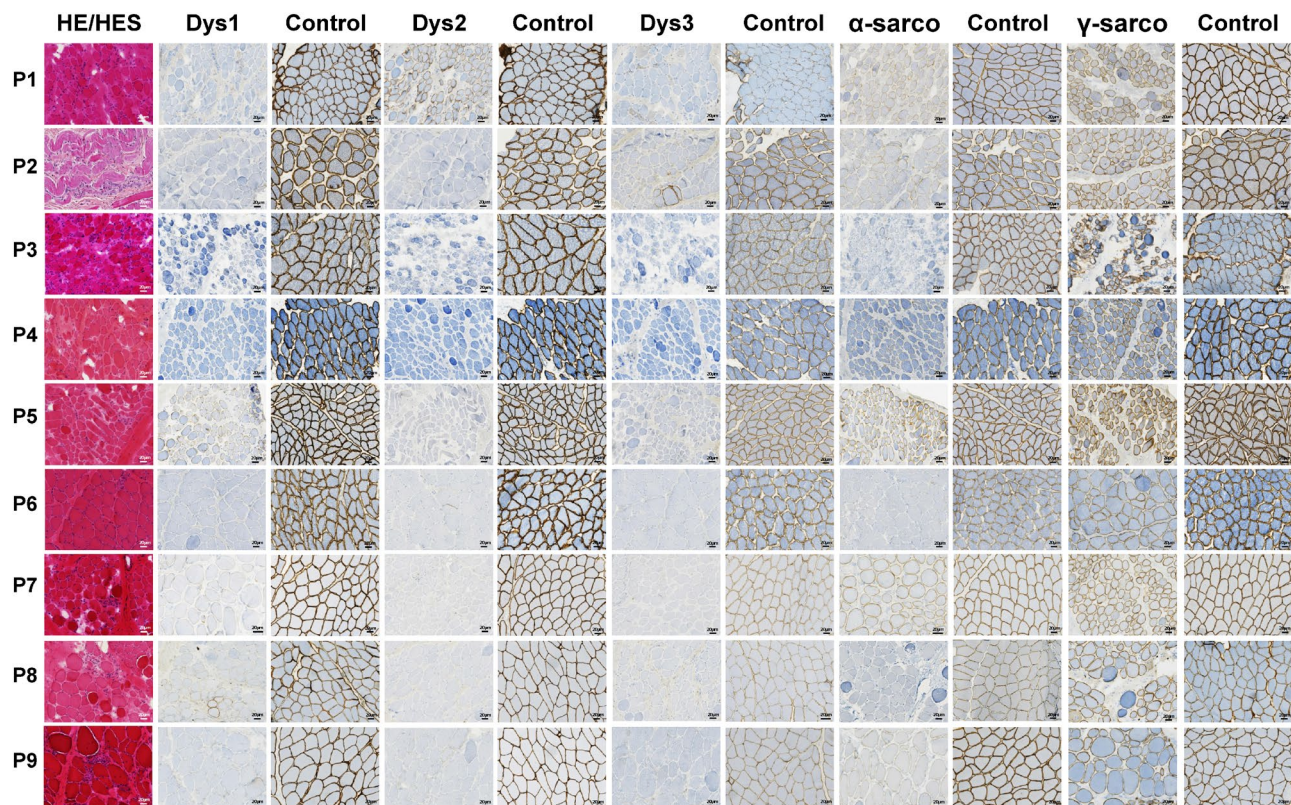
This work presents the first study ever published using MSI based on MALDI coupled with FTICR to explore muscle metabolic remodelling in DMD patients. Given the scope of the study and the high analytical superiority of the platform, this investigation allowed holistically parsing the complex metabolic remodelling in DMD tissues, revealing the DMD-associated molecular metabolic impairments and deregulations, aiming to open new avenues for deeper biological investigations to uncover new therapeutic targets, biomarkers and diagnostic tools.

## Results

**Histological findings.** Morphological lesions meeting DMD diagnostic criteria were observed in muscle biopsies including fiber necrosis with inflammatory response, diffuse variation in fiber size with rounded and hypercontracted fibers, basophilic regenerative fibers, and endomysial and perimysial fibrosis. Immunohistochemical studies revealed that six patients lacked DYS1, and three had severely decreased and irregular DYS1 immunoreactivity, all patients but one having no DYS2 and DYS3 (Fig. 1, Supplementary Tables 1 and 2). These expression patterns were confirmed by western blot analyses. Immunohistochemical or western blot techniques showed that absent or decreased DYS was associated with absent or decreased proportions of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$  sarcoglycans (Fig. 1, Supplementary Tables 1 and 2). Molecular analyses allowed for identifying different variants consisting of large deletions in the DMD gene sequence in 8 patients and a small frameshift deletion in one patient (Supplementary Tables 1 and 2).

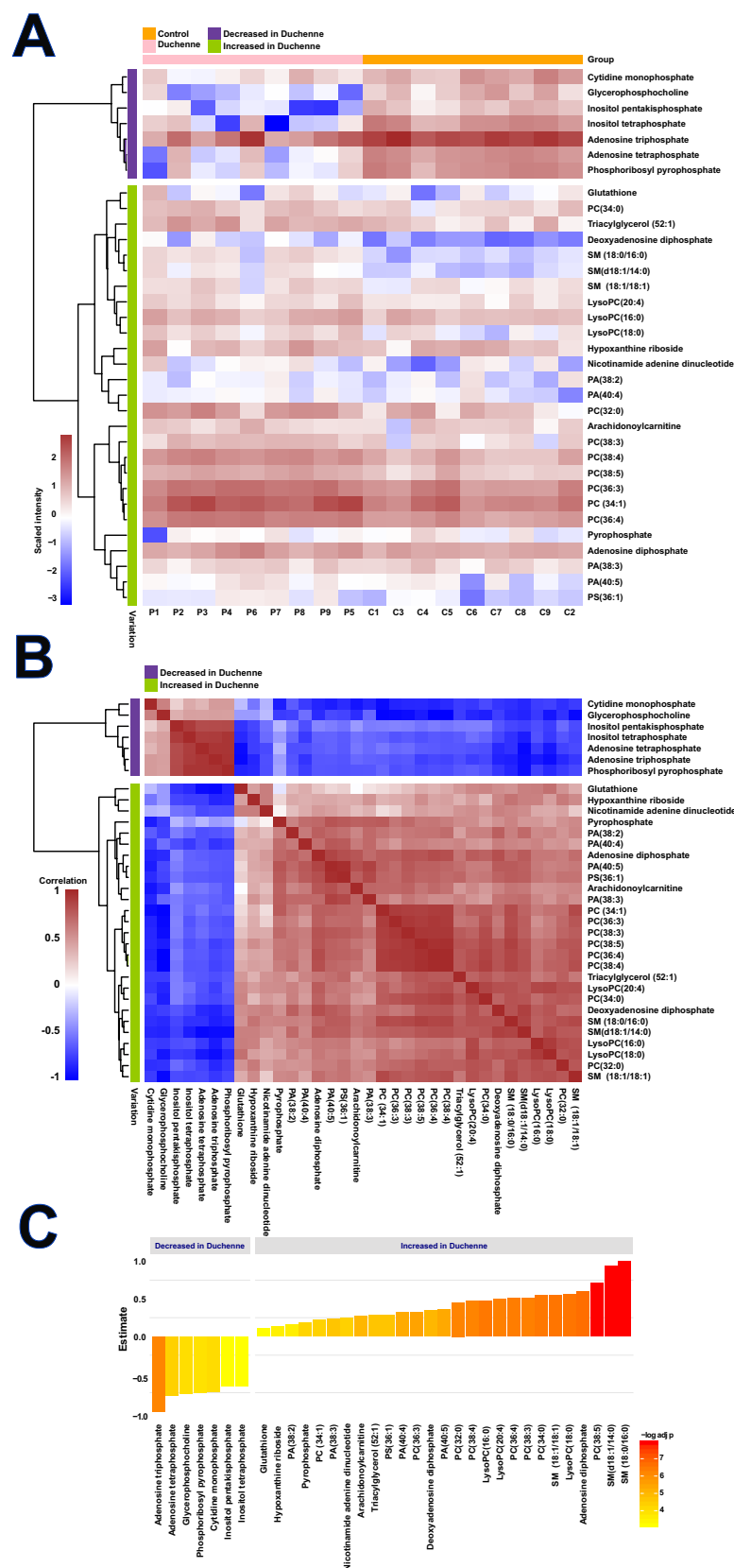
**Mass spectrometry-based metabolomics imaging.** This work explored the differential metabolic patterns between Duchenne and control biopsies by analyzing the spectral fingerprints extracted in MSI experiments from the analyzed muscle biopsies. These fingerprints were formed from ions generated by the mass spectrometry analysis used for data analysis and metabolite identification. Average spectra are presented in Supplementary Fig. 1. The first statistical analysis yielded 52 discriminant ions with significant differences between the Duchenne and control biopsies. Further annotation steps allowed filtering and cleaning this list to include only unambiguously identified metabolites. In positive-ion mode, the combination of high mass accuracy and MS/MS experiments highlighted phosphatidylcholine (PC) and sphingomyelin (SM) lipids due to their fragmentation patterns. PC and SM lipids can be detected in positive-ion mode owing to their quaternary amine groups. The fragmentation ions observed upon collisional activation of both  $[M+Na]^+$  and  $[M+K]^+$  of PC and SM lipids included the loss of trimethylamine (59.073499 Da) and phosphocholine (183.066045 Da). Supplementary



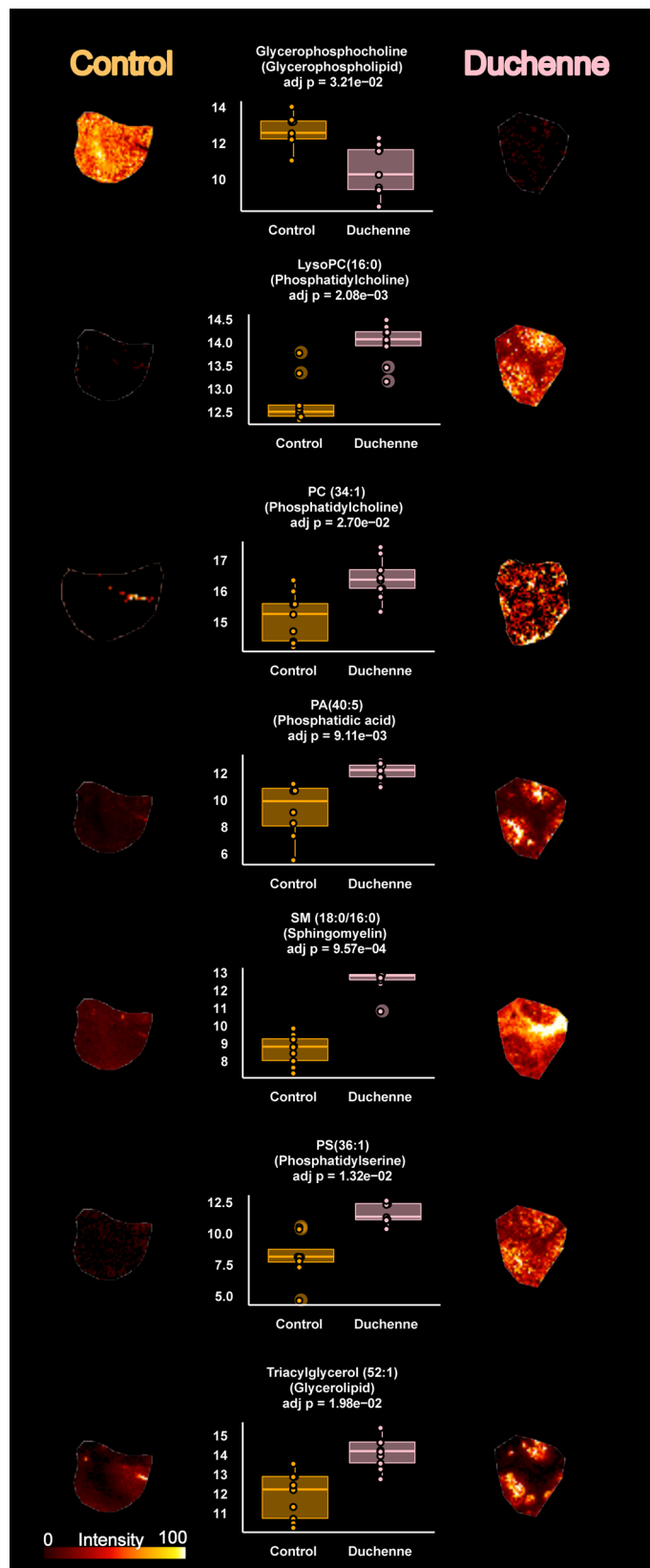


**Figure 1.** Morphological and immunohistochemical characteristics of DMD patients' muscles. Left column: Haematoxylin–Eosin (HE) (P1, P3, P4, P5, P6, P7, P8, P9) or Hemalun-Eosin-Safran (HES) (P2) displaying endo- and perimysial fibrosis, necrotic and atrophic fibres along with inflammatory infiltrates suggestive of progressive muscular dystrophy. Second to seventh columns : dystrophin immunolabeling (dys 1, dys 2 and dys 3 labeling the core, NH<sub>2</sub>-terminal and COOH-terminal domains of the protein respectively) showing severely decreased or absent immunoreactivity in patients compared to control muscle apposed on the same slide. Eighth to eleventh columns :  $\alpha$ - and  $\gamma$ -sarcoglycan immunolabelings showing either a decrease or normal immunoreactivity in patients compared to muscle control opposed on the same slide. Horizontal lines correspond to patients 1–9; scale bars: 20  $\mu$ .

Fig. 2A shows the fragmentation pattern of  $C_{28}H_{50}NO_7P$  at  $m/z$  566.32166  $[M+Na]^+$ . Given the FTICR high mass accuracy, a loss of  $m/z$  59.07351 and  $m/z$  183.06608 corresponded to trimethylamine and phosphocholine with mass errors of 186 ppb and 191 ppb, respectively. Therefore, the ion at  $m/z$  566.32166 with the molecular formula,  $C_{28}H_{50}NO_7P$ , was attributed to lysoPC(20:4)  $[M+Na]^+$ . Other PC and SM lipids were similarly unambiguously assigned. In negative-ion mode, other lipids included phosphatidic acid and phosphatidylserine. Adenosine triphosphate (ATP) at  $m/z$  505.98833 was annotated using MS/MS analysis by the loss of an adenine group at  $m/z$  272.95698 ( $C_5H_8O_9P_2$ ,  $[M-H]^-$ ) with an error of 362 ppb; Supplementary Fig. 2B). Other metabolites and lipids were similarly assigned with the mass accuracy. The final list included 34 unambiguously identified features with 21 and 13 in positive- and negative-ionization modes, respectively. Supplementary Table 3 presents the data matrix; Supplementary Table 4 presents the related statistics of the identified metabolites along with their annotation metrics. Supplementary Fig. 3 presents the boxplots of the different metabolites. More mass spectra are presented in Supplementary Figs. 4–10. Seven metabolites were decreased in the Duchenne biopsies: adenosine tetraphosphate, adenosine triphosphate, cytidine monophosphate, glycerophosphocholine, inositol pentakisphosphate, inositol tetraphosphate, and phosphoribosyl pyrophosphate. The other 27 metabolites were increased in the Duchenne biopsies, including SM, phosphatidylcholines, phosphatidic acids and phosphatidylserines. To explore the distribution of this signature and its expression across samples, we performed a clustering analysis using Euclidean distance, a similarity metric, between samples. Figure 2A shows two distinct clusters between the DMD and control biopsies due to the differential expression of the above-mentioned metabolites across samples. We also explored the covariation of these metabolites using Spearman correlation analysis. Figure 2B shows two main co-expression clusters that include upregulated and downregulated metabolites. Intraclass subclusters are observed such as a module that includes phosphatidylcholines, phosphatidic acids and phosphatidylserines. Another module includes SMs and lysophosphatidylcholines. Figure 2C presents the directional changes of these metabolites in the DMD samples along with their statistical significance. This figure reports adjusted p-values and estimate which indicates metabolite change direction. Figure 3 presents boxplots of the glycerophosphocholine, lysophosphatidylcholine, phosphatidylcholine, phosphatidic acid, SM, phosphatidylserine, along with their tissue distributions in the DMD and control biopsies with their related

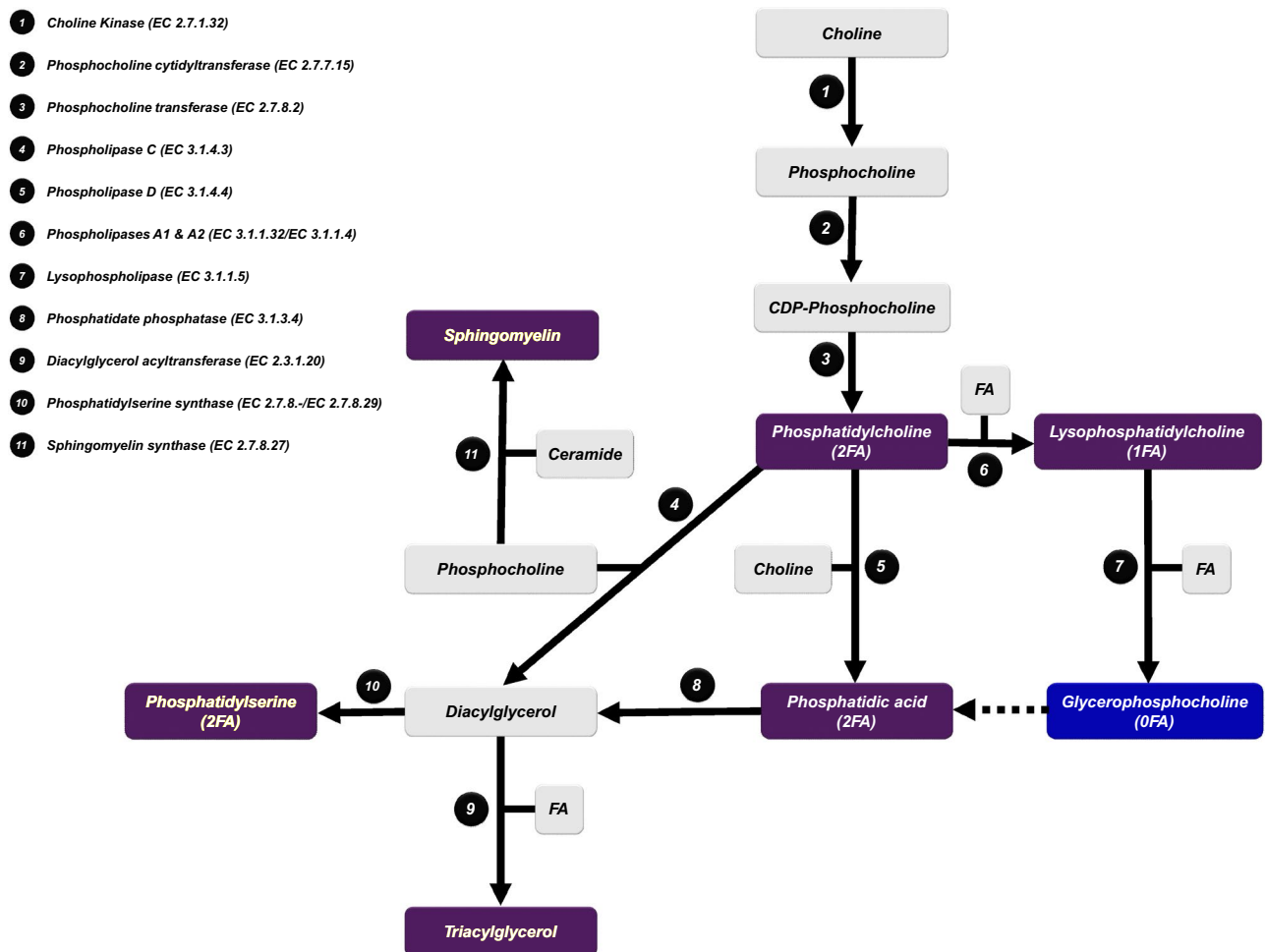


**Figure 2.** Metabolome variations between DMD and control tissues. (A) Unsupervised hierarchical clustering based on metabolic profiles (34 metabolites) detected in 9 DMD and 9 control tissues. C: Control, P: Patient; (B) Spearman correlation heatmap of the 34 differentially expressed metabolites between control and DMD tissues; (C) Barplots of the differentially expressed metabolites and their directional changes in the DMD tissues. Bar color is proportional to  $-\log(\text{adjusted } p\text{-value})$ . Red denotes high significance.



**Figure 3.** Tissue distribution of selected discriminant metabolites. Left) Tissue metabolite distribution in the control tissue. Right) Tissue metabolite distribution in the DMD tissue. Middle) Boxplot of the selected metabolites between DMD and control tissues with related adjusted p-values. The y-axis shows the log-scaled average intensity. Ion images were generated using SCiLS Lab software.





**Figure 4.** Phospholipid and triacylglycerol metabolism. Purple: increased metabolites in DMD. Blue: decreased metabolites in DMD. EC: Enzyme Commission number. FA: fatty acids.

adjusted p-values. Even though the figures don't show a cellular, sub-cellular or fiber level resolution, they highlight the heterogeneous distribution of intensities across the tissue section that mirrors the distribution of the related metabolites.

## Discussion

Defective dystrophin is known to be the main alteration in the DMD etiology, however, metabolic impairment has been reported in several tissues such as the skeletal and cardiac muscles, liver and brain<sup>27</sup>. Here, we explored the differential metabolic patterns of muscle biopsies from DMD patients and control samples using a hypothesis-free strategy based on MSI. Our results showed profound metabolic pathway remodelling in the muscles of DMD patients compared with the controls grouped in two distinct clusters corresponding to the upregulated and downregulated metabolites. The most discriminative metabolites were primarily phospholipids and energy metabolites, suggesting that both these metabolic pathways are important players in muscle pathology and possibly by extension cognitive/behavioral disabilities.

Phospholipids (PLs) are major components of all cell membranes, and glycerophospholipids (GLs) are the most abundant membrane PLs. GLs are composed of a glycerol backbone linked to a phosphate group in position 3. Acyl groups may be attached at positions 1 and 2 of the glycerol. Different moieties, such as choline, inositol, ethanolamine and serine, are linked to the phosphate group, contributing to GL diversity (e.g., PC, phosphatidylinositol [PI], phosphatidylethanolamine (PE), PS, and phosphatidylglycerol). PCs are the most abundant GL in mammalian cells. Other lipid classes, such as cholesterol and glycosphingolipids, play structural roles in cell membranes<sup>28,29</sup>. Disruption of PL metabolism has been previously reported in DMD<sup>10,11,13,20,21,23,30,31</sup>. We showed that several compounds belonging to PC, lysophosphatidylcholine (LPC), phosphatidic acid (PA), PS and SM classes, as well as triacylglycerols, are increased, while glycerophosphocholine (GPC) is decreased in DMD muscles compared with control muscles (Fig. 3). This metabolic remodelling could be attributed to a putative decrease in lysophospholipase activity (Fig. 4). Lysophospholipase catalyzes lysophosphatidylcholine deacylation and mediates GPC production<sup>32</sup>. Sharma et al. considered DMD to be mainly a GPC deficiency<sup>33</sup>. Consequently, this impairment could lead to an accumulation in phosphatidylcholine, which is redirected toward other metabolic pathways with an increase in PA, SM, phosphatidylserine and triacylglycerol (Fig. 4). It has

been shown that lysophospholipase activity in erythrocyte membranes of DMD patients was lower than that of healthy age-matched subjects<sup>32</sup>. Chalovich et al. compared the <sup>31</sup>P-NMR spectra of muscles from DMD patients, Werdnig-Hoffman (Spinal Muscular Atrophy Type 1) patients and controls. These authors observed that GPC levels were lower or near absent in DMD muscles compared with control muscles and dramatically increased (15-fold) in one Werdnig-Hoffman patient<sup>30</sup>. Increased triacylglycerol and SM in DMD mouse muscles have been interpreted as resulting from increased phospholipase C activity<sup>13</sup>. High phospholipid levels and high phospholipid-to-cholesterol ratios have been documented using <sup>1</sup>H-NMR spectroscopy, which helps distinguish DMD patients from healthy subjects<sup>34</sup>. Phospholipase D activity is reported to be increased in DMD muscles. Calcium homeostasis has been shown to be altered in DMD patients, with calcium concentration being increased in dystrophin-deficient muscle. Phospholipase D activity is enhanced under high concentrations of free calcium, resulting in increased PA levels<sup>35,36</sup>. In summary, profound phospholipid metabolism remodelling is associated with DYS deficiency; thus, DMD has been considered a glycerophosphocholine deficiency<sup>33,37</sup>. Morphological alterations of the mitochondria have been reported in DMD muscles with higher rates of swollen mitochondria compared with that of normal muscle<sup>38</sup>. PL alterations in DMD and a dysregulation of Ca<sup>2+</sup> homeostasis may induce mitochondrial membrane fragility and associated morphological changes with subsequent energy and oxidative metabolism disruptions<sup>39–42</sup>.

Energy metabolism impairments in DMD are major contributors to DYS-deficient muscle degeneration<sup>43</sup> as illustrated by mitochondrial function improvement upon the partial restoration of DYS expression<sup>44</sup>. Accordingly, we showed a significant increase in ADP and decreased ATP levels in DMD muscles compared with those of the control muscles (Supplementary Fig. 3). Several preclinical and clinical studies aimed at promoting energy metabolism are ongoing to treat DMD<sup>45</sup>. The need for studies with higher cellular or fiber level resolution would unveil deeper metabolic insights and further explore these results. Van Pelt et al. performed a multi-omics study in a Duchenne mouse model. They reported the impairment of glycolytic metabolism and phospholipids<sup>46</sup>. Two metabolites overlap with our results adenosine diphosphate and PC(36:3) (Supplementary Table 5). Exploring the potential release of the metabolites in less invasive biological fluids is relevant. Spitali et al. reported serum-based metabolomics results in patients affected by several multiple forms of muscular dystrophy. Fifteen metabolites have been reported belonging to energy, amino acid and testosterone metabolisms<sup>47</sup>. Lindsay et al. performed a urine-based metabolomics study in a Duchenne mouse model. They reported that five of seven detected Krebs cycle metabolites were depleted in these mice consistent with an impaired energy metabolism<sup>22</sup>. No overlap has been observed between our results and those reported in these studies (Supplementary Table 5).

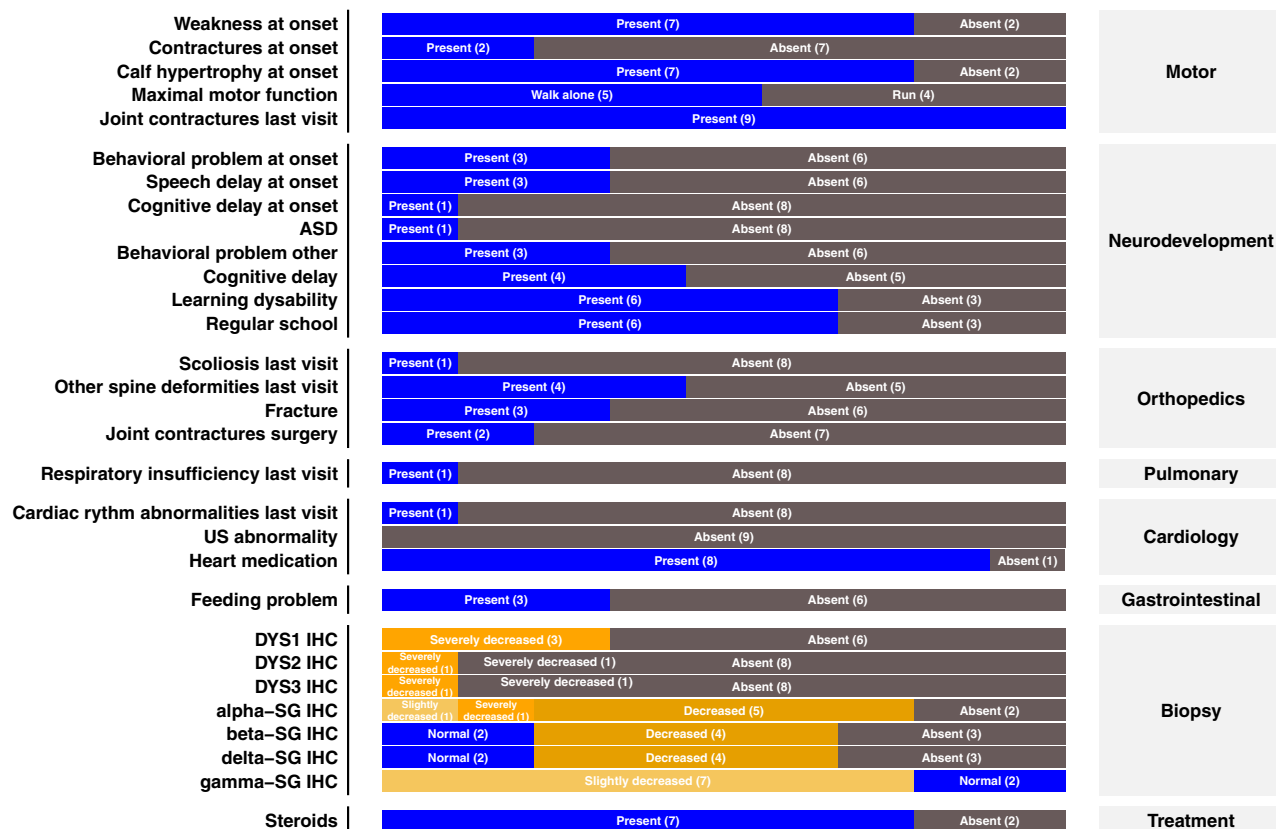
In conclusion, although metabolic impairments have been reported in DMD, this work describes for the first time the use of ultra-high-resolution MSI in DMD, which enabled more systematically and integratively exploring on-tissue metabolic disturbances. This powerful technique allowed investigating the different metabolic components in a single experiment. The present results highlight the potential use of MSI technology coupled with systems biology approaches to holistically explore metabolic impairments in DMD. This work lays the foundation for more mechanistic investigations of DMD and other metabolic diseases.

## Methods

The overall workflow is presented in Supplementary Fig. 11.

**Patients.** Nine patients with molecularly proven DMD over the last 10 years were selected for the study. All patients were referred to our neurology department, and biopsies were performed as a part of the diagnostic workup when the disease was suspected. Samples were collected before initiating treatment. Medical charts were reviewed for age at disease onset, age at diagnosis, age at walking unaided, age at walking loss, and age at last visit. Symptoms at disease onset were recorded as calf hypertrophy, muscle weakness, contractures, behavioral problems, speech delay, respiratory distress and cardiac arrhythmia. Disease activity parameters consisted of maximal motor function, maximal CPK, age of initial joint contractures and current joint contractures, surgical therapy and long-term physical therapy. Other parameters consisted of scoliosis occurrence or any other spinal deformities, along with their surgical and nonsurgical therapies, occurrence of bone fractures and other orthopedic surgeries, and mobility at last visit. Evaluated complications were respiratory insufficiency (e.g., IPPB, ventilation, tracheotomy), existing cardiac complications (e.g., rhythm abnormalities or heart abnormalities on ultrasound), digestive complications (e.g., nutritional problems, gastrostomy) and iatrogenic complications. Inclusion in therapeutic protocols, hospitalization frequency, cognitive delays and behavioral problems, including autism and school attendance, were also recorded.

The mean patient age at disease onset was  $4 \pm 1.5$  years, at last visit was  $10.5 \pm 2.2$  years, and at time of biopsy was  $5.5 \pm 1.9$  years. All patients are presently alive, and six (P1, P5, P6, P7, P8, and P9) were ambulant at the last visit. One non-ambulant patient (P2) lost the ability to walk at age 7; he was not given steroids, nor included in any therapeutic protocol. The other two patients (P3, P4) lost the ability to walk at 11 and 13 years of age. CK levels ranged from 5418–52,000. Symptoms at disease onset were muscle weakness with difficulty walking and climbing stairs and frequent falls. Eight patients had calf hypertrophy. Six patients presented no motor features at onset but had speech delays (P2, P5, P8) and/or behavioral/autistic disturbances (P5, P8, P9) that had likely led to delayed diagnosis. Four patients had cognitive delays (P2, P5, P8, P9). Two patients (P2, P5) developed behavioral abnormalities. Six patients had learning difficulties affecting speech, writing, attention and memory (P1, P2, P5, P7, P8, P9). Three patients (P4, P5, P9) had nutritional and feeding problems, with anorexia in two and obesity in one (P9). All but two patients were given steroids (P1, P2); one of these two patients were lost to follow up one year after diagnosis, and the other received no treatment owing to poor family compliance. Four patients (P1, P5, P7, P9) could run, the others could walk without help. Two patients had mild respiratory insufficiency that did not require ventilation. Eight patients had no significant cardiac arrhythmia or functional



**Figure 5.** Clinical characteristics of DMD patients. ASD: autistic spectrum disorders, DYS: dystrophin, IHC: immunohistochemistry, US: ultrasound, SG: sarcoglycan.

abnormalities. Patient P4 had rhythm abnormalities consisting of supraventricular extrasystoles 3.9% of the time, which did not require specific treatment. All but one patient received a prophylactic antiarrhythmic drug (perindopril), and one (P1) was included in a perindopril protocol (2-year double-blind treatment protocol). Three patients (P4, P5, P7) were included in exon-skipping protocols, and one (P6) was included in a givinostat (histone deacetylase inhibitor) protocol. Clinical data overview is presented in Fig. 5 and detailed clinical data are listed in Supplementary Tables 1 and 2.

**Muscle biopsies.** Each patient underwent a muscle biopsy at a mean age of  $5.5 \pm 1.9$  years, which was assessed according to standardized histochemical and histoenzymological methods<sup>48</sup>. For immunohistochemical studies, 6- $\mu$ m frozen sections were immunolabeled with antibodies against  $\beta$ -spectrin (diluted 1:50; Novocastra Leica Biosystems, Nanterre, France), dystrophin (DYS1, DYS2 and DYS3 correspond to amino acids 1181–1388, 3669–3685 and 321–494, of the dystrophin molecule and are diluted respectively at 1:10, 1:8 and 1:6—Novocastra Leica Biosystems), and  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$  sarcoglycans (diluted 1:100, 1:100, 1:100 and 1:50, respectively; Novocastra Leica Biosystems). Dystrophinopathy was morphologically diagnosed per the criteria of Dubowitz et al.<sup>48</sup>.

As age-matched control muscles were not available, nine male adults aged 28–58 years were selected as controls for comparative analyses. These patients presented myalgias, muscle weakness, and/or muscle fatigability. In one case, a polymyositis was suspected; Buschke scleroderma was suspected in another. In all control patients, routine biological tests, CK levels, EMGs, cardiac ultrasonography, pulmonary testing and muscle MRI were considered normal. Their muscle biopsies were also normal, and they were concluded to have probable fibromyalgia. One obvious potential limit of this work that needs to be highlighted consists in absence of age-matched control muscles could hamper interpretation of the results, as biochemical properties of muscles change over the lifetime. Even though paediatric muscle samples could be obtained during orthopaedic surgery, the location of the biopsy may change and may not entirely match with that of Duchenne patients.

**Reagents and chemicals.** Methanol, acetonitrile, ethanol and water of LC–MS grade were purchased from Fisher Scientific (Loughborough, UK); 2,5-dihydroxybenzoic acid (DHB) and 9-aminoacridine hydrochloride monohydrate (9-AA) MALDI matrices were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium trifluoroacetate (NaTFA, Sigma-Aldrich) at  $0.1 \text{ mg mL}^{-1}$  (ACN/H<sub>2</sub>O 50:50 [v/v]) was used as a calibrant before each analysis.

**Sample preparation.** Fresh frozen 10- $\mu\text{m}$  sections of human muscle were mounted on conductive ITO-coated glass slides  $75 \times 25 \text{ mm}$  (Bruker, Bremen, Germany) and stored at  $-80^\circ\text{C}$  until analysis. Matrix solutions were applied with an automatic microsprayer HTX TM-Sprayer (HTX Imaging, Chapel Hill, NC, USA) as previously described<sup>49</sup>. In the positive-ion mode, DHB matrix ( $30 \text{ mg mL}^{-1}$  in  $\text{MeOH}/\text{H}_2\text{O}$  50:50 [v/v]) was deposited with the following parameters: nozzle temperature  $80^\circ\text{C}$ , nozzle velocity  $1200 \text{ mm min}^{-1}$ ,  $\text{N}_2$  pressure 10 psi,  $\text{N}_2$  flow rate  $2 \text{ L min}^{-1}$ , number of passes 12, flow rate  $100 \mu\text{L min}^{-1}$  and track spacing 3 mm. In negative-ion mode, 9-AA matrix ( $10 \text{ mg mL}^{-1}$ ,  $\text{EtOH}/\text{H}_2\text{O}$  70:30 [v/v]) was sprayed using the following parameters: nozzle temperature  $90^\circ\text{C}$ , nozzle velocity  $1200 \text{ mm min}^{-1}$ ,  $\text{N}_2$  pressure 10 psi,  $\text{N}_2$  flow rate  $3 \text{ L min}^{-1}$ , number of passes 2, flow rate  $120 \mu\text{L min}^{-1}$ , track spacing 3 mm and drying time 30 s between passes. Each slide was vacuum-dried before analysis.

**Mass spectrometry instrumentation and data processing.** Data were acquired on a FTICR instrument (Solarix XR, Bruker, Bremen, Germany) equipped with a 12-T superconducting magnet and a dynamically harmonized ICR cell. This instrument is also equipped with both a laser desorption/ionization source (Smartbeam II, Nd:YAG  $\times 3$  laser at 355 nm, Bruker, Bremen, Germany) and an electrospray (ESI) source. Each MALDI spectrum for each position is the result of 1 scan and 500 consecutive laser shots. Spectra were acquired over an  $80 \mu\text{m}$  raster. Before imaging analyses, the instrument was externally calibrated in the required mode by NaTFA infusion via ESI source, then internally calibrated by assigning known metabolites from  $m/z$  150–1000 via MALDI source. The instrument was autocalibrated during image acquisition. In positive-ionization mode, calibration was performed by assigning  $\text{C}_7\text{H}_6\text{O}_4$  ( $m/z$  155.033885  $[\text{M} + \text{H}]^+$ , matrix peak),  $\text{C}_7\text{H}_{15}\text{NO}_3$  ( $m/z$  162.112470  $[\text{M} + \text{H}]^+$ , carnitine),  $\text{C}_7\text{H}_6\text{O}_4$  ( $m/z$  177.015829,  $[\text{M} + \text{Na}]^+$ , matrix peak),  $\text{C}_5\text{H}_{14}\text{NO}_4\text{P}$  ( $m/z$  184.073321,  $[\text{M} + \text{H}]^+$ , phosphocholine),  $\text{C}_9\text{H}_{17}\text{NO}_4$  ( $m/z$  204.123034,  $[\text{M} + \text{H}]^+$ , acetylcarnitine),  $\text{C}_{14}\text{H}_8\text{O}_6$  ( $m/z$  273.039364,  $[\text{M} + \text{H}]^+$ , matrix peak),  $\text{C}_{21}\text{H}_{12}\text{O}_9$  ( $m/z$  409.055408,  $[\text{M} + \text{H}]^+$ , matrix peak),  $\text{C}_{40}\text{H}_{80}\text{NO}_8\text{P}$  ( $m/z$  734.569432,  $[\text{M} + \text{H}]^+$ , lipid), and  $\text{C}_{42}\text{H}_{82}\text{NO}_8\text{P}$  ( $m/z$  798.54096,  $[\text{M} + \text{K}]^+$ , lipid). In negative-ion mode the assigned peaks were  $\text{C}_{13}\text{H}_{10}\text{N}_2$  ( $m/z$  193.77122,  $[\text{M} - \text{H}]^-$ , matrix peak),  $\text{C}_{17}\text{H}_{26}\text{N}_6\text{O}_6$  ( $m/z$  409.184106,  $[\text{M} - \text{H}]^-$ , amino acids),  $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{10}\text{P}_2$  ( $m/z$  426.022139,  $[\text{M} - \text{H}]^-$ , adenosine diphosphate),  $\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{13}\text{P}_3$  ( $m/z$  505.988470,  $[\text{M} - \text{H}]^-$ , adenosine triphosphate),  $\text{C}_{23}\text{H}_{39}\text{N}_7\text{O}_7$  ( $m/z$  524.283820,  $[\text{M} - \text{H}]^-$ , amino acids),  $\text{C}_{30}\text{H}_{46}\text{N}_6\text{O}_8$  ( $m/z$  599.319871,  $[\text{M} - \text{H}]^-$ , lipid) and  $\text{C}_{39}\text{H}_{77}\text{O}_9\text{P}$  ( $m/z$  701.512680,  $[\text{M} - \text{H}]^-$ , lipid). Data size was set at 2 million points for a transient length of 0.87 s, and spectra were acquired with a 97% data file reduction. A single MSI measurement has been performed by specimen. Images were captured using FTMS control and FlexImaging (v 5.0, Bruker) software. Images were processed with SCiLS Lab Pro software (Bruker Daltonics, Bremen, Germany). The total ion current method was used for normalization, and  $m/z$  intervals were automatically set at  $\pm 1$  ppm. Images were viewed using both FlexImaging and SCiLS Lab software (Bruker Daltonics, Bremen, Germany).

**Data analyses.** Ion intensities have been log-transformed. Univariate analyses were performed using t-tests to identify discriminatory ionic species between the assessed groups. Age has been taken into account by adding it as covariate. Spearman correlation analysis was performed using R software. Euclidean distance was used as a similarity measure in the clustering analysis. False discovery rates were corrected using the Benjamini–Hochberg–Yekutieli method, and  $p < 0.05$  was considered statistically significant.

**Metabolite annotation and identification.** Preliminary assignments based on accurate mass measurements were performed using the mass spectrometry databases, METLIN<sup>50</sup> and HMDB<sup>51</sup>, using a threshold of  $\pm 2$  ppm. For some metabolites, the precise raw formula led to one hit. Others were identified via “on-tissue” tandem mass spectrometry experiments using MALDI tandem MS/MS. Ions of interest were first isolated using a window of  $\pm 1$  Da, then fragmented by collision-induced dissociation with energy levels between 10 and 40 eV. For each MS/MS analysis, 50 scans were accumulated for better sensitivity. Spectra were reprocessed using Data Analysis 4.4 software (Bruker Daltonics, Bremen, Germany) and recalibrated with the single-point calibration option.

**Ethical statement.** All muscle samples used in this study belong to a collection declared to the French Ministry of Health (collection number DC-2015–2468, accession number AC-2015–2467) located in the Pathology Department (Prof. Annie Laquerrière) of Rouen University Hospital in accordance with the relevant guidelines and regulations and with permission of the local authorities. Written informed consents were obtained from the parents when the patient is under 18 or from the adult patient in order to perform any investigation related to their pathology. The study protocols have been approved by ethics committee of NORD WEST1 – Rouen University Hospital (collection number DC-2015–2468, accession number AC-2015–2467).

## Data availability

All the data that support the findings are presented in the manuscript and the supplementary material.

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## Author contributions

SB and AT conceived and designed the study. AL and FM provided the samples and histopathological data. CB tissue handling and technical assistance. CV, ID, SM and AL provided clinical data. ACB provided genetic data. FL performed Western blot experiments. JF, YB, VG, ISA and CA performed the mass spectrometry imaging data acquisition and raw data processing. AT performed statistical analyses, data visualization, illustrations and data interpretation. AT, ID, JF, FM, AL and SB wrote the manuscript. All authors read and approved the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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**Correspondence** and requests for materials should be addressed to S.B.

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## **Muscle metabolic remodelling patterns in Duchenne muscular dystrophy revealed by ultra-high resolution mass spectrometry imaging**

Ivana Dabaj<sup>1,2#</sup>, Justine Ferey<sup>3#</sup>, Florent Marguet<sup>2,4</sup>, Vianney Gilard<sup>3,5</sup>, Carole Basset<sup>4</sup>, Youssef Bahri<sup>6</sup>, Anne-Claire Brehin<sup>7</sup>, Catherine Vanhulle<sup>1</sup>, France Leturcq<sup>8</sup>, Stéphane Marret<sup>1,2</sup>, Annie Laquerrière<sup>2,4</sup>, Isabelle Schmitz-Afonso<sup>6</sup>, Carlos Afonso<sup>6</sup>, Soumeya Bekri<sup>2,3\*</sup>, Abdellah Tebani<sup>3</sup>

<sup>1</sup> Rouen University Hospital, Department of Neonatal Pediatrics, Intensive Care and Neuropediatrics, Rouen, 76031, France

<sup>2</sup> Normandie Univ, UNIROUEN, CHU Rouen, INSERM U1245, 76000 Rouen, France

<sup>3</sup> Rouen University Hospital, Metabolic Biochemistry Department, Rouen, 76031, France.

<sup>4</sup> Rouen University Hospital, Department of Pathology, Rouen, France.

<sup>5</sup> Rouen University Hospital, Department of Neurosurgery, Rouen, France.

<sup>6</sup> Normandie Univ, COBRA UMR 6014 et FR 3038 Univ Rouen; INSA Rouen; CNRS IRCOF, 1 Rue Tesnière, 76821 Mont-Saint-Aignan Cedex France

<sup>7</sup> Normandie Univ, UNIROUEN, Inserm U1245 and Rouen University Hospital, Department of Genetics and Reference Center for Developmental Disorders, F 76000, Normandy Center for Genomic and Personalized Medicine, Rouen, France

<sup>8</sup> APHP, Laboratoire de Génétique et Biologie moléculaire, HUPC Cochin, Paris, France.

#these authors contributed equally to this work

### **\*Corresponding author:**

Prof. Soumeya BEKRI  
Department of Metabolic Biochemistry,  
Rouen University Hospital 76031 ROUEN Cedex France  
soumeya.bekri@chu-rouen.fr  
Tel 00 33 2 32 88 81 24  
Fax 00 33 2 32 88 83 41

Other Supplementary Material provided as an Excel file for this manuscript includes the following:

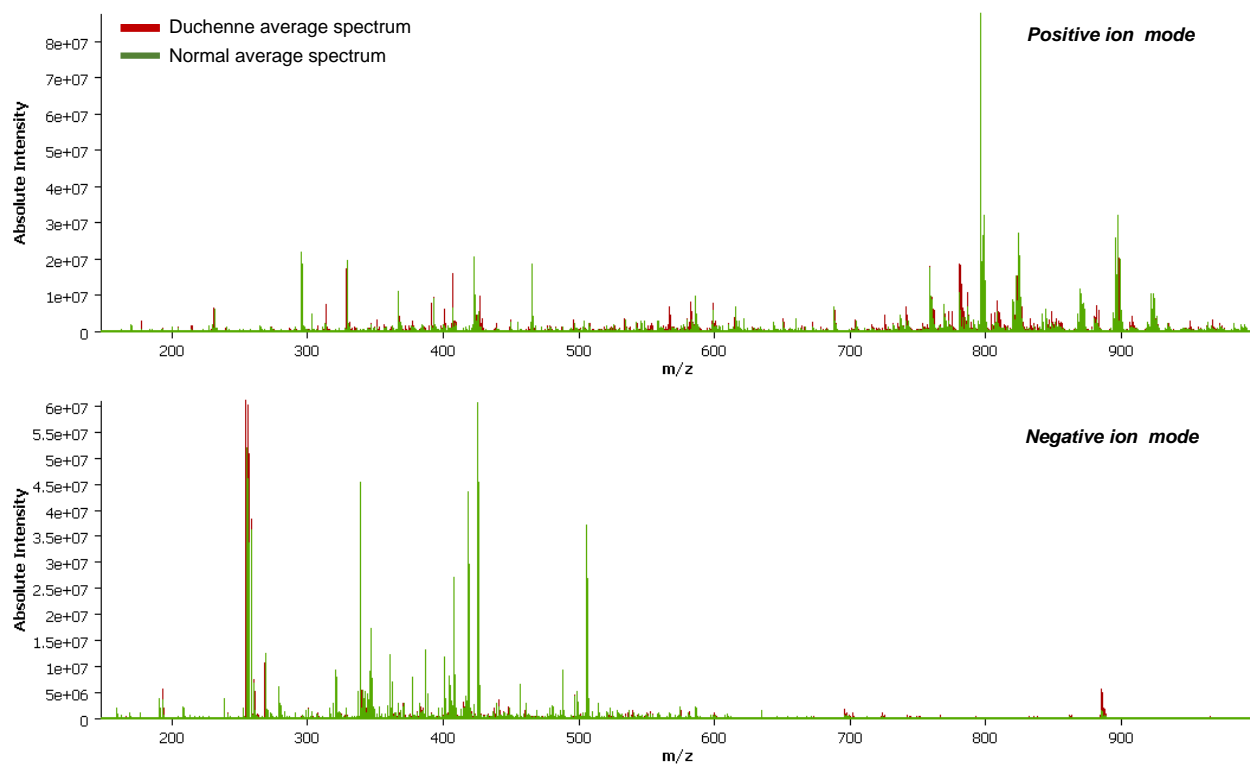
**Supplementary Table 1.** Clinical data for 9 Duchenne patients included in this study.

**Supplementary Table 2.** Descriptive statistics of the clinical data.

**Supplementary Table 3.** Complete intensity table of the annotated metabolites in the analysis's biopsies.

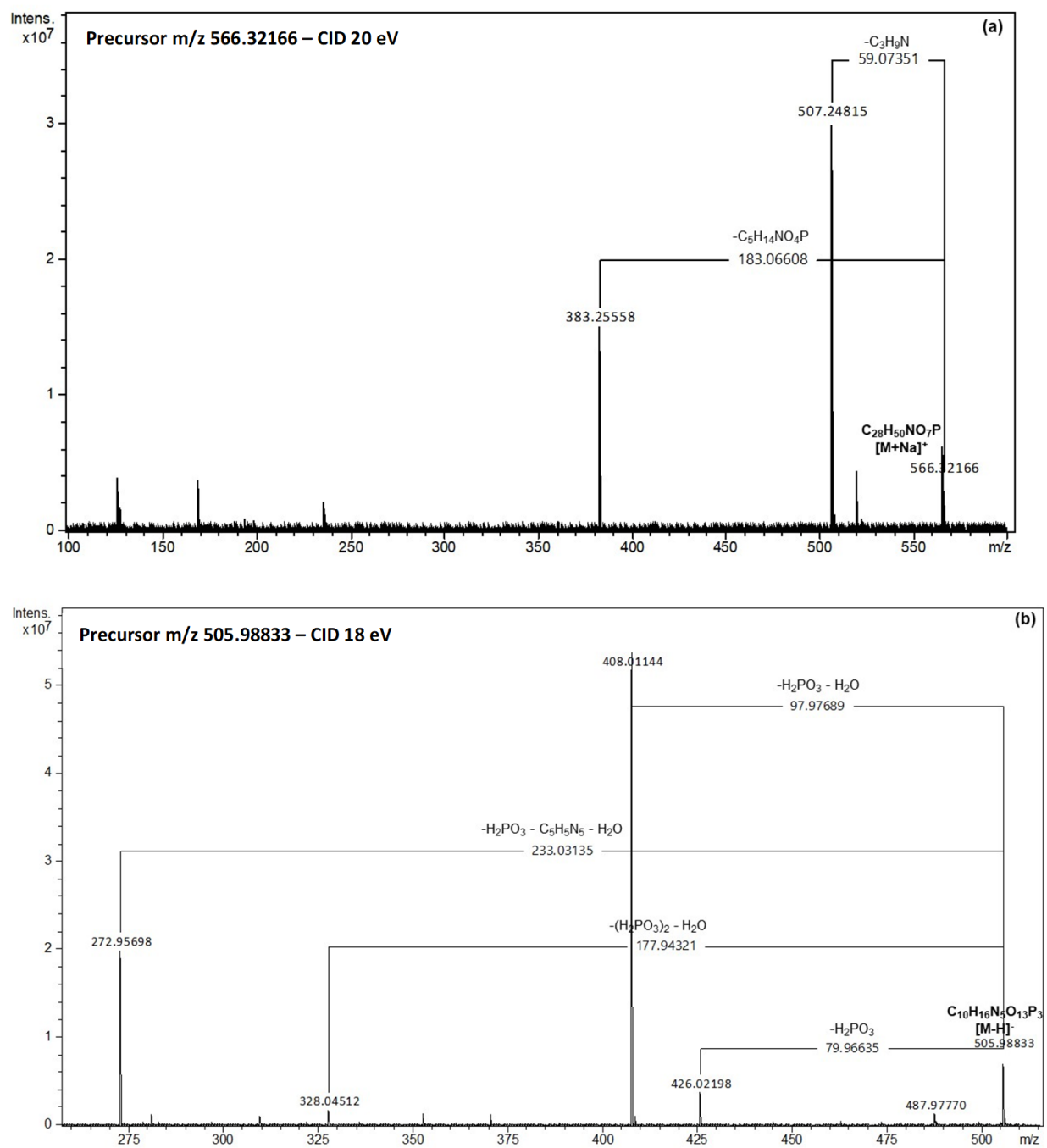
**Supplementary Table 4.** Complete statistical and annotation metrics of the included metabolites.

**Supplementary Table 5.** Metabolite comparison with reported literature.

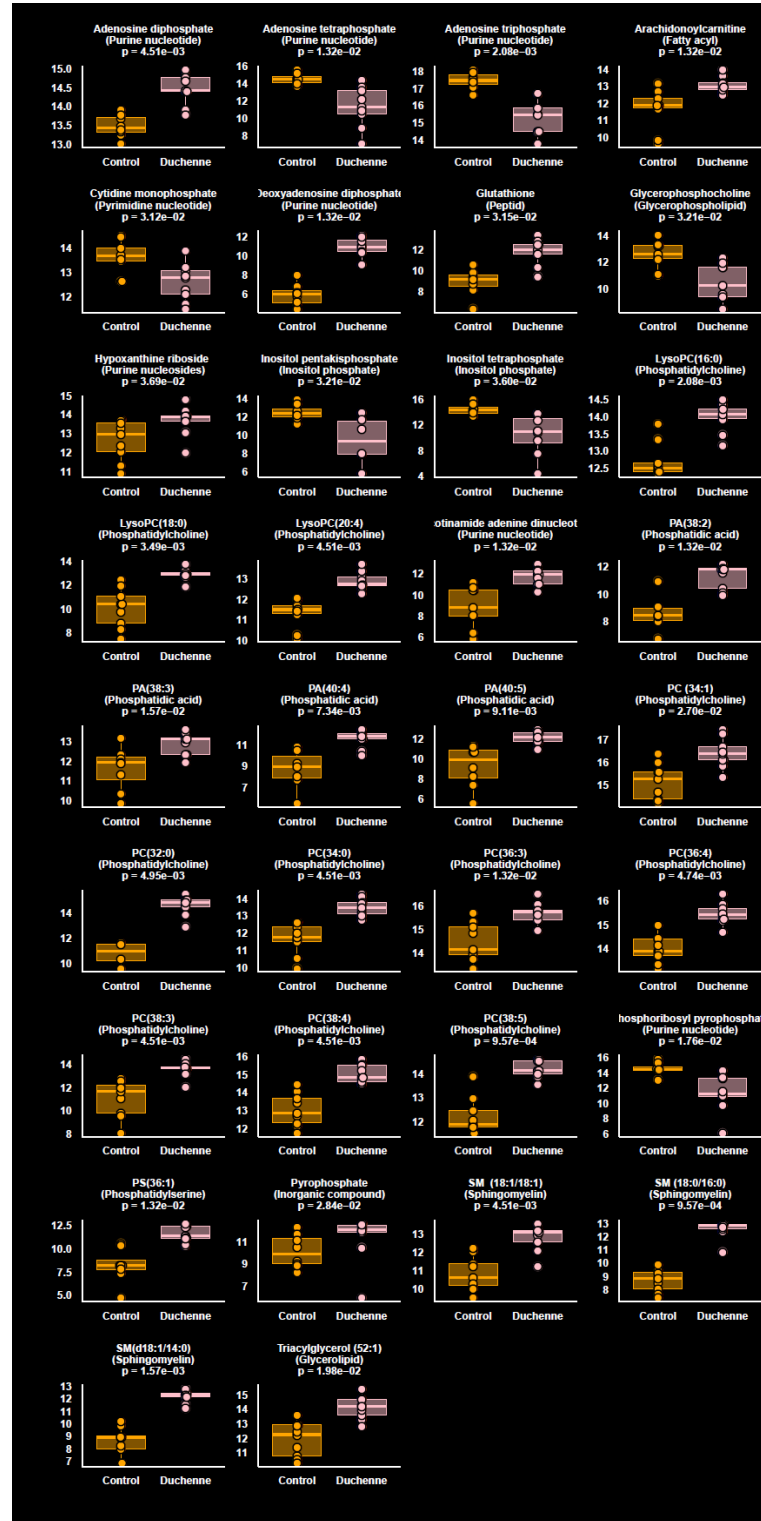


**Supplementary Figure 1.** Average spectra from Duchenne and Control samples in both Positive and Negative ionization mode.

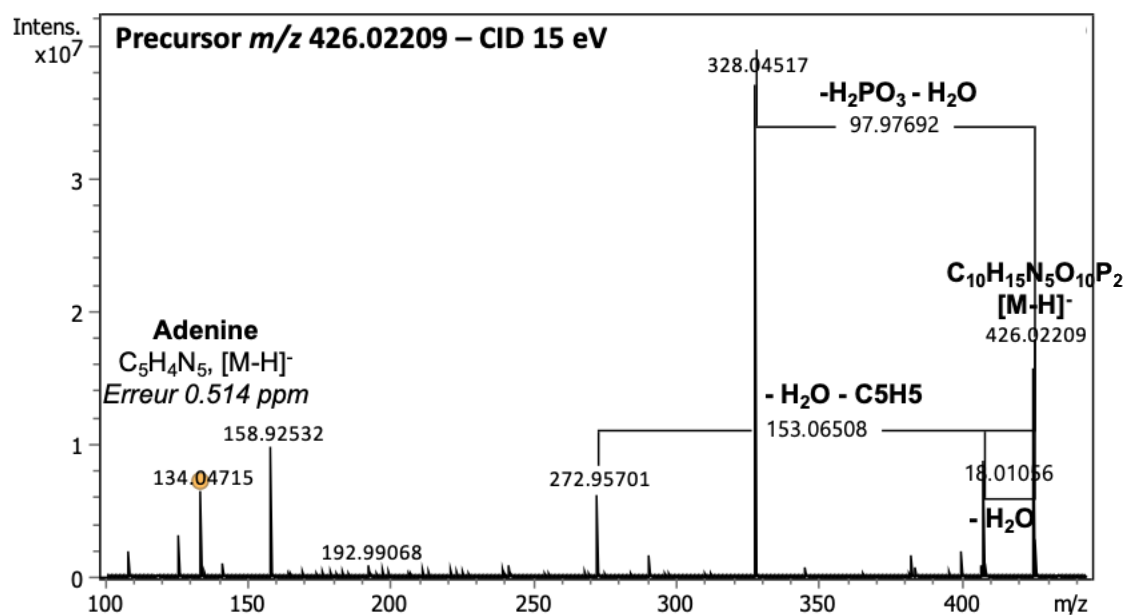




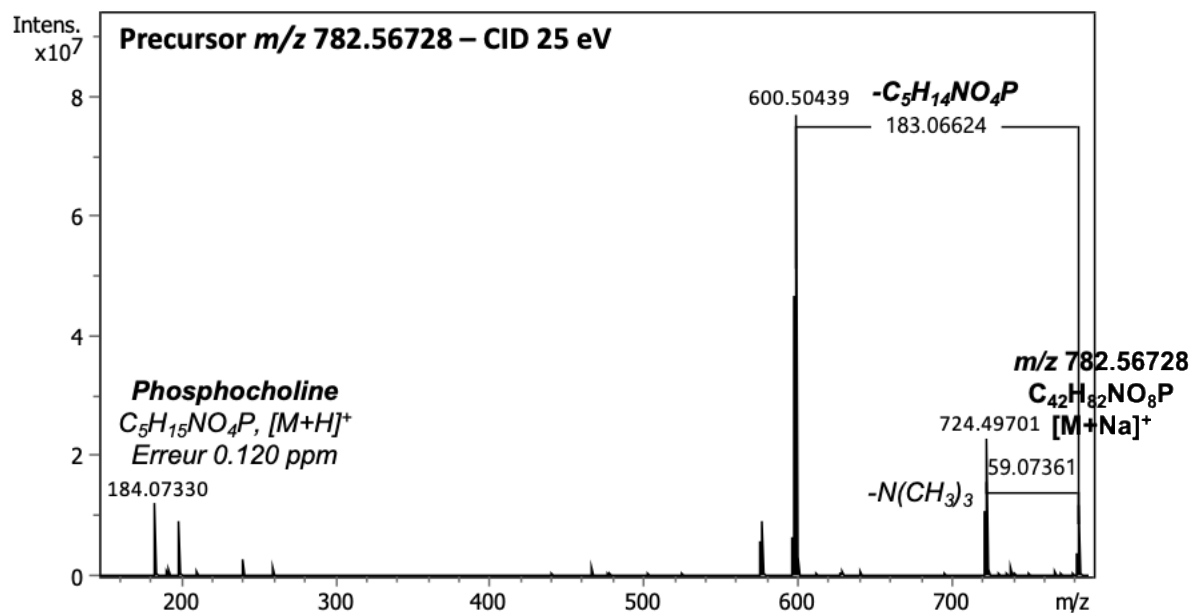
**Supplementary Figure 2.** MALDI-MS/MS spectra obtained from a) lysoPC(20:4) analyzed in positive ion mode by using DHB as a matrix and (b) Adenosine triphosphate (ATP) analyzed in negative ion mode by using 9-AA as a matrix. Spectra obtained using a Bruker Solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) was used for acquiring the MS/MS spectra.



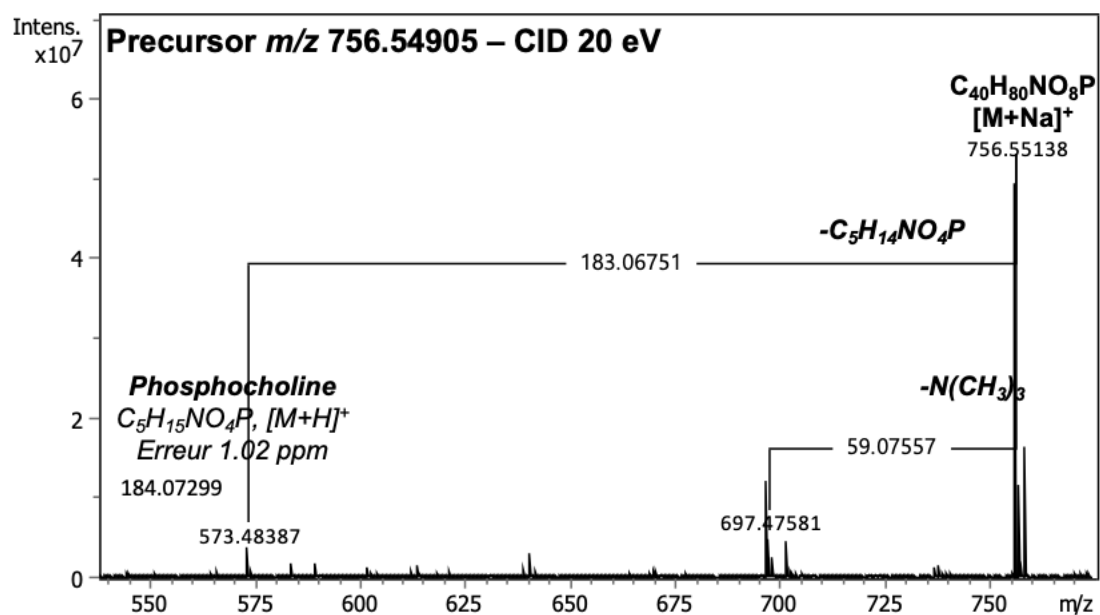
**Supplementary Figure 3.** Boxplots of the differentially expressed metabolites between Duchenne and control biopsies.



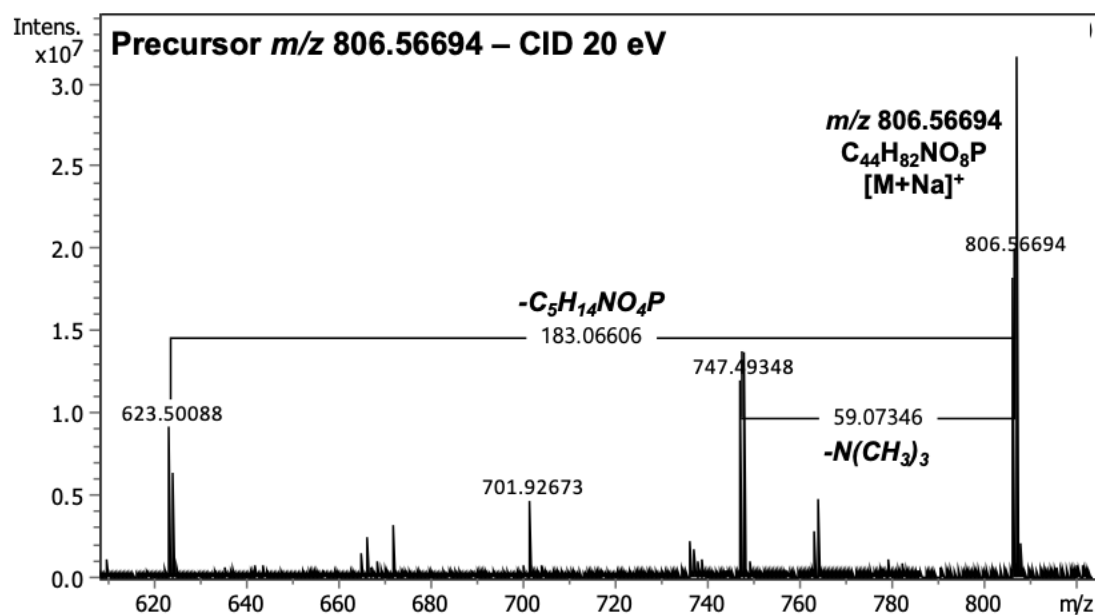
**Supplementary Figure 4.** MALDI-MS/MS spectra obtained from adenosine diphosphate (ADP, precursor  $m/z$  426.02209,  $[M-H]^-$ ) analyzed in negative ion mode by using 9-AA as a matrix. Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 15 eV was used for acquiring the MS/MS spectra.



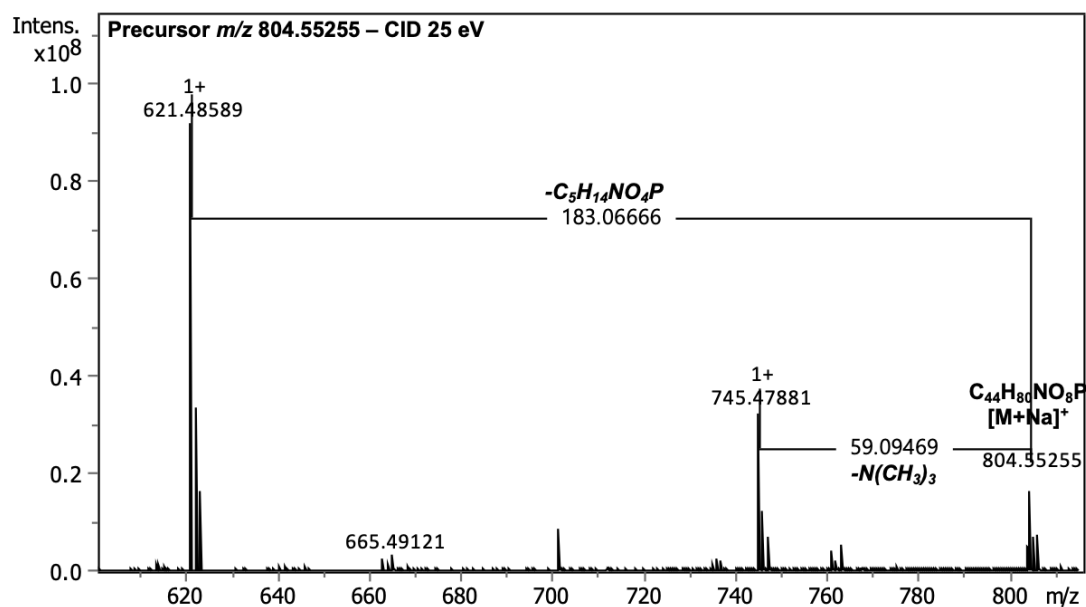
**Supplementary Figure 5.** MALDI-MS/MS spectra obtained from phosphatidylcholine 34:1 (PC 34:1, precursor  $m/z$  782.56728,  $[M+Na]^+$ ) analyzed in positive ion mode by using DHB as a matrix. Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 25 eV was used for acquiring the MS/MS spectra.



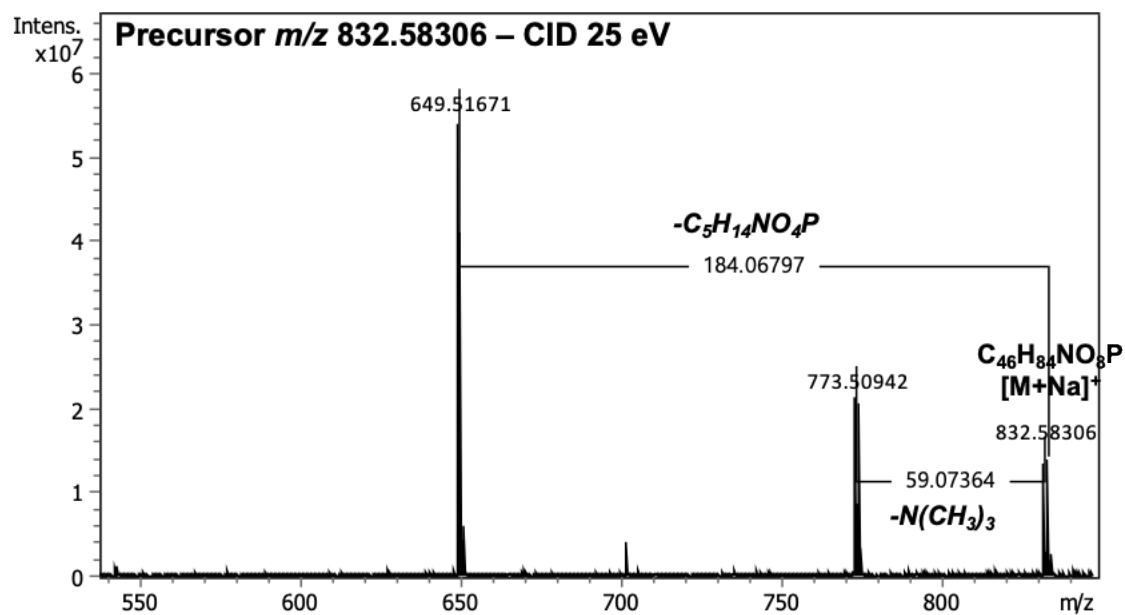
**Supplementary Figure 6.** MALDI-MS/MS spectra obtained from phosphatidylcholine 32:0 (PC 32:0, precursor  $m/z$  756.55138,  $[M+Na]^+$ ) analyzed in positive ion mode by using DHB as a matrix Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 20 eV was used for acquiring the MS/MS spectra.



**Supplementary Figure 7.** MALDI-MS/MS spectra obtained from phosphatidylcholine 32:0 (PC 32:0, precursor  $m/z$  756.55138, [M+Na]<sup>+</sup>) analyzed in positive ion mode by using DHB as a matrix Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 20 eV was used for acquiring the MS/MS spectra.

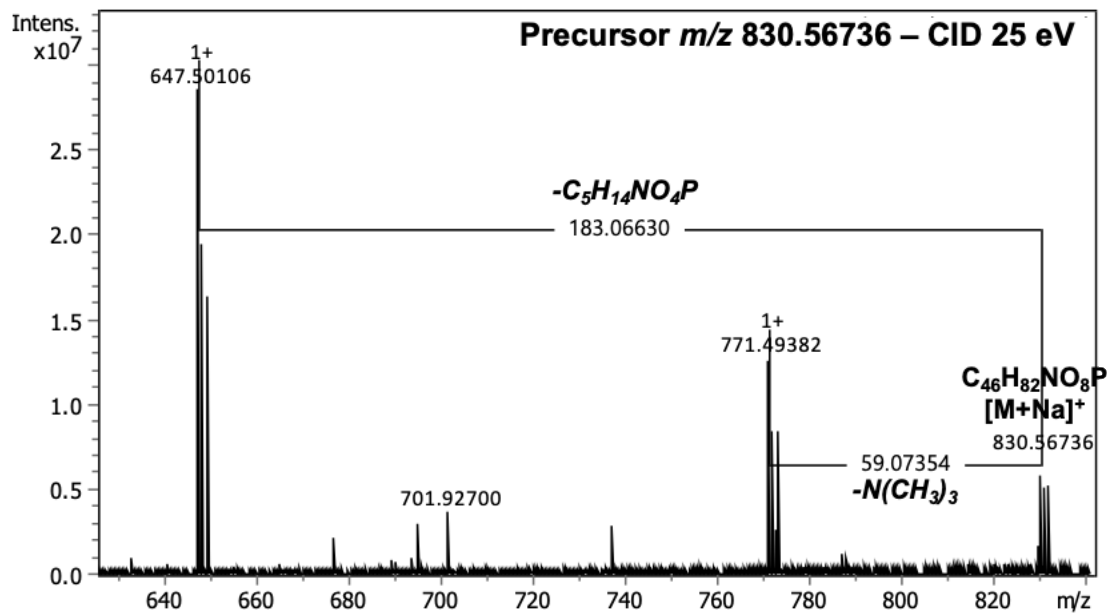


**Supplementary Figure 8.** MALDI-MS/MS spectra obtained from phosphatidylcholine 36:4 (PC 36:4, precursor  $m/z$  804.55255,  $[M+Na]^+$ ) analyzed in positive ion mode by using DHB as a matrix. Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 25 eV was used for acquiring the MS/MS spectra.



**Supplementary Figure 9.** MALDI-MS/MS spectra obtained from phosphatidylcholine 38:4 (PC 38:4, precursor  $m/z$  832.58306, [M+Na]<sup>+</sup>) analyzed in positive ion mode by using DHB as a matrix Spectra obtained using a Bruker solarix MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 25 eV was used for acquiring the MS/MS spectra.





**Supplementary Figure 10.** MALDI-MS/MS spectra obtained from phosphatidylcholine 38:4 (PC 38:4, precursor  $m/z$  832.58306, [M+Na]<sup>+</sup>) analyzed in positive ion mode by using DHB as a matrix Spectra obtained using a Bruker solariX MALDI-FTICR MS 12 Tesla. Collision induced dissociation (CID) at 25 eV was used for acquiring the MS/MS spectra.



**Supplementary Figure 11. General overview of mass spectrometry imaging workflow.** (1) Patient selection; (2) Tissue sampling; (3) Tissue sectioning using a cryomicrotome; (4) Tissue section fixed to the sample probe; (5) Homogenous deposition of matrices using an automatic sprayer; (6) MSI data acquisition using an FT-ICR 12T Solarix Bruker; (7) Mass spectrometry imaging datasets (raw data files) were processed; (8) Raw data were converted into data matrices, including ion mass-to-charge ratios ( $m/z$ ), spatial localization and relative intensity for each ion image, were generated using SCiLS Lab software; (9) Data analysis workflow for comparative analysis to extract the differential metabolic patterns; (10) Annotation of the discriminant features by interrogation mass spectrometry databases; (11) Interpretation of the data according to clinical and biological contexts.

## CHAPTER 2: OMICS IN SPINAL MUSCULAR ATROPHY

### I. Background

Spinal muscular atrophy (SMA), a neurodegenerative disorder due to degeneration of alpha motor neurons in the anterior horn of the spinal cord [60, 61, 366]. It is an autosomal recessive disorder caused by bi-allelic pathogenic variants in the *SMN1* gene, homozygous deletion in this gene is seen in 95% of the times [32, 367, 368]. The diagnosis is based on the clinical evaluation then confirmed by genetic testing. There is a clinical spectrum of disease severity with a large person-to-person variations, and the earlier the onset of symptoms, the more severe the disease. The number of *SMN2* copies (a shortened and partially functional form of *SMN* protein) is variable and inversely correlated to the disease severity [30, 369]. Until recently, like in many other rare diseases, the treatment was only symptomatic and based on multidisciplinary approach. However, targeted therapies are currently approved and are beneficial to patients [29, 33, 41]. Nusinersen is the first FDA and EMA approved drug in SMA. It is an *SMN2*-targeting antisense oligonucleotide (ASO) that links to intron 7 of the pre-mRNA *SMN2* which leads to the translation of a fully functional SMA protein [33, 66]. Despite all the advances in SMA treatment, this disease is still not curable, and more research is needed to understand the pathophysiology and response to treatment while taking into consideration the multitude of molecular and environmental factors. Moreover, probably second-generation treatment needs to be considered and developed to add to the current medication. That's why omics studies started its application in SMA which have provided insights to understand the pathogenesis and helped in identifying biomarkers for follow-up and in developing target therapies. Multiomics studies have been used to search for biomarkers, but more efforts are still needed [63-66].

In our study, we performed longitudinal multi-omics profiling of infantile spinal muscular atrophy metabolomic then proteomic (including NfH and NfL) studies will be done on plasma and CSF of SMA type 1, 2 and 3 patients from two French centers, before and after 6 months of Nusinersen treatment. Results were compared to non-neurological controls.

## II. Objectives

The primary objective is to search for metabolomic and proteomic changes that distinguish SMA naïve patients according to the phenotype severity from control individuals. The secondary objective is to perform a longitudinal study by comparing the omics profile of the patients before and after 6 months of Nusinersen treatment.

## III. Patients and Methods

### *Population*

Fifty-three SMA type 1, 2, and 3 plasmas were selected for the pre-treatment study and were compared with 74 control plasmas. Twenty-four plasmas on day 184 after the start of treatment were selected. CSF analyzed from 34 pretreatment SMA patients, 52 controls, and 24 SMA patients at day 184 after the start of treatment.

### *Omics*

Targeted metabolomic analysis using tandem mass spectrometry: the AbsoluteIDQ® p180 HR kit (Biocrates, Innsbruck, Austria) will be performed on the Api 4000 QTrap instrument (Sciex). This kit allows the quantification of 188 metabolites: amino acids (21), biogenic amines (21), hexoses (1), acylcarnitines (40), glycerophospholipids (90) and sphingolipids (15).

Targeted proteomic analysis: the targeted analysis of 276 proteins (Panels Neurology, Neuroexploratory and Immune response) will be performed using Olink® Biomarker technology (Olink, Uppsala, Sweden). Plasma and CSF NF-H protein will be measured using electrochemiluminescence assays (Meso Scale Discovery [MSD], Rockville, MD, USA). Data will be acquired using a SECTOR S 6000 plate reader (Meso Scale Diagnostics, Rockville, MD, USA).

### *Results*

SMA metabolic profiling investigations in plasma and CSF confirmed the presence of profound metabolic remodeling in SMA patients. Moreover, we demonstrated that these alterations are more pronounced in the severe phenotype. SMA proteomics studies are ongoing.

Further details are mentioned in the following article.

1. **Dabaj I**, Lagrue E, Ducatez F, Allouche S, Ausseil J, Seferian A, Benezit A, Plichet T, Pilon C, Vasseur S, Chapart M, Saugier-veber P, Quijanoroy S, Marret S, Tebani A, Bekri S. **Longitudinal metabolomics profiling of spinal muscular atrophy. in preparation.**

Article

# Longitudinal metabolomics profiling of spinal muscular atrophy

Ivana Dabaj <sup>1,2</sup>, Emmanuelle Lagrue <sup>3</sup>, Franklin Ducatez <sup>1,2</sup>, Stephane Allouche <sup>4</sup>, Jérôme Ausseil <sup>5</sup>, Andreea Seferian <sup>3</sup>, Audrey Benezit <sup>6</sup>, Thomas Plichet <sup>7</sup>, Carine Pilon <sup>7</sup>, Aurelie Phelep <sup>3</sup>, Stephane Vasseur <sup>8</sup>, Maud Chapart <sup>8</sup>, Pascale Saugier-veber <sup>9</sup>, Susana Quijanoroy <sup>6</sup>, Stephane Marret <sup>1,2</sup>, Abdellah Tebani <sup>2,7</sup>, Soumeiya Bekri <sup>2,7</sup>

<sup>1</sup> Department of Neonatology, Pediatric Intensive Care and Neuropediatrics, CHU de Rouen, F-76000 Rouen, France; ivana.dabaj@chu-rouen.fr; franklin.ducatez@chu-rouen.fr; stephane.marret@chu-rouen.fr

<sup>2</sup> Normandie Univ, UNIROUEN, CHUOUEN, INSERM U1245, F-76000 Rouen, France; ivana.dabaj@chu-rouen.fr; franklin.ducatez@chu-rouen.fr; stephane.marret@chu-rouen.fr; abdellah.tebani@chu-rouen.fr; soumeiya.bekri@chu-rouen.fr

<sup>3</sup> Institut I-MOTION, Hôpital Armand Trousseau, Paris cedex 12, 75571, Paris, France; a.seferian@institut-myologie.org; a.phelep@institut-myologie.org; lagrue@univ-tours.fr

<sup>4</sup> Department of Biochemistry, CHU Caen, EA 4650, UNICAEN, Normandie Université, Caen, France; allouche-s@chu-caen.fr

<sup>5</sup> Service de Biochimie, Institut Fédératif de Biologie, Centre Hospitalier Universitaire de Toulouse, Toulouse, France; jerome.ausseil@inserm.fr

<sup>6</sup> APHP Université Paris-Saclay, Pediatric Neuromuscular Unit, Hôpital Universitaire Raymond-Poincaré, Université de Versailles Saint-Quentin-en-Yvelines, Garches, France; audrey.benezit@aphp.fr; susana.quijano-roy@aphp.fr

<sup>7</sup> Department of Metabolic Biochemistry, CHUOUEN, F-76000 Rouen, France; thomas.plichet@chu-rouen.fr; carine.pilon@chu-rouen.fr; abdellah.tebani@chu-rouen.fr; soumeiya.bekri@chu-rouen.fr

<sup>8</sup> MyoBank AFM-Institut de Myologie, Paris, France; s.vasseur@institut-myologie.org; m.chapart@institut-myologie.org

<sup>9</sup> Normandie Univ, UNIROUEN, CHU Rouen, INSERM U1245, 76000 Rouen, Normandy Center for Genomic and Personalized Medicine, Rouen, France.; pascale.saugier-veber@chu-rouen.fr

<sup>10</sup> APHP Université Paris-Saclay, Pediatric Neuromuscular Unit, Hôpital Universitaire Raymond-Poincaré, Université de Versailles Saint-Quentin-en-Yvelines, Garches, France.

\* Correspondence: ivana.dabaj@chu-rouen.fr

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**Abstract:** (1) Background: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by pathogenic variants in the *SMN1* gene and characterized by degeneration of anterior horn cells of the spinal cord. It is a disabling disease with a spectrum of severity. In the past few years, innovative therapies have been developed to treat SMA. Nusinersen was the first drug approved in France. This study aims to identify biomarkers in CSF and plasma that correlated to the clinical status of naïf patients and the evolution under treatment. (2) Methods: To systematically address this issue, we performed targeted metabolomics-based investigations on plasma and CSF samples from patients with SMA before and after six months of treatment. (3) Results: This analysis identified 65 differentially expressed metabolites in CSF and 89 in plasma. These metabolites include mainly Glycerophospholipids, Acylcarnitines, Aminoacids, and Biogenic Amines. Important to note that 28 metabolites vary in CSF and plasma according to the SMA type and *SMN2* copy number. (4) Conclusion: Overall, our study demonstrates systemic metabolic rewiring in SMA that correlates to the *SMN2* copy numbers and the degree of severity.

**Keywords:** spinal muscular atrophy; metabolomics; biomarkers; liquid chromatography; tandem mass spectrometry; Nusinersen.

## 1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by pathogenic variants in the Survival Motor Neuron 1 (*SMN1*) gene, which encodes the SMN protein (survival motor neuron protein). This disease is characterized by progressive degeneration of the motor neurons resulting in generalized muscular weakness and secondary moderate to severe impairment, with an incidence of 1:8,400 births in Europe and 1:11,000 births in the USA [1-5]. Clinically, there is a broad spectrum of age onset, disease course, and severity. The current classification differentiates five subtypes based on the age of onset, severity, and maximal motor milestone reached: type 0 is the most severe, with death shortly after birth; type 1 non-sitters, type 2 sitters, type 3 walkers, type 4 adult onset [3, 6, 7]. Cognitive functions are usually preserved, even if in the most severely affected patients, neurodegeneration could reach not only the anterior horn cells but is also widespread in the cerebral cortex, basal ganglia, brainstem and cerebellum [8].

The *SMN1* gene homozygous deletions or loss of function variants are the main SMA-determining cause, while its homologous gene *SMN2* is the major modifier of the disease severity [4]. Both genes are located on chromosome 5q13. *SMN1* and *SMN2* sequences are very similar; only changes in 5 nucleotides occur, one in exon 7 of the *SMN2* gene resulting in alternative splicing. As a result, 90 % of the expressed *SMN2* protein is truncated, lacking exon 7. This protein is unstable and easily degraded. Of note, *SMN2* is present in at least one copy, but the number of copies varies among individuals. Usually, the *SMN2* copy number is inversely correlated with SMA disease severity [4, 6], but other factors, such as hypoxia and Plastin 3 expression, could influence the phenotype [9]. In the past few years, innovative therapies have been developed to treat SMA. Nusinersen (Spinraza), the first drug approved, is a synthetic antisense oligonucleotide (ASO) that binds to intron 7 of the *SMN2* pre-mRNA, which leads to the translation of a fully functional SMN protein [1, 3, 7, 10]. It is administered intrathecally with a loading dose in the first two months (day 1, 15, 29, 64) and maintenance doses every fourth month [10]. The earlier the drug is given, the higher the success rate [6, 11]. Other therapies are Onasemnogene Apeparvovec (Zolgensma), a one-time *SMN1* gene replacement therapy given intravenously or intrathecally, and Risdiplam (RG7916), a small molecule splicing modifier given orally [3, 11]. The current innovative therapy can significantly alter the disease course. With the experience in Nusinersen, we now know that there is an urge to make an early diagnosis and to find biomarkers of disease activity and treatment response. Regarding early diagnosis, newborn screening programs have been developed in several countries: USA, Germany, Belgium, and Australia [3]. These programs aim to allow presymptomatic initiation of therapy [6]. These tools are important to detect affected children, but some patients show very mild disease course with late onset of symptoms. Therefore, predictive measures of the disease severity were developed to help clinicians decide to treat it. The correlation between the phenotype and the genotype is not absolute, and the main determinant of the different subtypes relates to the number of copies of the gene *SMN2* [1, 5, 7, 12, 13]. The phenotype is related to the *SMN2* copy number rather than the expression levels of *SMN2* in the blood [12]. The main genetic modifiers other than *SMN2* studied are Plastin 3, Coronin 1C, Neurocalcin Delta, Small EDRK-Rich Factor 1, NLR Family Apoptosis Inhibitory Protein, General Transcription Factor IIH subunit 2, and Tolloid Like 2 [7, 14-16]. Functional outcome measures have been widely used to assess the response to treatment. The Biomarker for SMA study showed that Motor Function measure (MFM), pulmonary function testing,

strength, electroneuromyography, muscle MRI and workspace volume discriminate between SMA types 1, 2, 3, and 4. MyoSet devices and Active-seated discriminate type 2 and 3 [17]. Body composition was also evaluated as a potential biomarker, but the correlation with the motor function was weak in SMA type 2 and moderated in SMA type 1 [18]. For electrophysiological biomarkers, the NeuroNEXT study showed that compound muscle action potential (CMAP) is a potential biomarker [13]. In addition to CMAP, Motor unit number estimation (MUNE) has been studied, and the amplitude of both declined with age in SMA 1 and 2. These measurements delineate motor function and may reveal abnormalities before symptoms appear. The stability of these two electrophysiological biomarkers indicates a capacity for prolonged motor neuron survival. While CMAP may remain stable over time, MUNE has been shown to increase [5, 14, 19]. Circulatory biomarkers have gained significant attention for therapeutic monitoring. The first one is SMN protein that can be measured from biological samples and could be useful to monitor treatments that increase SMN levels [14]. Czech et al. study showed that the expression of SMN mRNA and protein in the blood greatly overlaps between different disease types and thus cannot be used as a marker for clinical severity [12]. The second potential biomarkers are neurofilaments, intermediate filaments of the neuronal axons. Phosphorylated neurofilament heavy chain (pNfH) and neurofilament light chain (NfL) have been associated with axonal degeneration, and their levels tend to increase in cerebrospinal fluid (CSF), plasma/serum of patients with motor neuronopathy [1, 7, 14]. There is a positive correlation between CSF NfL levels and the age of treatment as well as the duration of disease, but no correlation was found with CSF pNfH. Note that there is a slight but significant decrease in both neurofilaments type before and after six months of treatment with Nusinersen [1]. Although plasma neurofilaments seem to be reliable biomarkers in infants that are correlated to motor function improvement [10], contradicting results in adult patients [7]. Another neuron-specific structural protein Tau. It is considered a cortical neuronal marker. It was high in CSF of SMA children and decreased with Nusinersen treatment, but it was not altered in SMA type 3 patients [7]. Serum creatinine could be an interesting biomarker of muscle deterioration; however, the range of variability is very wide. The increased tendency of creatinine concentration with the decrease in creatine kinase activity during Nusinersen treatment suggests decreased muscle mass wasting and an improvement in muscular energy metabolism. However, prospective studies are needed [20, 21]. Some potential imaging biomarkers have been studied: muscle MRI and ultrasound, electrical impedance myography, MR neurography, imaging flow cytometry combined with spot analysis, and magnetization transfer ratio [22-24]. Splicing regulators like miRNAs could be interesting as biomarkers and therapeutic targets for neurodegenerative diseases like SMA [25]. MyomiR serological levels decrease after Nusinersen treatment in the infantile SMA [26]. Another regulator is SMN2 methylation, that could change the disease phenotype by modulating its transcription. Differences in methylation patterns in certain genes through genome-wide methylation studies could be involved in the pathogenesis of SMA [14]. Multiomics studies have been used to search for biomarkers, but more efforts are still needed [2, 27-29]. In our study, we performed metabolomic studies on plasma and CSF of SMA type 1, 2, and 3 patients from two French centers before and after six months of Nusinersen treatment. The results are compared to control subjects. This study aims to identify biomarkers in CSF and plasma correlated to clinical status and the evolution of SMA under treatment.

## 2. Materials and Methods

### 2.1. Patients and Samples

Patients with genetically confirmed SMA diagnosis, who underwent treatment with Nusinersen in two French centers were included. Plasma and CSF samples were collected during the regular visits for treatment (before treatment, day 15, day 30, day 64, day 184, every four months). Different scales to evaluate motor function (Motor Function Measurement, HINE, HFMSE, CHOP intend), respiratory function (vital capacity), and digestive and orthopedic statuses were used to evaluate the severity of the disease and efficacy of treatment. Fifty-three plasmas SMA types 1, 2, and 3 were selected for the study before treatment and were compared to 74 plasmas from controls (from two French centers: Caen University Hospital and Toulouse University Hospital). Twenty-four plasmas at day 184 after treatment initiation were selected. CSF samples were analyzed in 34 SMA patients before treatment, and 24 SMA patients at day 184 after treatment initiation. Fifty-two CSF control samples (Caen University Hospital and Toulouse University Hospital) have been used. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

### 2.2. Targeted Metabolomics

#### 2.2.1. Reagents

All reagents, internal and calibration standards, quality controls, test mix, and a patented 96-well filter plate required for the AbsoluteIDQ®p180 analysis are included in the kit or provided by Biocrates Life Science AG (Innsbruck, Austria). All metabolites were identified and quantified using isotopically labeled internal standards and multiple reaction monitoring (MRM) as optimized and provided by Biocrates Life Sciences AG (Innsbruck, Austria).

#### 2.2.2. Metabolite quantitation

All reagents, internal and calibration standards, quality controls, test mix, and a patented 96-well filter plate required for the AbsoluteIDQ®p180 analysis are included in the kit or provided by Biocrates Life Science AG (Innsbruck, Austria). Sample preparation was carried out according to the manufacturer's protocol [30, 31]. Briefly, 10 µL of plasma was transferred to the upper 96-well plate and dried under a nitrogen stream. After that, 50 µL of a 5% PITC solution was added to derivatize amino acids and biogenic amines. After incubation, the spots were dried again before the metabolites were extracted using 5 mM ammonium acetate in methanol (300 µL) into the lower 96-well plate for analysis after further dilution using the MS running solvent A. Quantification was carried out using isotopically labeled internal standards and a calibration curve [30, 31]. The complete list of 188 measured metabolites is presented in **Supplementary Table S1**. The AbsoluteIDQ® p180 kit is a fully automated assay based on phenylisothiocyanate (PITC) derivatization of the target analytes in bodily fluids using internal standards for quantitation. Amino acids and biogenic amines are determined in LC-MS mode, acylcarnitines, phospholipids, sphingomyelins, and the sum of hexoses are analyzed in flow injection analysis (FIA). The analyses were performed following the instructions of the kit manufacturer using liquid chromatography instrument prominence Shimadzu UFLC System (Shimadzu, Prominence, Kyoto, Japan) coupled to the 4000 Qtrap mass spectrometer (Sciex, Framingham, MA, USA) with an electrospray ion source. Data acquisition and processing were performed using the Analyst 1.6.3 software (Sciex, Framingham, MA, USA).

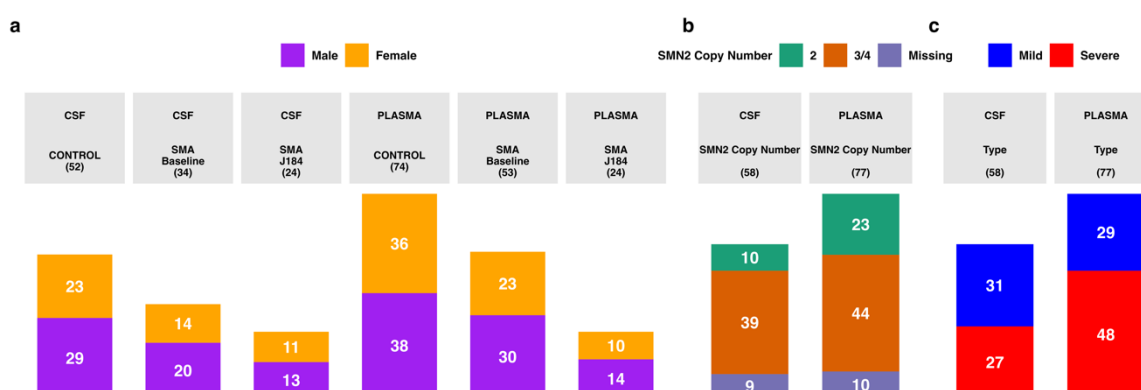


### 2.2.3. Data analysis

The Data matrix was log-transformed and pareto-scaled. Missing values were imputed using nearest neighbor averaging algorithm using the `impute.knn` function in the `impute` R package. Univariate analyses were performed using t-tests to identify discriminatory features between the assessed groups. Limma package was used for differential analysis with sex, and age are considered by adding it as a covariate. Spearman correlation analysis was performed using R software. Euclidean distance was used as a similarity measure in the clustering analysis. False discovery rates were corrected using the Benjamini–Hochberg–Yekutieli method and  $p < 0.05$  was considered statistically significant. All data analyses and visualizations were performed using R software.

## 3. Results

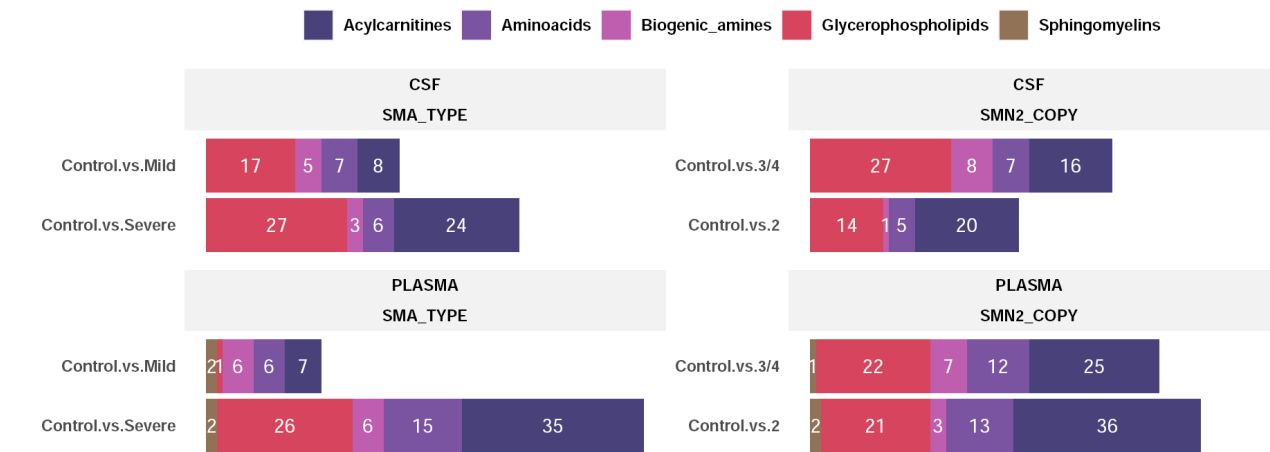
This work aims to explore metabolomics profile differences in CSF and plasma samples between (i) SMA samples at baseline before Nusinersen treatment and control samples and (ii) SMA samples at baseline before Nusinersen treatment and samples at day184 after treatment. An overview of the cohort is presented in **Figure 1**.



**Figure 1.** Overview of the cohort. a) Sex distribution across matrix and SMA groups. b) SMN2 Copy number overview across plasma and cerebrospinal fluid matrices. c) Cohort description regarding clinical severity.

The full data matrix with sample characteristics is presented in **Supplementary Table S2**. To analyse the data, we performed a differential analysis between (i) two baseline SMA groups according to the clinical severity (severe (type 1) and mild phenotype (types 2 and 3) versus control samples. The analysis identified 45 metabolites that are differentially expressed in CSF and 70 in plasma (ii) two baseline SMA groups according to SMN2 copy number (group 1: SMN2 copy number = 2, group 2: SMN2 copy number  $\geq 3$ ). This analysis identified 65 metabolites differentially expressed in CSF and 89 in plasma (**Figure 2**).

209



210 **Figure 2.** Overview of metabolomics differential expression analysis in plasma and CSF according to  
211 clinical type (severe/mild) and *SMN2* copy numbers (2 and 3 / 4).

212  
213 These metabolites include mainly Glycerophospholipids, Acylcarnitines, Aminoacids, and  
214 Biogenic Amines (**Figure 2**). Interestingly, 28 metabolites vary in CSF and plasma according to the  
215 SMA type and *SMN2* copy number (**Figure 3 and Supplementary Table S3**).

216 Regarding the glycerophospholipids, the concentrations of phosphatidylcholine species are  
217 often increased, while those of lysophosphatidylcholine are mainly decreased. Acylcarnitine  
218 concentrations are elevated in SMA patients while the concentrations of the assessed amino acid are  
219 decreased except for Glutamine and Citrulline. The concentrations of biogenic amines are either  
220 decreased (e.g., Dopamine, Kynurenine, nitrotyrosine, hydroxyproline, creatinine) or increased  
221 (spermidine, putrescine).

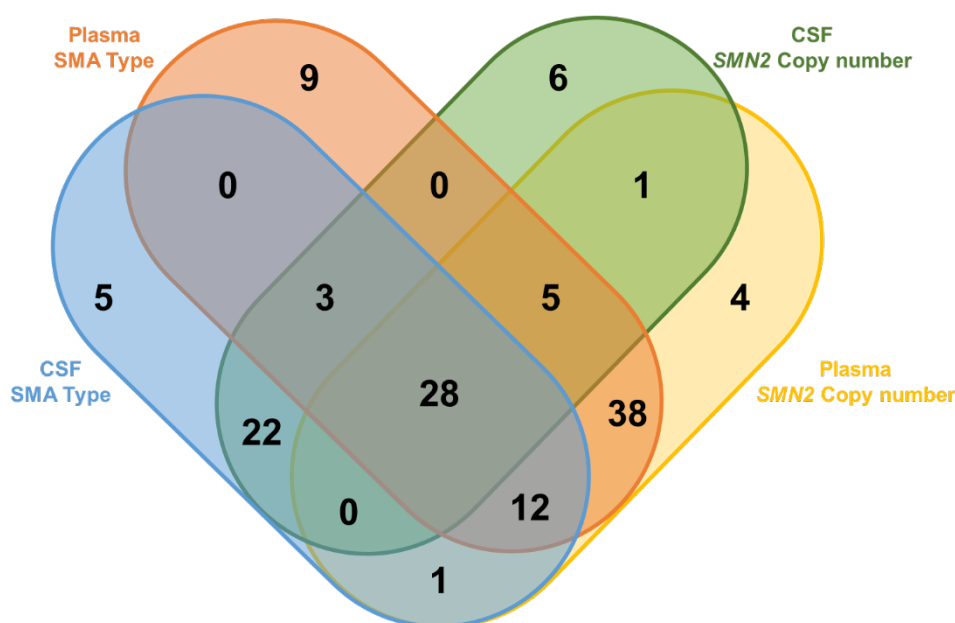


Figure 3. Differential expressed metabolites in plasma and CSF according to clinical type (severe/mild) and SMN2 copy numbers (2 and 3 / 4).

The complete list of metabolites and their related statistics are presented in **Supplementary Table S3**. The differentially expressed metabolites are shown in boxplots (**Supplementary Figures 1 and 2**).

The concentration of several metabolites varies according to the clinical severity (SMA type) or SMN2 copy number (**Figure 4**).

The comparison between the SMA baseline and SMA day184 (6 months) after treatment did not reveal any significant changes.



**Figure 4.** Venn diagram showing the overlap and the intersections between the differentially expressed metabolites in plasma and CSF according to clinical type (severe/ mild) and SMN2 copy numbers (2 and 3 / 4).

#### 4. Discussion

SMA disease is a neuromuscular disorder that displays clinical heterogeneity. Currently, there are no reliable diagnostic and follow-up biomarkers for SMA. The availability of new specific treatments requires developing tools for early diagnosis and treatment monitoring. These specific biomarkers may enable the stratification of patients. To achieve this goal, we conducted a targeted metabolomic study in a cohort of SMA patients prior to and after Nusinersen treatment versus control individuals. 188 metabolites, including acylcarnitines, biogenic amines, amino acids, glycerophospholipids, and sphingomyelins, were quantified in plasma and CSF samples.

This study showed a profound metabolic remodeling in naive SMA patients, with notably an increase in the concentration of acylcarnitines, a decrease in that of amino acids, and a modulation of glycerophospholipid metabolism. These results are broadly consistent with those previously described in the literature. Metabolic abnormalities (alteration of fatty acid metabolism, glucose tolerance impairment, and mitochondrial defects) were reported in SMA animals and patients, and there is a possible impact of SMN deficiency on metabolic abnormalities; therefore metabolomics studies could help enlightening the underlying mechanisms of the disease and in finding potential biomarkers [32].

Indeed, fat metabolism alterations in SMA patients have been characterized since the seventies [33]. Several reports mentioned dyslipidemia with increased cardiovascular risk factor [34, 35], liver steatosis [34–36], elevated concentrations of acylcarnitines with a decrease in free plasma carnitine [37]. The deregulation of fatty acid metabolism may be due to denervation, which results in loss of muscle fiber function and, thus a decrease in the flux of the beta-oxidation [38]. In this study, the decreased concentration of several amino acids (BCAAs, phenylalanine, alanine, aspartic acid, serine, histidine, methionine, glycine, glutamic acid, proline, lysine, ornithine, arginine), as well as some biogenic amines (e.g., hydroxyproline), may reflect a decrease in the flux of protein metabolism. A decrease in the concentration of several amino acids (alanine, phenylalanine, branched-chain amino acids (BCAAs)) with hypoglycemia upon fasting has been described [39, 40]. Interestingly, BCAA concentration was significantly reduced in the mouse SMA model, and BCAA supplementation had a beneficial impact on survival [41].

Furthermore, we compared the metabolomic profiles of controls and SMA patients according to the SMA clinical phenotype (severe: type 1 and moderate types 2/3) and according to the copy number of the *SMN2* gene. The expression of several metabolites varies with severity based on clinical type or *SMN2* copy number (Figure 3). This may suggest that metabolic remodeling is closely related to phenotypic severity and thus may drive disease worsening. In addition, this approach shows that the comparison using the *SMN2* copy number criterion yielded a higher number of differentially expressed metabolites (Figure 3). This may indicate that this item enables a better stratification of SMA patients compared to the clinical type-based analysis. Notably, the metabolomic profiles failed to distinguish SMA-naïve from SMA patients after six months of treatment. This may be because this metabolic remodelling requires a more extended period of treatment to normalize if it occurs.

## 5. Conclusions

This work confirms the presence of profound metabolic remodelling in SMA patients. Moreover, we demonstrated that these alterations are more pronounced in the severe phenotype and are therefore related to the degree of severity.

*SMN2* copy number is inversely correlated with SMA severity. Our results indicate that a patient stratification based on *SMN2* copy numbers would be more reliable and better reflect the severity degree. This classification could therefore replace the classification based on the clinical phenotype that may be subjective and dependent on the practitioner who examines the patient.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx](http://www.mdpi.com/xxx) Table S1: Biocrates Metabolic Panel with 188 measured metabolites; Table S2 : Samples' characteristics, Table S3: Differentially expressed metabolites.

**Author Contributions:** ID, AT and SB conceived and designed the study. SV and MC provided the samples of patients. JA, SA provided samples of controls. AP, EL, AS provided the clinical data of one center; ID, AB, SQR provided the clinical data of the other center. TP, CP provided sample handling and technical assistance. TP provided raw data processing. PSV performed the molecular analyses. FD, AT performed statistical analyses, data visualization, illustrations, and data interpretation. ID and SB wrote the original draft. SM and AT edited the final manuscript. All authors read and approved the manuscript

**Conflicts of Interest:** The authors declare no conflict of interest

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## 384 Supplementary tables

## 385 Table S1: Biocrates Metabolic Panel with 188 measured metabolites

| Amino Acids (21) |            |     |               |
|------------------|------------|-----|---------------|
| Ala              | Alanine    | Lys | Lysine        |
| Arg              | Arginine   | Met | Methionine    |
| Asn              | Asparagine | Om  | Omithine      |
| Asp              | Aspartate  | Phe | Phenylalanine |
| Cit              | Citrulline | Pro | Proline       |
| Glu              | Glutamate  | Ser | Serine        |
| Gln              | Glutamine  | Thr | Threonine     |
| Gly              | Glycine    | Trp | Tryptophan    |
| His              | Histidine  | Tyr | Tyrosine      |
| Ile              | Isoleucine | Val | Valine        |
| Leu              | Leucine    |     |               |

| Biogenic Amines (21) |                             |                         |                            |
|----------------------|-----------------------------|-------------------------|----------------------------|
| Ac-Om                | Acetylornithine             | Met-SO                  | Methionine sulfoxide       |
| alpha-AAA            | alpha-Aminoadipic acid      | Nitro-Tyr <sup>1)</sup> | Nitrotyrosine              |
| ADMA                 | Asymmetric dimethylarginine | PEA                     | Phenylethylamine           |
| Carnosine            | Carnosine                   | Putrescine              | Putrescine                 |
| Creatinine           | Creatinine                  | Sarcosine               | Sarcosine                  |
| DOPA                 | Dihydroxyphenylalanine      | Serotonin               | Serotonin                  |
| Dopamine             | Dopamine                    | Spermidine              | Spermidine                 |
| Histamine            | Histamine                   | Spermine                | Spermine                   |
| c4-OH-Pro            | cis-4-Hydroxyproline        | SDMA                    | Symmetric dimethylarginine |
| t4-OH-Pro            | trans-4-Hydroxyproline      | Taurine                 | Taurine                    |
| Kynurenine           | Kynurenine                  |                         |                            |

| Monosaccharides (1) |                             |
|---------------------|-----------------------------|
| H1                  | Hexoses (including glucose) |

| Acylcarnitines (40) |  |                 |  |
|---------------------|--|-----------------|--|
| C0                  | Carnitine                                    | C5-M-DC         | Methylglutaryl carnitine                           |
| C2                  | Acetylcarnitine                              | C5-OH (C3-DC-M) | Hydroxyvaleryl carnitine (Methylmalonyl carnitine) |
| C3                  | Propionyl carnitine                          | C5:1            | Tiglyl carnitine                                   |
| C3-OH               | Hydroxypropionyl carnitine                   | C5:1-DC         | Glutaconyl carnitine                               |
| C3:1                | Propenyl carnitine                           | C6 (C4:1-DC)    | Hexanoyl carnitine (Fumaryl carnitine)             |
| C4                  | Butyryl carnitine                            | C6:1            | Hexenoyl carnitine                                 |
| C4-OH (C3-DC)       | Hydroxybutyryl carnitine (Malonyl carnitine) | C7-DC           | Pimeloyl carnitine                                 |
| C4:1                | Butyryl carnitine                            | C8              | Octanoyl carnitine                                 |

| Acylcarnitines (40) |                                  |          |                                 |
|---------------------|----------------------------------|----------|---------------------------------|
| C10:1               | Decenoyl carnitine               | C16      | Hexadecanoyl carnitine          |
| C10:2               | Decadienyl carnitine             | C16-OH   | Hydroxyhexadecanoyl carnitine   |
| C12                 | Dodecanoyl carnitine             | C16:1    | Hexadecenoyl carnitine          |
| C12-DC              | Dodecanedioyl carnitine          | C16:1-OH | Hydroxyhexadecenoyl carnitine   |
| C12:1               | Dodecenoyl carnitine             | C16:2    | Hexadecadienyl carnitine        |
| C14                 | Tetradecanoyl carnitine          | C16:2-OH | Hydroxyhexadecadienyl carnitine |
| C14:1               | Tetradecenoyl carnitine          | C18      | Octadecanoyl carnitine          |
| C14:1-OH            | Hydroxytetradecenoyl carnitine   | C18:1    | Octadecenoyl carnitine          |
| C14:2               | Tetradecadienyl carnitine        | C18:1-OH | Hydroxyoctadecenoyl carnitine   |
| C14:2-OH            | Hydroxytetradecadienyl carnitine | C18:2    | Octadecadienyl carnitine        |

| Glycerophospholipids (90) |                           |             |             |
|---------------------------|---------------------------|-------------|-------------|
| lysoPC a C14:0            | PC aa C34:1               | PC aa C42:0 | PC ae C38:2 |
| lysoPC a C16:0            | PC aa C34:2               | PC aa C42:1 | PC ae C38:3 |
| lysoPC a C16:1            | PC aa C34:3               | PC aa C42:2 | PC ae C38:4 |
| lysoPC a C17:0            | PC aa C34:4               | PC aa C42:4 | PC ae C38:5 |
| lysoPC a C18:0            | PC aa C36:0               | PC aa C42:5 | PC ae C38:6 |
| lysoPC a C18:1            | PC aa C36:1               | PC aa C42:6 | PC ae C40:1 |
| lysoPC a C18:2            | PC aa C36:2               | PC ae C30:0 | PC ae C40:2 |
| lysoPC a C20:3            | PC aa C36:3               | PC ae C30:1 | PC ae C40:3 |
| lysoPC a C20:4            | PC aa C36:4               | PC ae C30:2 | PC ae C40:4 |
| lysoPC a C24:0            | PC aa C36:5               | PC ae C32:1 | PC ae C40:5 |
| lysoPC a C26:0            | PC aa C36:6               | PC ae C32:2 | PC ae C40:6 |
| lysoPC a C26:1            | PC aa C38:0               | PC ae C34:0 | PC ae C42:0 |
| lysoPC a C28:0            | PC aa C38:1 <sup>2)</sup> | PC ae C34:1 | PC ae C42:1 |
| lysoPC a C28:1            | PC aa C38:3               | PC ae C34:2 | PC ae C42:2 |
| PC aa C24:0               | PC aa C38:4               | PC ae C34:3 | PC ae C42:3 |
| PC aa C26:0               | PC aa C38:5               | PC ae C36:0 | PC ae C42:4 |
| PC aa C28:1               | PC aa C38:6               | PC ae C36:1 | PC ae C42:5 |
| PC aa C30:0               | PC aa C40:1               | PC ae C36:2 | PC ae C44:3 |
| PC aa C30:2 <sup>2)</sup> | PC aa C40:2               | PC ae C36:3 | PC ae C44:4 |
| PC aa C32:0               | PC aa C40:3               | PC ae C36:4 | PC ae C44:5 |
| PC aa C32:1               | PC aa C40:4               | PC ae C36:5 | PC ae C44:6 |
| PC aa C32:2               | PC aa C40:5               | PC ae C38:0 |             |
| PC aa C32:3               | PC aa C40:6               | PC ae C38:1 |             |

| Sphingomyelins (15) |               |                        |               |
|---------------------|---------------|------------------------|---------------|
| SM (OH) C14:1       | SM C18:0      | SM (OH) C22:2          | SM (OH) C24:1 |
| SM C16:0            | SM C18:1      | SM C22:3 <sup>2)</sup> | SM C26:0      |
| SM C16:1            | SM C20:2      | SM C24:0               | SM C26:1      |
| SM (OH) C16:1       | SM (OH) C22:1 | SM C24:1               |               |





**Table S2:** Samples' characteristics

| Patient | Matrix | Time | Site    | Sex | Age (years) | SMA type | Number copies SMN2 | Survival |
|---------|--------|------|---------|-----|-------------|----------|--------------------|----------|
| P1      | PLASMA | J1   | Imotion | M   | 0,4         | 1        | 2                  | Y        |
| P1      | PLASMA | J184 | Imotion | M   | 1,0         | 1        | 2                  | Y        |
| P2      | CSF    | J1   | Imotion | M   | 0,3         | 1        | 2                  | Y        |
| P2      | PLASMA | J1   | Imotion | M   | 0,3         | 1        | 2                  | Y        |
| P3      | CSF    | J1   | Imotion | M   | 0,4         | 1        | 2                  | Y        |
| P3      | PLASMA | J1   | Imotion | M   | 0,4         | 1        | 2                  | Y        |
| P4      | CSF    | J1   | Imotion | M   | 0,7         | 1        | 2                  | Y        |
| P4      | PLASMA | J1   | Imotion | M   | 0,7         | 1        | 2                  | Y        |
| P5      | CSF    | J1   | Imotion | M   | 1,0         | 1        | 3                  | Y        |
| P5      | PLASMA | J1   | Imotion | M   | 1,0         | 1        | 3                  | Y        |
| P5      | CSF    | J184 | Imotion | M   | 1,5         | 1        | 3                  | Y        |
| P5      | PLASMA | J184 | Imotion | M   | 1,5         | 1        | 3                  | Y        |
| P6      | CSF    | J1   | Imotion | F   | 0,2         | 1        | 2                  | Y        |
| P6      | PLASMA | J1   | Imotion | F   | 0,2         | 1        | 2                  | Y        |
| P7      | CSF    | J1   | Imotion | M   | 2,7         | 1        | 3                  | Y        |
| P7      | PLASMA | J1   | Imotion | M   | 2,7         | 1        | 3                  | Y        |
| P7      | CSF    | J184 | Imotion | M   | 3,2         | 1        | 3                  | Y        |
| P7      | PLASMA | J184 | Imotion | M   | 3,2         | 1        | 3                  | Y        |
| P8      | CSF    | J1   | Imotion | F   | 1,2         | 1        | 2                  | Y        |
| P8      | PLASMA | J1   | Imotion | F   | 1,2         | 1        | 2                  | Y        |
| P8      | CSF    | J184 | Imotion | F   | 1,9         | 1        | 2                  | Y        |
| P8      | PLASMA | J184 | Imotion | F   | 1,9         | 1        | 2                  | Y        |
| P9      | CSF    | J1   | Imotion | M   | 2,3         | 1        | 3                  | Y        |
| P9      | PLASMA | J1   | Imotion | M   | 2,3         | 1        | 3                  | Y        |
| P9      | PLASMA | J184 | Imotion | M   | 2,8         | 1        | 3                  | Y        |
| P10     | CSF    | J1   | Imotion | F   | 1,5         | 1        | 3                  | Y        |
| P10     | PLASMA | J1   | Imotion | F   | 1,5         | 1        | 3                  | Y        |
| P10     | CSF    | J184 | Imotion | F   | 2,1         | 1        | 3                  | Y        |
| P10     | PLASMA | J184 | Imotion | F   | 2,1         | 1        | 3                  | Y        |
| P11     | CSF    | J1   | Imotion | M   | 0,7         | 1        | 3                  | Y        |
| P11     | PLASMA | J1   | Imotion | M   | 0,7         | 1        | 3                  | Y        |
| P12     | CSF    | J1   | Imotion | M   | 1,7         | 1        | 3                  | Y        |
| P12     | PLASMA | J1   | Imotion | M   | 1,7         | 1        | 3                  | Y        |
| P13     | CSF    | J1   | Imotion | F   | 0,4         | 1        | 3                  | Y        |
| P13     | PLASMA | J1   | Imotion | F   | 0,4         | 1        | 3                  | Y        |
| P13     | CSF    | J184 | Imotion | F   | 1,0         | 1        | 3                  | Y        |

**Table S2:** Samples' characteristics (continued)

| Patient | Matrix | Time | Site    | Sex | Age (years) | SMA type | Number copies SMN2 | Survival |
|---------|--------|------|---------|-----|-------------|----------|--------------------|----------|
| P13     | PLASMA | J184 | Imotion | F   | 1,0         | 1        | 3                  | Y        |
| P14     | CSF    | J1   | Imotion | M   | 2,5         | 1        | 2                  | Y        |
| P14     | PLASMA | J1   | Imotion | M   | 2,5         | 1        | 2                  | Y        |
| P15     | CSF    | J1   | Imotion | F   | 3,5         | 1        | 3                  | Y        |
| P15     | PLASMA | J1   | Imotion | F   | 3,5         | 1        | 3                  | Y        |
| P16     | PLASMA | J1   | Imotion | M   | 0,3         | 1        | 2                  | Y        |
| P17     | PLASMA | J1   | Imotion | M   | 0,3         | 1        | 2                  | Y        |
| P18     | PLASMA | J1   | Imotion | F   | 1,1         | 1        | 3                  | Y        |
| P19     | CSF    | J1   | Imotion | F   | 4,4         | 1        | 3                  | Y        |
| P19     | PLASMA | J1   | Imotion | F   | 4,4         | 1        | 3                  | Y        |
| P19     | CSF    | J184 | Imotion | F   | 4,9         | 1        | 3                  | Y        |
| P19     | PLASMA | J184 | Imotion | F   | 4,9         | 1        | 3                  | Y        |
| P20     | PLASMA | J1   | Imotion | F   | 0,8         | 1        | 2                  | Y        |
| P21     | PLASMA | J1   | Imotion | M   | 6,4         | 1        | 3                  | Y        |
| P22     | PLASMA | J1   | Imotion | F   | 2,1         | 1        | 2                  | Y        |
| P22     | PLASMA | J184 | Imotion | F   | 2,9         | 1        | 2                  | Y        |
| P22     | PLASMA | J184 | Imotion | F   | 2,9         | 1        | 2                  | Y        |
| P23     | PLASMA | J1   | Imotion | F   | 3,0         | 1        | 3                  | Y        |
| P24     | PLASMA | J1   | Imotion | M   | 1,0         | 1        | 3                  | Y        |
| P25     | PLASMA | J1   | Imotion | M   | 0,7         | 1        | 2                  | Y        |
| P26     | PLASMA | J1   | Imotion | M   | 2,2         | 1        | 2                  | Y        |
| P27     | PLASMA | J1   | Imotion | M   | 3,0         | 1        | 3                  | N        |
| P28     | CSF    | J1   | Imotion | F   | 1,6         | 1        | 2                  | Y        |
| P28     | PLASMA | J1   | Imotion | F   | 1,6         | 1        | 2                  | Y        |
| P29     | PLASMA | J1   | Imotion | F   | 0,7         | 1        | 2                  | Y        |
| P30     | PLASMA | J1   | Imotion | F   | 0,4         | 1        | 2                  | Y        |
| P31     | PLASMA | J1   | Imotion | F   | 0,5         | 1        | 2                  | Y        |
| P32     | PLASMA | J1   | Imotion | F   | 2,4         | 1        | 3                  | Y        |
| P33     | CSF    | J1   | Imotion | M   | 4,0         | 1        | 3                  | Y        |
| P33     | PLASMA | J1   | Imotion | M   | 4,0         | 1        | 3                  | Y        |
| P34     | PLASMA | J1   | Imotion | M   | 3,7         | 2        | 3                  | Y        |
| P34     | CSF    | J184 | Imotion | M   | 4,3         | 2        | 3                  | Y        |
| P34     | PLASMA | J184 | Imotion | M   | 4,3         | 2        | 3                  | Y        |
| P35     | PLASMA | J1   | Imotion | M   | 2,2         | 2        | UK                 | Y        |
| P35     | CSF    | J184 | Imotion | M   | 2,7         | 2        | UK                 | Y        |
| P35     | PLASMA | J184 | Imotion | M   | 2,7         | 2        | UK                 | Y        |
| P36     | PLASMA | J1   | Imotion | F   | 5,9         | 2        | 3                  | Y        |
| P36     | CSF    | J184 | Imotion | F   | 6,6         | 2        | 3                  | Y        |

**Table S2:** Samples' characteristics (continued)

| Patient | Matrix | Time | Site    | Sex | Age (years) | SMA type | Number copies SMN2 | Survival |
|---------|--------|------|---------|-----|-------------|----------|--------------------|----------|
| P36     | PLASMA | J184 | Imotion | F   | 6,6         | 2        | 3                  | Y        |
| P37     | CSF    | J1   | Imotion | F   | 3,6         | 2        | UK                 | Y        |
| P37     | PLASMA | J1   | Imotion | F   | 3,6         | 2        | UK                 | Y        |
| P37     | CSF    | J184 | Imotion | F   | 4,2         | 2        | UK                 | Y        |
| P37     | PLASMA | J184 | Imotion | F   | 4,2         | 2        | UK                 | Y        |
| P38     | PLASMA | J1   | Imotion | M   | 3,3         | 2        | 3                  | Y        |
| P38     | CSF    | J184 | Imotion | M   | 3,8         | 2        | 3                  | Y        |
| P39     | PLASMA | J184 | Imotion | M   | 3,8         | 2        | 3                  | Y        |
| P40     | PLASMA | J1   | Imotion | F   | 3,3         | 1        | 2                  | Y        |
| P41     | CSF    | J1   | Imotion | F   | 10,3        | 2        | UK                 | Y        |
| P41     | PLASMA | J1   | Imotion | F   | 10,3        | 2        | UK                 | Y        |
| P42     | CSF    | J1   | Imotion | M   | 1,3         | 2        | 3                  | Y        |
| P42     | PLASMA | J1   | Imotion | M   | 1,3         | 2        | 3                  | Y        |
| P43     | CSF    | J1   | Garches | M   | 9,2         | 2        | 3                  | Y        |
| P43     | PLASMA | J1   | Garches | M   | 9,2         | 2        | 3                  | Y        |
| P43     | CSF    | J184 | Garches | M   | 9,7         | 2        | 3                  | Y        |
| P43     | PLASMA | J184 | Garches | M   | 9,7         | 2        | 3                  | Y        |
| P44     | CSF    | J1   | Garches | M   | 11,7        | 3        | 4                  | Y        |
| P44     | CSF    | J184 | Garches | M   | 12,3        | 3        | 4                  | Y        |
| P44     | PLASMA | J184 | Garches | M   | 12,3        | 3        | 4                  | Y        |
| P45     | CSF    | J1   | Garches | M   | 9,5         | 1        | 2                  | Y        |
| P45     | PLASMA | J1   | Garches | M   | 9,5         | 1        | 2                  | Y        |
| P45     | CSF    | J184 | Garches | M   | 10,0        | 1        | 2                  | Y        |
| P46     | CSF    | J1   | Garches | F   | 8,1         | 2        | 3                  | Y        |
| P46     | PLASMA | J1   | Garches | F   | 8,1         | 2        | 3                  | Y        |
| P46     | CSF    | J184 | Garches | F   | 8,7         | 2        | 3                  | Y        |
| P46     | PLASMA | J184 | Garches | F   | 8,7         | 2        | 3                  | Y        |
| P47     | CSF    | J1   | Garches | F   | 16,8        | 2        | 3                  | Y        |
| P47     | PLASMA | J1   | Garches | F   | 16,8        | 2        | 3                  | Y        |
| P47     | CSF    | J184 | Garches | F   | 17,3        | 2        | 3                  | Y        |
| P48     | CSF    | J1   | Garches | M   | 7,4         | 3        | 3                  | Y        |
| P48     | PLASMA | J1   | Garches | M   | 7,4         | 3        | 3                  | Y        |
| P48     | CSF    | J184 | Garches | M   | 7,9         | 3        | 3                  | Y        |
| P48     | PLASMA | J184 | Garches | M   | 7,9         | 3        | 3                  | Y        |
| P49     | CSF    | J1   | Garches | M   | 4,3         | 2        | UK                 | Y        |
| P49     | PLASMA | J1   | Garches | M   | 4,3         | 2        | UK                 | Y        |
| P49     | CSF    | J184 | Garches | M   | 4,8         | 2        | UK                 | Y        |
| P49     | PLASMA | J184 | Garches | M   | 4,8         | 2        | UK                 | Y        |

**Table S2:** Samples' characteristics (continued)

| Patient | Matrix | Time | Site    | Sex | Age (years) | SMA type | Number copies SMN2 | Survival |
|---------|--------|------|---------|-----|-------------|----------|--------------------|----------|
| P50     | CSF    | J1   | Garches | M   | 7,9         | 2        | UK                 | Y        |
| P50     | PLASMA | J1   | Garches | M   | 7,9         | 2        | UK                 | Y        |
| P50     | CSF    | J184 | Garches | M   | 8,4         | 2        | UK                 | Y        |
| P50     | PLASMA | J184 | Garches | M   | 8,4         | 2        | UK                 | Y        |
| P51     | CSF    | J1   | Garches | M   | 15,0        | 1        | 3                  | Y        |
| P51     | PLASMA | J1   | Garches | M   | 15,0        | 1        | 3                  | Y        |
| P51     | CSF    | J184 | Garches | M   | 15,6        | 1        | 3                  | Y        |
| P51     | PLASMA | J184 | Garches | M   | 15,6        | 1        | 3                  | Y        |
| P52     | CSF    | J1   | Garches | F   | 12,5        | 2        | 3                  | Y        |
| P52     | PLASMA | J1   | Garches | F   | 12,5        | 2        | 3                  | Y        |
| P52     | CSF    | J184 | Garches | F   | 13,0        | 2        | 3                  | Y        |
| P53     | CSF    | J1   | Garches | F   | 13,7        | 2        | 3                  | Y        |
| P53     | CSF    | J184 | Garches | F   | 14,2        | 2        | 3                  | Y        |
| P53     | PLASMA | J184 | Garches | F   | 14,2        | 2        | 3                  | Y        |
| P54     | CSF    | J1   | Garches | F   | 13,0        | 2        | 3                  | Y        |
| P54     | PLASMA | J1   | Garches | F   | 13,0        | 2        | 3                  | Y        |
| P54     | CSF    | J184 | Garches | F   | 13,5        | 2        | 3                  | Y        |
| P55     | CSF    | J1   | Garches | M   | 20,2        | 2        | UK                 | Y        |
| P56     | CSF    | J1   | Garches | M   | 11,6        | 2        | 3                  | Y        |
| P56     | PLASMA | J1   | Garches | M   | 11,6        | 2        | 3                  | Y        |
| P56     | CSF    | J184 | Garches | M   | 12,1        | 2        | 3                  | Y        |
| P56     | PLASMA | J184 | Garches | M   | 12,1        | 2        | 3                  | Y        |
| P57     | PLASMA | J1   | Rouen   | M   | 0,3         | 1        | UK                 | Y        |

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391 **Table S3:** Differentially expressed metabolites.

| clinical_data | matrice | group1  | group2 | Name                      | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|---------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | CSF     | Control | Mild   | Dodecanedioylcarnitine    | Acylcarnitines       | 5,44      | 1,58E-07    |
| SMA_TYPE      | CSF     | Control | Mild   | total DMA                 | Biogenic_amines      | 4,34      | 4,22E-05    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C42:1               | Glycerophospholipids | 4,24      | 6,75E-05    |
| SMA_TYPE      | CSF     | Control | Mild   | Histidine                 | Aminoacids           | 3,90      | 2,92E-04    |
| SMA_TYPE      | CSF     | Control | Mild   | Decadienylcarnitine       | Acylcarnitines       | 3,60      | 4,77E-04    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C42:5               | Glycerophospholipids | 3,70      | 6,46E-04    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C40:4               | Glycerophospholipids | 3,40      | 1,99E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Tiglylcarnitine           | Acylcarnitines       | 3,18      | 2,18E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Hexanoylcarnitine         | Acylcarnitines       | 3,16      | 2,36E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C44:4               | Glycerophospholipids | 3,24      | 3,60E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C30:0               | Glycerophospholipids | 3,02      | 3,82E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Serine                    | Aminoacids           | 3,01      | 3,97E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Spermidine                | Biogenic_amines      | 3,15      | 4,86E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Lysine                    | Aminoacids           | 2,95      | 6,48E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | Methylglutaryl carnitine  | Acylcarnitines       | 2,81      | 7,43E-03    |
| SMA_TYPE      | CSF     | Control | Mild   | lysoPC a C18:1            | Glycerophospholipids | 2,61      | 1,80E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Kynurenine                | Biogenic_amines      | 2,73      | 1,89E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C36:1               | Glycerophospholipids | 2,73      | 1,89E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C26:0               | Glycerophospholipids | 2,46      | 2,06E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C24:0               | Glycerophospholipids | 2,40      | 2,45E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C34:4               | Glycerophospholipids | 2,37      | 2,64E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Taurine                   | Biogenic_amines      | 2,62      | 2,67E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Methionine                | Aminoacids           | 2,61      | 2,70E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C36:3               | Glycerophospholipids | 2,33      | 2,96E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Asparagine                | Aminoacids           | 2,58      | 3,00E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Arginine                  | Aminoacids           | 2,32      | 3,03E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C44:6               | Glycerophospholipids | 2,56      | 3,15E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C38:1               | Glycerophospholipids | 2,55      | 3,21E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C40:1               | Glycerophospholipids | 2,44      | 3,36E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Glutaconylcarnitine       | Acylcarnitines       | 2,27      | 3,50E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Threonine                 | Aminoacids           | 2,51      | 3,62E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Dopamine                  | Biogenic_amines      | 2,23      | 3,88E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC ae C30:1               | Glycerophospholipids | 2,20      | 4,22E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | PC aa C36:0               | Glycerophospholipids | 2,17      | 4,49E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Hydroxyvaleryl carnitine  | Acylcarnitines       | 1,99      | 4,62E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | lysoPC a C16:1            | Glycerophospholipids | 2,41      | 4,72E-02    |
| SMA_TYPE      | CSF     | Control | Mild   | Propionylcarnitine        | Acylcarnitines       | 2,13      | 4,99E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Hydroxypropionylcarnitine | Acylcarnitines       | 4,68      | 8,73E-06    |
| SMA_TYPE      | CSF     | Control | Severe | Hydroxyvaleryl carnitine  | Acylcarnitines       | 4,62      | 1,14E-05    |
| SMA_TYPE      | CSF     | Control | Severe | Methylglutaryl carnitine  | Acylcarnitines       | 4,50      | 2,06E-05    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                         | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|------------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | CSF     | Control | Severe | Serine                       | Aminoacids           | 4,38      | 3,62E-05    |
| SMA_TYPE      | CSF     | Control | Severe | Decadienylcarnitine          | Acylcarnitines       | 4,26      | 6,18E-05    |
| SMA_TYPE      | CSF     | Control | Severe | Butyrylcarnitine             | Acylcarnitines       | 4,07      | 1,38E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Acetylcarnitine              | Acylcarnitines       | 4,01      | 1,85E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Propionylcarnitine           | Acylcarnitines       | 3,94      | 2,40E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Nonaylcarnitine              | Acylcarnitines       | 3,81      | 4,23E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Dodecenoylcarnitine          | Acylcarnitines       | 3,80      | 4,43E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Histidine                    | Aminoacids           | 3,60      | 4,79E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Tiglylcarnitine              | Acylcarnitines       | 3,76      | 5,08E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Dodecanedioylcarnitine       | Acylcarnitines       | 3,45      | 8,37E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Hexanoylcarnitine            | Acylcarnitines       | 3,62      | 8,99E-04    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C42:1                  | Glycerophospholipids | 3,41      | 9,62E-04    |
| SMA_TYPE      | CSF     | Control | Severe | Hexenoylcarnitine            | Acylcarnitines       | 3,58      | 1,05E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Glutaconylcarnitine          | Acylcarnitines       | 3,47      | 1,58E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Glutaryl carnitine           | Acylcarnitines       | 3,44      | 1,72E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Tetradecanoylcarnitine       | Acylcarnitines       | 3,42      | 1,85E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C36:0                  | Glycerophospholipids | 3,40      | 2,05E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C30:0                  | Glycerophospholipids | 3,39      | 2,10E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Hydroxyhexadecenoylcarnitine | Acylcarnitines       | 3,32      | 2,65E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C38:0                  | Glycerophospholipids | 3,28      | 3,09E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Valerylcarnitine             | Acylcarnitines       | 3,28      | 3,15E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Methionine sulfoxide         | Biogenic_ amines     | 3,21      | 3,98E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C44:5                  | Glycerophospholipids | 3,19      | 4,32E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C40:4                  | Glycerophospholipids | 2,85      | 6,47E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Citrulline                   | Aminoacids           | 3,03      | 7,24E-03    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C32:2                  | Glycerophospholipids | 2,96      | 9,11E-03    |
| SMA_TYPE      | CSF     | Control | Severe | Hexadecadienylcarnitine      | Acylcarnitines       | 2,93      | 1,01E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Hydroxyhexadecanoylcarnitine | Acylcarnitines       | 2,93      | 1,03E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C26:0                  | Glycerophospholipids | 2,92      | 1,04E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Arginine                     | Aminoacids           | 2,92      | 1,05E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C44:4                  | Glycerophospholipids | 2,69      | 1,06E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C24:0                  | Glycerophospholipids | 2,90      | 1,12E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C30:0                  | Glycerophospholipids | 2,89      | 1,15E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Phenylalanine                | Aminoacids           | 2,85      | 1,32E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Hydroxyoctadecenoylcarnitine | Acylcarnitines       | 2,85      | 1,33E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C40:6                  | Glycerophospholipids | 2,78      | 1,63E-02    |
| SMA_TYPE      | CSF     | Control | Severe | lysoPC a C18:1               | Glycerophospholipids | 2,51      | 1,80E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C36:0                  | Glycerophospholipids | 2,74      | 1,86E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C34:4                  | Glycerophospholipids | 2,73      | 1,88E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C36:1                  | Glycerophospholipids | 2,48      | 1,98E-02    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                      | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|---------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | CSF     | Control | Severe | Octadecanoylcarnitine     | Acylcarnitines       | 2,71      | 2,05E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C38:4               | Glycerophospholipids | 2,68      | 2,21E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C38:3               | Glycerophospholipids | 2,66      | 2,36E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C28:1               | Glycerophospholipids | 2,59      | 2,87E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C36:3               | Glycerophospholipids | 2,44      | 2,96E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Dopamine                  | Biogenic_amines      | 2,57      | 3,03E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C36:4               | Glycerophospholipids | 2,56      | 3,18E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C42:5               | Glycerophospholipids | 2,29      | 3,28E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C40:1               | Glycerophospholipids | 2,28      | 3,36E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Hexadecenoylcarnitine     | Acylcarnitines       | 2,51      | 3,62E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC aa C38:5               | Glycerophospholipids | 2,48      | 3,90E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Glutamate                 | Aminoacids           | 2,48      | 3,91E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C30:1               | Glycerophospholipids | 2,28      | 4,22E-02    |
| SMA_TYPE      | CSF     | Control | Severe | lysoPC a C26:0            | Glycerophospholipids | 2,45      | 4,31E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Decenoylcarnitine         | Acylcarnitines       | 2,45      | 4,32E-02    |
| SMA_TYPE      | CSF     | Control | Severe | PC ae C42:0               | Glycerophospholipids | 2,45      | 4,34E-02    |
| SMA_TYPE      | CSF     | Control | Severe | Taurine                   | Biogenic_amines      | 2,16      | 4,66E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | Hydroxypropionylcarnitine | Acylcarnitines       | 2,87      | 6,15E-03    |
| SMA_TYPE      | CSF     | Mild    | Severe | Lysine                    | Aminoacids           | 2,85      | 6,48E-03    |
| SMA_TYPE      | CSF     | Mild    | Severe | Butyrylcarnitine          | Acylcarnitines       | 2,66      | 1,18E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | total DMA                 | Biogenic_amines      | 2,58      | 1,48E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | Propenoylcarnitine        | Acylcarnitines       | 2,47      | 4,06E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | Tetradecanoylcarnitine    | Acylcarnitines       | 2,20      | 4,18E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | PC ae C36:4               | Glycerophospholipids | 2,18      | 4,37E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | Hydroxyvaleryl carnitine  | Acylcarnitines       | 2,01      | 4,62E-02    |
| SMA_TYPE      | CSF     | Mild    | Severe | Hexadecenoylcarnitine     | Acylcarnitines       | 2,13      | 4,99E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Creatinine                | Biogenic_amines      | 5,74      | 1,43E-08    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Octadecenoylcarnitine     | Acylcarnitines       | 4,52      | 9,49E-06    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Octadecadienylcarnitine   | Acylcarnitines       | 4,11      | 6,03E-05    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Kynurenine                | Biogenic_amines      | 3,67      | 7,26E-04    |
| SMA_TYPE      | PLASMA  | Control | Mild   | total DMA                 | Biogenic_amines      | 3,65      | 7,92E-04    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Methylglutaryl carnitine  | Acylcarnitines       | 3,40      | 1,00E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Phenylalanine             | Aminoacids           | 3,36      | 1,17E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Glutaryl carnitine        | Acylcarnitines       | 3,09      | 2,96E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Aspartate                 | Aminoacids           | 3,06      | 3,29E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Methionine                | Aminoacids           | 3,25      | 3,43E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Hexadecanoylcarnitine     | Acylcarnitines       | 2,93      | 5,11E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Arginine                  | Aminoacids           | 3,10      | 5,87E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | cis-4-Hydroxyproline      | Biogenic_amines      | 2,83      | 7,06E-03    |
| SMA_TYPE      | PLASMA  | Control | Mild   | trans-4-Hydroxyproline    | Biogenic_amines      | 2,98      | 8,60E-03    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                         | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|------------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | PLASMA  | Control | Mild   | SM (OH) C22:2                | Sphingomyelins       | 2,71      | 1,02E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | SM (OH) C16:1                | Sphingomyelins       | 2,70      | 1,03E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Glycine                      | Aminoacids           | 2,65      | 1,21E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Nitrotyrosine                | Biogenic_amines      | 2,65      | 1,21E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Valerylcarnitine             | Acylcarnitines       | 2,75      | 1,78E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Octadecanoylcarnitine        | Acylcarnitines       | 2,45      | 2,14E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | PC ae C36:2                  | Glycerophospholipids | 2,29      | 3,29E-02    |
| SMA_TYPE      | PLASMA  | Control | Mild   | Leucine                      | Aminoacids           | 2,15      | 4,77E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Creatinine                   | Biogenic_amines      | 7,12      | 3,27E-12    |
| SMA_TYPE      | PLASMA  | Control | Severe | Phenylalanine                | Aminoacids           | 7,06      | 5,11E-12    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hexanoylcarnitine            | Acylcarnitines       | 5,80      | 2,03E-08    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C16:0               | Glycerophospholipids | 5,57      | 7,58E-08    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C20:4               | Glycerophospholipids | 5,47      | 1,36E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Octadecadienylcarnitine      | Acylcarnitines       | 5,46      | 1,40E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C18:1               | Glycerophospholipids | 5,40      | 2,05E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C18:0               | Glycerophospholipids | 5,34      | 2,86E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C20:3               | Glycerophospholipids | 5,30      | 3,39E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Octadecanoylcarnitine        | Acylcarnitines       | 5,26      | 4,42E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Leucine                      | Aminoacids           | 5,21      | 5,74E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Aspartate                    | Aminoacids           | 5,18      | 6,74E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Octadecenoylcarnitine        | Acylcarnitines       | 5,18      | 6,79E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Acetylcarnitine              | Acylcarnitines       | 5,13      | 8,74E-07    |
| SMA_TYPE      | PLASMA  | Control | Severe | Decanoylcarnitine            | Acylcarnitines       | 5,09      | 1,09E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C16:1               | Glycerophospholipids | 5,09      | 1,10E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Isoleucine                   | Aminoacids           | 4,95      | 2,20E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Decadienylcarnitine          | Acylcarnitines       | 4,93      | 2,53E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hexadecadienylcarnitine      | Acylcarnitines       | 4,86      | 3,48E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hexenoylcarnitine            | Acylcarnitines       | 4,77      | 5,56E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C17:0               | Glycerophospholipids | 4,72      | 7,14E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxyoctadecenoylcarnitine | Acylcarnitines       | 4,66      | 9,27E-06    |
| SMA_TYPE      | PLASMA  | Control | Severe | Alanine                      | Aminoacids           | 4,58      | 1,42E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Tetradecadienylcarnitine     | Acylcarnitines       | 4,47      | 2,33E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Tetradecenoylcarnitine       | Acylcarnitines       | 4,46      | 2,51E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C28:0               | Glycerophospholipids | 4,35      | 4,02E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | cis-4-Hydroxyproline         | Biogenic_amines      | 4,32      | 4,60E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Propionylcarnitine           | Acylcarnitines       | 4,27      | 5,89E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Tetradecanoylcarnitine       | Acylcarnitines       | 4,25      | 6,46E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxyhexadecenoylcarnitine | Acylcarnitines       | 4,23      | 6,93E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hexadecenoylcarnitine        | Acylcarnitines       | 4,23      | 6,96E-05    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxyhexadecanoylcarnitine | Acylcarnitines       | 3,90      | 2,88E-04    |



**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                            | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|---------------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxytetradecadienylcarnitine | Acylcarnitines       | 3,89      | 3,03E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxytetradecenoylcarnitine   | Acylcarnitines       | 3,82      | 3,93E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C36:4                     | Glycerophospholipids | 3,72      | 5,98E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | Glutamate                       | Aminoacids           | 3,71      | 6,10E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C24:0                  | Glycerophospholipids | 3,71      | 6,19E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxybutyrylcarnitine         | Acylcarnitines       | 3,53      | 6,19E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | Histidine                       | Aminoacids           | 3,65      | 7,94E-04    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C38:4                     | Glycerophospholipids | 3,37      | 1,13E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C34:3                     | Glycerophospholipids | 3,55      | 1,14E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Proline                         | Aminoacids           | 3,53      | 1,26E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C14:0                  | Glycerophospholipids | 3,48      | 1,48E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C34:2                     | Glycerophospholipids | 3,48      | 1,51E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Decenoylcarnitine               | Acylcarnitines       | 3,26      | 1,66E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxyhexadecadienylcarnitine  | Acylcarnitines       | 3,45      | 1,67E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Glycine                         | Aminoacids           | 3,42      | 1,85E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Lysine                          | Aminoacids           | 3,41      | 1,97E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C26:0                  | Glycerophospholipids | 3,37      | 2,27E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Ornithine                       | Aminoacids           | 3,32      | 2,71E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Dodecenoylcarnitine             | Acylcarnitines       | 3,32      | 2,74E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Butyrylcarnitine                | Acylcarnitines       | 3,11      | 2,77E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Taurine                         | Biogenic_amines      | 3,11      | 2,82E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Nonaylcarnitine                 | Acylcarnitines       | 3,29      | 3,01E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxypropionylcarnitine       | Acylcarnitines       | 3,09      | 3,11E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C40:4                     | Glycerophospholipids | 3,19      | 4,27E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Tiglylcarnitine                 | Acylcarnitines       | 3,19      | 4,30E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Glutaconylcarnitine             | Acylcarnitines       | 3,19      | 4,31E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hexadecanoylcarnitine           | Acylcarnitines       | 3,06      | 5,11E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Propenoylcarnitine              | Acylcarnitines       | 2,89      | 5,71E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C40:4                     | Glycerophospholipids | 3,09      | 5,93E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C28:1                     | Glycerophospholipids | 3,04      | 7,02E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | lysoPC a C18:2                  | Glycerophospholipids | 3,04      | 7,13E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | SM C16:1                        | Sphingomyelins       | 3,03      | 7,40E-03    |
| SMA_TYPE      | PLASMA  | Control | Severe | Methionine                      | Aminoacids           | 2,71      | 1,02E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C32:1                     | Glycerophospholipids | 2,92      | 1,06E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Dodecanedioylcarnitine          | Acylcarnitines       | 2,91      | 1,08E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Nitrotyrosine                   | Biogenic_amines      | 2,79      | 1,21E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Octanoylcarnitine               | Acylcarnitines       | 2,86      | 1,25E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C30:2                     | Glycerophospholipids | 2,85      | 1,31E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C36:3                     | Glycerophospholipids | 2,61      | 1,35E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | trans-4-Hydroxyproline          | Biogenic_amines      | 2,58      | 1,48E-02    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                             | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|----------------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | PLASMA  | Control | Severe | Pimeloylcarnitine                | Acylcarnitines       | 2,74      | 1,52E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Glutaryl carnitine               | Acylcarnitines       | 2,42      | 1,55E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C38:3                      | Glycerophospholipids | 2,55      | 1,60E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Valine                           | Aminoacids           | 2,74      | 1,82E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Hydroxyvaleryl carnitine         | Acylcarnitines       | 2,50      | 1,84E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Glutamine                        | Aminoacids           | 2,71      | 2,01E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Spermine                         | Biogenic_amines      | 2,67      | 2,30E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | SM C24:0                         | Sphingomyelins       | 2,65      | 2,41E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | Citrulline                       | Aminoacids           | 2,65      | 2,44E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC ae C36:5                      | Glycerophospholipids | 2,53      | 3,41E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C38:4                      | Glycerophospholipids | 2,44      | 4,44E-02    |
| SMA_TYPE      | PLASMA  | Control | Severe | PC aa C36:1                      | Glycerophospholipids | 2,40      | 4,97E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Glutaryl carnitine               | Acylcarnitines       | 4,51      | 1,95E-05    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Methylglutaryl carnitine         | Acylcarnitines       | 4,20      | 8,12E-05    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C17:0                   | Glycerophospholipids | 3,94      | 1,20E-04    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C16:1                   | Glycerophospholipids | 3,72      | 2,95E-04    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxybutyryl carnitine         | Acylcarnitines       | 3,86      | 3,41E-04    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxyhexadecanoyl carnitine    | Acylcarnitines       | 3,53      | 6,15E-04    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C16:0                   | Glycerophospholipids | 3,37      | 1,13E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C38:4                      | Glycerophospholipids | 3,37      | 1,13E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hexenoyl carnitine               | Acylcarnitines       | 3,31      | 1,41E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Butyryl carnitine                | Acylcarnitines       | 3,48      | 1,51E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Decenoyl carnitine               | Acylcarnitines       | 3,39      | 1,66E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C20:4                   | Glycerophospholipids | 3,12      | 2,69E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Taurine                          | Biogenic_amines      | 3,20      | 2,82E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Decadienyl carnitine             | Acylcarnitines       | 3,10      | 2,91E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C38:3                      | Glycerophospholipids | 3,29      | 3,02E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxypropionyl carnitine       | Acylcarnitines       | 3,08      | 3,11E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxytetradecadienyl carnitine | Acylcarnitines       | 3,05      | 3,49E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C14:0                   | Glycerophospholipids | 3,01      | 3,86E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C36:4                      | Glycerophospholipids | 2,98      | 4,29E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C34:2                      | Glycerophospholipids | 3,19      | 4,33E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C18:0                   | Glycerophospholipids | 2,97      | 4,51E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C20:3                   | Glycerophospholipids | 2,95      | 4,71E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Decanoyl carnitine               | Acylcarnitines       | 2,95      | 4,77E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Propenoyl carnitine              | Acylcarnitines       | 2,94      | 5,71E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C36:3                      | Glycerophospholipids | 3,04      | 7,07E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hexadecadienyl carnitine         | Acylcarnitines       | 2,81      | 7,50E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxyoctadecenoyl carnitine    | Acylcarnitines       | 2,78      | 8,19E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | SM (OH) C16:1                    | Sphingomyelins       | 2,96      | 9,21E-03    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                         | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|------------------------------|----------------------|-----------|-------------|
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hydroxyvalerylcarnitine      | Acylcarnitines       | 2,95      | 9,55E-03    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Arginine                     | Aminoacids           | 2,71      | 1,01E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | SM (OH) C22:2                | Sphingomyelins       | 2,89      | 1,02E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Tiglylcarnitine              | Acylcarnitines       | 2,61      | 1,34E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Pimeloylcarnitine            | Acylcarnitines       | 2,57      | 1,52E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Propionylcarnitine           | Acylcarnitines       | 2,57      | 1,53E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Hexanoylcarnitine            | Acylcarnitines       | 2,56      | 1,56E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C18:1               | Glycerophospholipids | 2,54      | 1,64E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | SM C16:1                     | Sphingomyelins       | 2,54      | 1,68E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Acetylcarnitine              | Acylcarnitines       | 2,52      | 1,77E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC aa C40:4                  | Glycerophospholipids | 2,47      | 2,05E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | SM C18:1                     | Sphingomyelins       | 2,70      | 2,06E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Tetradecenoylcarnitine       | Acylcarnitines       | 2,45      | 2,13E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Octanoylcarnitine            | Acylcarnitines       | 2,44      | 2,22E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | lysoPC a C18:2               | Glycerophospholipids | 2,44      | 2,22E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C40:4                  | Glycerophospholipids | 2,43      | 2,24E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Alanine                      | Aminoacids           | 2,43      | 2,25E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Kynurenine                   | Biogenic_amines      | 2,32      | 3,01E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Nonaylcarnitine              | Acylcarnitines       | 2,29      | 3,27E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C36:2                  | Glycerophospholipids | 2,49      | 3,29E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | total DMA                    | Biogenic_amines      | 2,22      | 3,98E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | PC ae C34:0                  | Glycerophospholipids | 2,45      | 4,23E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Glutaconylcarnitine          | Acylcarnitines       | 2,19      | 4,24E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Dodecenoylcarnitine          | Acylcarnitines       | 2,15      | 4,68E-02    |
| SMA_TYPE      | PLASMA  | Mild    | Severe | Tetradecadienylcarnitine     | Acylcarnitines       | 2,13      | 4,96E-02    |
| SMN2_COPY     | CSF     | 2       | 3/4    | Tetradecanoylcarnitine       | Acylcarnitines       | 2,49      | 1,91E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Decadienylcarnitine          | Acylcarnitines       | 4,26      | 3,06E-05    |
| SMN2_COPY     | CSF     | Control | 2      | Tetradecanoylcarnitine       | Acylcarnitines       | 3,77      | 4,92E-04    |
| SMN2_COPY     | CSF     | Control | 2      | Methylglutaryl carnitine     | Acylcarnitines       | 3,64      | 8,26E-04    |
| SMN2_COPY     | CSF     | Control | 2      | Hydroxyvalerylcarnitine      | Acylcarnitines       | 3,61      | 9,26E-04    |
| SMN2_COPY     | CSF     | Control | 2      | Serine                       | Aminoacids           | 3,41      | 9,71E-04    |
| SMN2_COPY     | CSF     | Control | 2      | Dodecanedioylcarnitine       | Acylcarnitines       | 3,29      | 1,51E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Citrulline                   | Aminoacids           | 3,39      | 2,13E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Hydroxyhexadecenoylcarnitine | Acylcarnitines       | 3,34      | 2,54E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C30:0                  | Glycerophospholipids | 3,08      | 3,08E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Tiglylcarnitine              | Acylcarnitines       | 2,97      | 4,39E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C42:1                  | Glycerophospholipids | 2,92      | 5,22E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Propionylcarnitine           | Acylcarnitines       | 2,91      | 5,43E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C24:0                  | Glycerophospholipids | 2,90      | 5,58E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Histidine                    | Aminoacids           | 2,86      | 6,32E-03    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                          | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|-------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | CSF     | Control | 2      | Hydroxypropionylcarnitine     | Acylcarnitines       | 3,06      | 6,66E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Hexanoylcarnitine             | Acylcarnitines       | 2,83      | 7,05E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Acetylcarnitine               | Acylcarnitines       | 3,04      | 7,16E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C44:5                   | Glycerophospholipids | 2,81      | 7,34E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C36:4                   | Glycerophospholipids | 3,00      | 7,99E-03    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C26:0                   | Glycerophospholipids | 2,78      | 8,05E-03    |
| SMN2_COPY     | CSF     | Control | 2      | Valerylcarnitine              | Acylcarnitines       | 2,70      | 1,03E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C40:4                   | Glycerophospholipids | 2,61      | 1,38E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Glutaryl carnitine            | Acylcarnitines       | 2,83      | 1,38E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C36:0                   | Glycerophospholipids | 2,53      | 1,70E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Hydroxyhexadecanoylcarnitine  | Acylcarnitines       | 2,72      | 1,95E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Glutaconylcarnitine           | Acylcarnitines       | 2,47      | 2,02E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C42:5                   | Glycerophospholipids | 2,42      | 2,30E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Phenylalanine                 | Aminoacids           | 2,40      | 2,48E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C36:3                   | Glycerophospholipids | 2,39      | 2,55E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Glutamate                     | Aminoacids           | 2,34      | 2,87E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C36:1                   | Glycerophospholipids | 2,33      | 3,01E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C38:3                   | Glycerophospholipids | 2,52      | 3,18E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Hydroxyoctadecenoylcarnitine  | Acylcarnitines       | 2,54      | 3,36E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Tetradecadienylcarnitine      | Acylcarnitines       | 2,36      | 3,44E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC aa C32:2                   | Glycerophospholipids | 2,27      | 3,45E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Hydroxytetradecenoylcarnitine | Acylcarnitines       | 2,50      | 3,70E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Butyrylcarnitine              | Acylcarnitines       | 2,48      | 3,95E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Hexenoylcarnitine             | Acylcarnitines       | 2,21      | 4,06E-02    |
| SMN2_COPY     | CSF     | Control | 2      | PC ae C36:0                   | Glycerophospholipids | 2,17      | 4,53E-02    |
| SMN2_COPY     | CSF     | Control | 2      | Dopamine                      | Biogenic_amines      | 2,16      | 4,59E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Dodecanedioylcarnitine        | Acylcarnitines       | 5,75      | 2,73E-08    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C42:1                   | Glycerophospholipids | 5,46      | 1,41E-07    |
| SMN2_COPY     | CSF     | Control | 3/4    | total DMA                     | Biogenic_amines      | 4,90      | 2,90E-06    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hexanoylcarnitine             | Acylcarnitines       | 4,52      | 1,82E-05    |
| SMN2_COPY     | CSF     | Control | 3/4    | Decadienylcarnitine           | Acylcarnitines       | 4,45      | 2,52E-05    |
| SMN2_COPY     | CSF     | Control | 3/4    | Tiglylcarnitine               | Acylcarnitines       | 4,39      | 3,41E-05    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C36:1                   | Glycerophospholipids | 4,29      | 5,30E-05    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C30:0                   | Glycerophospholipids | 4,20      | 8,16E-05    |
| SMN2_COPY     | CSF     | Control | 3/4    | Serine                        | Aminoacids           | 3,93      | 2,50E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C24:0                   | Glycerophospholipids | 3,88      | 3,08E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | Valerylcarnitine              | Acylcarnitines       | 3,80      | 4,26E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C26:0                   | Glycerophospholipids | 3,79      | 4,57E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | Histidine                     | Aminoacids           | 3,69      | 6,76E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C30:1                   | Glycerophospholipids | 3,68      | 7,06E-04    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                             | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|----------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C36:3                      | Glycerophospholipids | 3,60      | 9,48E-04    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C38:0                      | Glycerophospholipids | 3,59      | 1,01E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C42:5                      | Glycerophospholipids | 3,57      | 1,06E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Glutaconylcarnitine              | Acylcarnitines       | 3,45      | 1,67E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C44:5                      | Glycerophospholipids | 3,43      | 1,80E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Kynurenine                       | Biogenic_amines      | 3,41      | 1,94E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Nitrotyrosine                    | Biogenic_amines      | 3,40      | 1,99E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Methylglutaryl carnitine         | Acylcarnitines       | 3,20      | 2,04E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hydroxyvaleryl carnitine         | Acylcarnitines       | 3,17      | 2,32E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | alpha-Aminoadipic acid           | Biogenic_amines      | 3,31      | 2,77E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Propionyl carnitine              | Acylcarnitines       | 3,29      | 2,98E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C40:4                      | Glycerophospholipids | 3,26      | 3,29E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hexenoyl carnitine               | Acylcarnitines       | 3,26      | 3,32E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C34:4                      | Glycerophospholipids | 3,25      | 3,46E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C42:0                      | Glycerophospholipids | 3,24      | 3,59E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Dopamine                         | Biogenic_amines      | 3,14      | 5,10E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Phenylalanine                    | Aminoacids           | 3,06      | 6,54E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C42:6                      | Glycerophospholipids | 3,05      | 6,81E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hydroxyhexadecenoyl carnitine    | Acylcarnitines       | 2,82      | 7,18E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Taurine                          | Biogenic_amines      | 2,99      | 8,45E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C40:1                      | Glycerophospholipids | 2,94      | 9,82E-03    |
| SMN2_COPY     | CSF     | Control | 3/4    | Spermidine                       | Biogenic_amines      | 2,92      | 1,05E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C36:0                      | Glycerophospholipids | 2,91      | 1,09E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hexadecanoyl carnitine           | Acylcarnitines       | 2,91      | 1,09E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C42:4                      | Glycerophospholipids | 2,84      | 1,36E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Acetyl carnitine                 | Acylcarnitines       | 2,61      | 1,37E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hydroxypropionyl carnitine       | Acylcarnitines       | 2,55      | 1,63E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C40:2                      | Glycerophospholipids | 2,77      | 1,68E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Arginine                         | Aminoacids           | 2,77      | 1,69E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C36:0                      | Glycerophospholipids | 2,75      | 1,70E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | lysoPC a C18:1                   | Glycerophospholipids | 2,76      | 1,71E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Asparagine                       | Aminoacids           | 2,75      | 1,78E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C32:2                      | Glycerophospholipids | 2,69      | 2,16E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Citrulline                       | Aminoacids           | 2,43      | 2,24E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | lysoPC a C26:0                   | Glycerophospholipids | 2,66      | 2,37E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Hydroxytetradecadienyl carnitine | Acylcarnitines       | 2,65      | 2,44E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC ae C40:1                      | Glycerophospholipids | 2,62      | 2,62E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Glutamate                        | Aminoacids           | 2,44      | 2,87E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C38:3                      | Glycerophospholipids | 2,30      | 3,18E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | Tetradecadienyl carnitine        | Acylcarnitines       | 2,27      | 3,44E-02    |

**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                         | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | CSF     | Control | 3/4    | Methionine sulfoxide         | Biogenic_amines      | 2,53      | 3,46E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C38:1                  | Glycerophospholipids | 2,51      | 3,58E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C38:5                  | Glycerophospholipids | 2,48      | 3,90E-02    |
| SMN2_COPY     | CSF     | Control | 3/4    | PC aa C34:3                  | Glycerophospholipids | 2,47      | 4,07E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | PC aa C28:1                  | Glycerophospholipids | 2,76      | 8,71E-03    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | total DMA                    | Biogenic_amines      | 2,57      | 1,53E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Nitrotyrosine                | Biogenic_amines      | 2,45      | 2,13E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Alanine                      | Aminoacids           | 2,15      | 3,19E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Dodecanoylcarnitine          | Acylcarnitines       | 2,27      | 3,45E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Glutaryl carnitine           | Acylcarnitines       | 2,25      | 3,65E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Decenoylcarnitine            | Acylcarnitines       | 2,25      | 3,71E-02    |
| SMN2_COPY     | PLASMA  | 2       | 3/4    | Hydroxyhexadecanoylcarnitine | Acylcarnitines       | 2,21      | 4,05E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Creatinine                   | Biogenic_amines      | 6,03      | 2,40E-09    |
| SMN2_COPY     | PLASMA  | Control | 2      | Phenylalanine                | Aminoacids           | 5,99      | 3,32E-09    |
| SMN2_COPY     | PLASMA  | Control | 2      | Octadecadienyl carnitine     | Acylcarnitines       | 5,13      | 8,54E-07    |
| SMN2_COPY     | PLASMA  | Control | 2      | Aspartate                    | Aminoacids           | 4,84      | 3,84E-06    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C16:0               | Glycerophospholipids | 4,84      | 3,85E-06    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hexanoylcarnitine            | Acylcarnitines       | 4,82      | 4,41E-06    |
| SMN2_COPY     | PLASMA  | Control | 2      | Octadecanoylcarnitine        | Acylcarnitines       | 4,58      | 6,95E-06    |
| SMN2_COPY     | PLASMA  | Control | 2      | Octadecenoylcarnitine        | Acylcarnitines       | 4,50      | 1,00E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C20:3               | Glycerophospholipids | 4,64      | 1,05E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C20:4               | Glycerophospholipids | 4,56      | 1,53E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Acetylcarnitine              | Acylcarnitines       | 4,55      | 1,64E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C18:1               | Glycerophospholipids | 4,55      | 1,64E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C18:0               | Glycerophospholipids | 4,41      | 1,98E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Leucine                      | Aminoacids           | 4,46      | 2,49E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hexenoylcarnitine            | Acylcarnitines       | 4,44      | 2,65E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C16:1               | Glycerophospholipids | 4,43      | 2,82E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Decanoylcarnitine            | Acylcarnitines       | 4,23      | 7,08E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Decadienyl carnitine         | Acylcarnitines       | 4,21      | 7,74E-05    |
| SMN2_COPY     | PLASMA  | Control | 2      | Alanine                      | Aminoacids           | 4,13      | 1,09E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C17:0               | Glycerophospholipids | 4,11      | 1,19E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Isoleucine                   | Aminoacids           | 4,09      | 1,30E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hexadecenoylcarnitine        | Acylcarnitines       | 3,91      | 1,40E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Tetradecenoylcarnitine       | Acylcarnitines       | 3,99      | 1,99E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC aa C28:1                  | Glycerophospholipids | 3,96      | 2,27E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Tetradecanoylcarnitine       | Acylcarnitines       | 3,93      | 2,60E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxyoctadecenoylcarnitine | Acylcarnitines       | 3,89      | 3,03E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Tetradecadienyl carnitine    | Acylcarnitines       | 3,74      | 5,60E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hexadecadienyl carnitine     | Acylcarnitines       | 3,67      | 6,84E-04    |



**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                            | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|---------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | PLASMA  | Control | 2      | Propionylcarnitine              | Acylcarnitines       | 3,66      | 7,38E-04    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxyhexadecanoylcarnitine    | Acylcarnitines       | 3,57      | 1,06E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxyhexadecenoylcarnitine    | Acylcarnitines       | 3,31      | 1,40E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | cis-4-Hydroxyproline            | Biogenic_amines      | 3,22      | 1,90E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxytetradecadienylcarnitine | Acylcarnitines       | 3,40      | 2,00E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Histidine                       | Aminoacids           | 3,38      | 2,19E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Citrulline                      | Aminoacids           | 3,36      | 2,36E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxytetradecenoylcarnitine   | Acylcarnitines       | 3,17      | 2,61E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Glycine                         | Aminoacids           | 3,32      | 2,71E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hexadecanoylcarnitine           | Acylcarnitines       | 3,09      | 3,01E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxybutyrylcarnitine         | Acylcarnitines       | 3,27      | 3,25E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Proline                         | Aminoacids           | 3,08      | 6,29E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Glutamate                       | Aminoacids           | 2,80      | 7,68E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Butyrylcarnitine                | Acylcarnitines       | 2,99      | 8,38E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | Decenoylcarnitine               | Acylcarnitines       | 2,94      | 9,88E-03    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC aa C34:2                     | Glycerophospholipids | 2,70      | 1,04E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Octanoylcarnitine               | Acylcarnitines       | 2,92      | 1,05E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Dodecenoylcarnitine             | Acylcarnitines       | 2,91      | 1,07E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Tiglylcarnitine                 | Acylcarnitines       | 2,90      | 1,13E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Lysine                          | Aminoacids           | 2,89      | 1,16E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C14:0                  | Glycerophospholipids | 2,87      | 1,25E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Pimeloylcarnitine               | Acylcarnitines       | 2,85      | 1,30E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C26:0                  | Glycerophospholipids | 2,62      | 1,32E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC ae C36:4                     | Glycerophospholipids | 2,80      | 1,51E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C28:0                  | Glycerophospholipids | 2,57      | 1,52E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Ornithine                       | Aminoacids           | 2,79      | 1,60E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C18:2                  | Glycerophospholipids | 2,71      | 2,02E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | SM C24:0                        | Sphingomyelins       | 2,44      | 2,22E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Glutaconylcarnitine             | Acylcarnitines       | 2,41      | 2,40E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Nonaylcarnitine                 | Acylcarnitines       | 2,65      | 2,41E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC ae C30:2                     | Glycerophospholipids | 2,54      | 2,46E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Dodecanoylcarnitine             | Acylcarnitines       | 2,64      | 2,50E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC ae C34:3                     | Glycerophospholipids | 2,38      | 2,62E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | SM C16:1                        | Sphingomyelins       | 2,60      | 2,76E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Putrescine                      | Biogenic_amines      | 2,35      | 2,80E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC ae C38:4                     | Glycerophospholipids | 2,60      | 2,81E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Valine                          | Aminoacids           | 2,57      | 3,02E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC aa C40:2                     | Glycerophospholipids | 2,56      | 3,10E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxyhexadecadienylcarnitine  | Acylcarnitines       | 2,31      | 3,17E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxypropionylcarnitine       | Acylcarnitines       | 2,52      | 3,50E-02    |

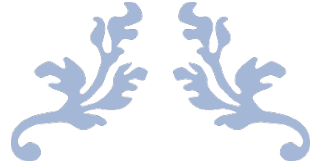
**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                          | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|-------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | PLASMA  | Control | 2      | Hydroxyvalerylcarnitine       | Acylcarnitines       | 2,52      | 3,52E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Propenoylcarnitine            | Acylcarnitines       | 2,51      | 3,57E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Glutaryl carnitine            | Acylcarnitines       | 2,27      | 3,65E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC ae C40:4                   | Glycerophospholipids | 2,24      | 3,78E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | Dodecanedioylcarnitine        | Acylcarnitines       | 2,17      | 4,53E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | lysoPC a C24:0                | Glycerophospholipids | 2,15      | 4,70E-02    |
| SMN2_COPY     | PLASMA  | Control | 2      | PC aa C32:1                   | Glycerophospholipids | 2,41      | 4,81E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Creatinine                    | Biogenic_amines      | 6,93      | 1,28E-11    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Phenylalanine                 | Aminoacids           | 5,98      | 3,32E-09    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Octadecenoylcarnitine         | Acylcarnitines       | 5,80      | 1,99E-08    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | cis-4-Hydroxyproline          | Biogenic_amines      | 5,32      | 3,10E-07    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Octadecanoylcarnitine         | Acylcarnitines       | 5,19      | 6,27E-07    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Octadecadienylcarnitine       | Acylcarnitines       | 4,98      | 9,47E-07    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Nitrotyrosine                 | Biogenic_amines      | 5,04      | 1,37E-06    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C16:0                | Glycerophospholipids | 4,52      | 9,22E-06    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | total DMA                     | Biogenic_amines      | 4,63      | 1,07E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C20:4                | Glycerophospholipids | 4,39      | 1,67E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C18:0                | Glycerophospholipids | 4,36      | 1,98E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hexanoylcarnitine             | Acylcarnitines       | 4,29      | 2,63E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C18:1                | Glycerophospholipids | 4,21      | 3,86E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Leucine                       | Aminoacids           | 4,04      | 8,07E-05    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hexadecenoylcarnitine         | Acylcarnitines       | 3,96      | 1,40E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C20:3                | Glycerophospholipids | 3,85      | 1,76E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hydroxyhexadecenoylcarnitine  | Acylcarnitines       | 4,02      | 1,78E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Acetylcarnitine               | Acylcarnitines       | 3,83      | 1,95E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Decadienylcarnitine           | Acylcarnitines       | 3,82      | 2,02E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C24:0                | Glycerophospholipids | 3,90      | 2,83E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C28:0                | Glycerophospholipids | 3,89      | 2,95E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Isoleucine                    | Aminoacids           | 3,68      | 3,54E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hexadecadienylcarnitine       | Acylcarnitines       | 3,51      | 6,84E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Aspartate                     | Aminoacids           | 3,49      | 7,20E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Propionylcarnitine            | Acylcarnitines       | 3,49      | 7,38E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | trans-4-Hydroxyproline        | Biogenic_amines      | 3,64      | 8,23E-04    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hydroxyoctadecenoylcarnitine  | Acylcarnitines       | 3,37      | 1,14E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Decanoylcarnitine             | Acylcarnitines       | 3,36      | 1,19E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Tetradecadienylcarnitine      | Acylcarnitines       | 3,32      | 1,36E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Methionine                    | Aminoacids           | 3,45      | 1,66E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hexadecanoylcarnitine         | Acylcarnitines       | 3,39      | 2,08E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hydroxytetradecenoylcarnitine | Acylcarnitines       | 3,13      | 2,61E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Tetradecanoylcarnitine        | Acylcarnitines       | 3,08      | 3,06E-03    |



**Table S3:** Differentially expressed metabolites (continued)

| clinical_data | matrice | group1  | group2 | Name                           | Class                | statistic | adj_p_value |
|---------------|---------|---------|--------|--------------------------------|----------------------|-----------|-------------|
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC aa C34:2                    | Glycerophospholipids | 3,27      | 3,22E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hexenoylcarnitine              | Acylcarnitines       | 3,06      | 3,34E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Kynurenine                     | Biogenic_amines      | 3,24      | 3,65E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Glycine                        | Aminoacids           | 3,03      | 3,66E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Glutamate                      | Aminoacids           | 3,16      | 4,66E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C16:1                 | Glycerophospholipids | 2,94      | 4,96E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | SM C24:0                       | Sphingomyelins       | 3,05      | 6,90E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Tetradecenoylcarnitine         | Acylcarnitines       | 2,83      | 6,96E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Citrulline                     | Aminoacids           | 2,75      | 8,87E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Valerylcarnitine               | Acylcarnitines       | 2,94      | 9,80E-03    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Dodecanedioylcarnitine         | Acylcarnitines       | 2,90      | 1,12E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C26:0                 | Glycerophospholipids | 2,81      | 1,32E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C17:0                 | Glycerophospholipids | 2,61      | 1,34E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C44:6                    | Glycerophospholipids | 2,83      | 1,38E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Pimeloylcarnitine              | Acylcarnitines       | 2,54      | 1,66E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC aa C38:4                    | Glycerophospholipids | 2,75      | 1,79E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Lysine                         | Aminoacids           | 2,47      | 2,01E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | lysoPC a C14:0                 | Glycerophospholipids | 2,45      | 2,15E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Glutaconylcarnitine            | Acylcarnitines       | 2,67      | 2,27E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Putrescine                     | Biogenic_amines      | 2,66      | 2,34E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C30:2                    | Glycerophospholipids | 2,40      | 2,46E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C34:3                    | Glycerophospholipids | 2,47      | 2,62E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C40:2                    | Glycerophospholipids | 2,62      | 2,64E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Tiglylcarnitine                | Acylcarnitines       | 2,36      | 2,71E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C36:4                    | Glycerophospholipids | 2,35      | 2,85E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Hydroxyhexadecadienylcarnitine | Acylcarnitines       | 2,46      | 3,17E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Alanine                        | Aminoacids           | 2,25      | 3,19E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Nonacylcarnitine               | Acylcarnitines       | 2,29      | 3,30E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC aa C40:4                    | Glycerophospholipids | 2,51      | 3,60E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC aa C32:0                    | Glycerophospholipids | 2,50      | 3,68E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C40:4                    | Glycerophospholipids | 2,33      | 3,78E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | PC ae C36:5                    | Glycerophospholipids | 2,43      | 4,56E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Glutamine                      | Aminoacids           | 2,40      | 4,85E-02    |
| SMN2_COPY     | PLASMA  | Control | 3/4    | Arginine                       | Aminoacids           | 2,40      | 4,91E-02    |



## *CONCLUSION & PERSPECTIVES*



Omics studies are revolutionizing research in the health field by enhancing the power of discovery and providing deeper understanding with a more holistic view. This new field is moving very quickly, opening our eyes to discoveries and making us change our clinical practice to adopt a more personalized medicine in the omics era.

Patients want to know whether they will be cured of the disease, whether the disease will worsen and whether they would benefit from any therapy, and what to expect with each treatment suggested. Answering these questions is extremely difficult, and physicians face this daily challenge.

Multiomics uses multiple approaches by layering various omics on top of each other for a multidimensional view of human biology to understand a single problem. With this holistic approach, novel insights are uncovered, and we are witnessing a revolution in research. The adoption of multiomics assays includes the understanding that cells are very closely connected, even though they may differ. Using -omics studies enable the discovery of biomarkers and novel pathways. With each omics study, we look at the same problem from a different angle, and with multiomics, we can build a multidimensional picture that mimics the setup in real life. With these sciences, we go from large scale to depth. Therefore, these studies are the essential key to precision medicine. Omics studies in neuromuscular disorders are still poorly explored. They help identify novel biomarkers and new drug targets and improve understanding of responses to current therapies. They give us clues to choose the most adapted therapy regimen for each patient and the diagnosis earlier, which helps in early management. To date, strategies to reduce mortality and morbidity associated with pediatric neuromuscular disease have focused primarily on tertiary interventions, initiated more often after a diagnosis to improve the symptom burden for the child and family. At the dawn of a new era of patient-centered precision medicine, the advent of new omics technologies, drug discovery, and biomarker development offer unprecedented opportunities to challenge and fundamentally alter the traditional approach to these diseases. As a result, primary strategies aimed at reducing the risk of disease onset, and secondary strategies emphasizing early detection of disease and early therapeutic intervention, are rapidly emerging as new models of care that have the potential to alter the course of the pediatric neuromuscular disease at the individual and epidemiologic levels.

Although there are many pediatric neuromuscular diseases, this thesis focused on the two most common neuromuscular diseases: SMA and DMD.

### ***Duchenne Muscular Dystrophy***

For DMD, many potentially interesting biomarkers that reflect the primary feature of myonecrosis, the associated inflammation, and oxidative stress, as well as secondary disturbances such as fibrosis, have been demonstrated in muscle tissue samples and biological fluids, mainly blood and urine. However, very few are used in clinical routine. For example, a widely used classical clinical blood biomarker for DMD is the enzyme creatine kinase (CK), which is elevated in patients and rodent and dog models of DMD but can be highly variable. Identifying biomarkers in biological fluids that reflect primary events of myonecrosis, oxidative stress, or inflammation requires an accurate assessment of necrosis in muscle. Although the specific events that trigger sarcolemmal injury and subsequent myonecrosis in DMD are not fully understood, membrane disruption, increased intracellular calcium, inflammation, and oxidative stress are strongly implicated and are closely linked. Myonecrosis causes histological changes that can be quantified; however, the underlying molecular events are not fully understood. Dystrophic muscles secrete several molecule types into the bloodstream.

Although it is easy to obtain human blood and urine samples for analysis, tracking changes in the muscles could be informative, as this tissue is the site of structural and metabolic alterations in Duchenne dystrophy. In this context, we have explored muscle biopsies from 9 DMD patients and 9 control individuals using ultra-high resolution mass spectrometry metabolomic imaging. Clinical metrics et genetic data were collected and integrated using systems biology tools.

Differential metabolic patterns of muscle biopsies of patients were detected with significant changes in 34 key metabolites. The profound metabolic alterations in DMD biopsies are mainly related to phospholipid and energy metabolism. Phospholipids are major components of cell membranes, and glycerophospholipids are the most abundant membrane phospholipids. Among these glycerophospholipids, phosphocholine is the most abundant in mammalian cells. In our study, several metabolites belonging to phospholipid and

triacylglycerol are increased except glycerophosphocholine, which is decreased. This metabolic remodeling could be related to an alteration of the enzyme lysophospholipase, which catalyzes glycerophosphocholine production. A putative decrease in this enzyme would lead to the accumulation of the upstream components in phospholipids and triacylglycerol metabolism and the reduction of glycerophosphocholine levels. Phospholipid alterations and the dysregulation of  $\text{Ca}^{2+}$  homeostasis reported in DMD may induce mitochondrial membrane fragility with subsequent energy and oxidative metabolism disruptions. Accordingly, we showed a significant increase in ADP and decreased ATP levels in DMD muscles compared to control powers. These alterations in energy metabolism could be multifactorial. Indeed, dystrophin deficiency may induce mitochondrial alteration as a partial restoration of dystrophin expression improves mitochondrial functions [370] .

### ***Spinal Muscular Atrophy***

SMA is caused by a decreased level of functional SMN protein due to alteration in the *SMN* gene. The severity of SMA varies along a spectrum and is modulated by the copy number of the *SMN2* paralog gene in humans.

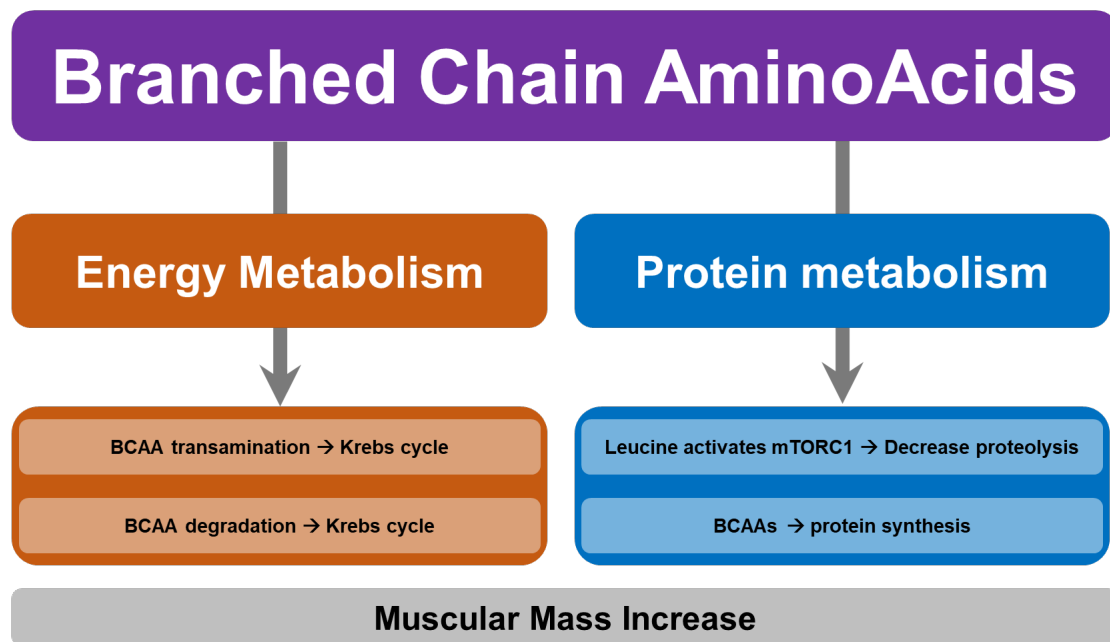
The availability of specific therapies for SMA makes it mandatory to identify biomarkers that reflect the clinical course. *SMN2* copy number is inversely correlated with disease severity and predicts the disease burden. *SMN* mRNA and SMN protein can be measured in the blood or CSF of patients receiving specific treatments and may reflect their efficacy. Neurofilaments are robust disease progression markers for infants with SMA on therapy but not for adolescents and adults. It is not yet known which proteins released by motor neurons (NfL, Nf-H, or others) will be more sensitive in detecting the early stages of degeneration. These proteins could benefit the presymptomatic patient, helping to guide therapeutic decisions. Further evaluation of NFs in SMA populations and assessment of potential correlations with efficacy outcomes is needed.

In this context, developing a battery of biomarkers seems crucial to better answer questions regarding safety and optimal modality or even discontinuation of therapeutic intervention. This project aimed to identify differential metabolic patterns in naïve SMA (J0) patients after six months (J184) of Nusinersen treatment. Two omics approaches (metabolomics and proteomics) were planned on 53 plasma J0, 24 plasma (J184), 74 plasma controls, 34 CSF J0,

24 CSF 184, and 52 CSF controls. Clinical metrics and genetic data were collected and integrated using systems biology tools. In this thesis, metabolomics results are reported.

Profound metabolic remodeling in blood and CSF of naïve SMA patients is observed. We noted an increase in the concentration of several acylcarnitines (plasma and CSF) in SMA patients compared to the control. This alteration in fatty acid metabolism may be linked to a decrease in the beta-oxidation flux. This metabolic deficiency may be related to denervation, which leads to muscle degeneration and mitochondrial alterations with a consequent defect in the beta-oxidation of fatty acids. On the other hand, the concentrations of the assessed amino acids (all except for Glutamine and Citrulline) and of most of the biogenic amines (Dopamine, Kynurenine, nitrotyrosine, hydroxyproline, creatinine) are decreased, which could suggest a decrease in the flux of proteins related to the decreased muscular mass. Patients with SMA are known to have glucose tolerance impairment with hypoglycemia upon fasting and a reduction in the concentration of several amino acids such as alanine, phenylalanine, Branched Chain Amino Acids (Leucine, Isoleucine, and Valine – BCAAs [371, 372]. BCAAs represent a metabolic hub for building and maintaining muscle mass and for anabolism processes in the whole body. Several metabolic pathways are involved in this process (i) BCAAs are substrates for protein synthesis, (ii) leucine is a crucial activator of mTORC1 and promotes anabolism, (iii) BCAA modulates insulin and glucagon secretion [373] (iv) BCAAs contribute to energy metabolism by providing several intermediates of the Krebs cycle (**Figure 10**). Therefore, any alteration in the BCAA level induces mTORC1 deregulation. Subsequently, proteolysis activation leads to muscle wasting and the absence of muscle protection. Another consequence of the decreased concentration of amino acids (especially BCAAs and Glutamate) is the alteration of the Krebs cycle, which consequently yields a decrease in the function of the respiratory chain and mitochondrial dysfunction. Notably, the BCAA level controls the KLF15 (Krüppel-like factor 15) expression. KLF15 is a transcription factor and a key regulator of metabolism highly expressed in the muscles. Besides, KLF15 and BCAAs are modulated by glucocorticoid (GC) levels, which are subjected to the circadian rhythm. Thus, The GC-KLF15-BCAA pathway may be a therapeutic target in SMA patients. Preclinical studies with supplementation of BCAAs or glucocorticoids or targeting overexpression of KLF15 genetically lead to improvement in SMA mice phenotype [374]. These encouraging results pave the way for therapies targeting altered metabolic pathways, which could be given in

conjunction with specific treatments. Of note, KLF15 expression is dramatically decreased in DMD. Thus, the implication of KLF15 in the pathogenesis of DMD could explain the improvement of the phenotype upon administration of glucocorticoids as a result of GC-KLF15-BCAA signaling pathway activation [375].



**Figure 10:** BCAA roles in skeletal muscle

Abnormalities in deacylation-reacylation of phospholipids have been associated with several neurological disorders [376-378]. In our study, the concentrations of phosphatidylcholine species are mainly increased in CSF, while those of lysophosphatidylcholine are mainly decreased in plasma. Glycerophospholipids, including phosphatidylcholines (PCs), have a structural role and are major components of cell membranes while their deacylated forms (lysoPLs) are bioactive lipids involved in signaling through their interaction with G protein coupled receptors. Phospholipase A2 is responsible for the deacylation of PC into LysoPC [376]. Thus, our results could be consistent with a decreased activity of phospholipase A2 in SMA patients. To confirm this hypothesis, we are aiming to assess phospholipase A2 activity in CSF according to the method used by Fonteh et al. [379].

Our findings confirm those previously described in the literature where alteration of fatty acid metabolism, glucose tolerance impairment, and mitochondrial defects was documented.

Another interesting result is the correlation between metabolomic profiles and clinical severity and the SMN2 copy number: a higher number of differentially expressed metabolites is observed when the phenotype is more severe (SMA type 2 and 3) and inversely correlated to the SMN2 copy number. However, no changes were observed after six months of treatment (day 184) with Nusinersen. This could be due to the short duration of therapy, as the patients received only the loading dose, which could be insufficient to induce a modulation in metabolic pathways. Clinically, the treatment efficacy is correlated to the disease severity and the time delay between the initiation of treatment and the onset of symptoms (onset of motor neuron degeneration). Treatment is considered helpful if it stops disease progression, in other words, if it helps protect the motor neurons that were not yet altered by the disease or if it helps restore motor neurons which yield to gaining motor function and prevents regression in motor milestones. Some cases help in reaching new milestones. Our findings at 6 months are coherent with the clinical practice where the poor response to treatment is yet seen at six months. The omics investigations should be done after a more extended period of treatment by Nusinersen to explore the metabolic alterations. We also suggest performing the same studies using the plasma of patients treated by gene therapy and Risdiplam, which could be helpful in the choice of the appropriate treatment for every patient.

Scientists have been pursuing deeper insights into disease and therapies. We can store and mine data that was inconceivable a few years ago in scope, breadth, and depth. The challenge is to work effectively with the data lakes needed to paint a clear picture of how disease occurs and how we can stop it.

Omics studies can also help in clinical trial design. We can target the patients who most likely suffer from the disease's adverse outcomes. In that case, we can reduce the number of patients involved in the trial, reduce the trial duration, and increase the probability of success. Neuromuscular disorders are heterogenous, and the diagnosis is still challenging despite genetic advances. Consequently, adequate management and treatment still need to be improved. Understanding diseases will not be possible without research, and valuable data in clinical research will not be likely without the implication of physicians. Therefore, joint efforts between researchers and clinicians are mandatory to develop precision medicine and look at the individual level rather than an average level. With the adoption of precision medicine, we can diagnose and treat patients more accurately. This approach is valuable in all domains and,



more precisely, in the neuromuscular diseases field, where innovative treatments are being produced for some diseases. With innovative treatments and appropriate management, we are witnessing changes in the natural history of rare neuromuscular diseases. Omics approaches may enable understanding the new phenotypes and guide their follow-up. In conclusion, the metabolic signatures unveiled in this work attest that these diseases are primarily disorders of the motor unit, but they are also systemic. This observation should be considered for keeping a global management of these patients.

## References

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## Explorations Multi-omiques dans les maladies neuromusculaires

### Résumé

Les maladies neuromusculaires pédiatriques sont relatives à des atteintes du muscle, de la jonction neuromusculaire, du nerf périphérique et de la cellule de la corne antérieure. La multitude de sites, ainsi que l'hétérogénéité et la rareté de ces troubles, rendent leur diagnostic et leur traitement difficiles. Par conséquent, les efforts conjoints des chercheurs et des cliniciens sont nécessaires pour diagnostiquer et traiter les patients avec plus de précision en utilisant des outils de la biologie des systèmes. Cette approche permet d'analyser différents types de biomolécules et permet, ainsi, d'étudier de manière exhaustive les changements moléculaires en tenant compte des caractéristiques individuelles du patient (contexte clinique, environnement...). Il y a actuellement une recherche très active dans le domaine des maladies neuromusculaires qui a abouti à la mise à disposition de traitements innovants nécessitant une meilleure identification et stratification des patients pour améliorer la prise en charge. Les objectifs de cette thèse sont d'appliquer les techniques omiques afin d'étudier les deux maladies neuromusculaires les plus courantes (l'amyotrophie spinale infantile (SMA) et la myopathie de Duchenne), d'explorer leurs mécanismes physiopathologiques, d'identifier des biomarqueurs de diagnostic potentiels et de mieux stratifier les patients. Le second objectif est d'identifier des biomarqueurs pour suivre la réponse au traitement dans la SMA. Une étude métabolomique sur des biopsies musculaires de patients atteints de la maladie de Duchenne a révélé des changements significatifs dans les métabolismes énergétique et phospholipidique. Par ailleurs, l'étude métabolomique des plasma et LCR de patients SMA a confirmé la présence d'un remodelage métabolique profond chez les patients SMA. De plus, nous avons démontré que ces altérations sont plus prononcées dans le phénotype sévère. L'étude protéomique de ces échantillons est en cours. En conclusion, les outils de biologie des systèmes sont pertinents pour l'étude des maladies neuromusculaires car ils permettent la découverte de biomarqueurs, la compréhension de la physiopathologie et l'identification de nouvelles cibles thérapeutiques.

**Mots clés:** maladies neuromusculaires, omiques, médecine de précision, biologie des systèmes, métabolomique, protéomique, classification, biomarqueurs, amyotrophie spinale infantile, ASI, myopathie de Duchenne, DMD.

## Multiomics in neuromuscular disorders

### Abstract

Pediatric neuromuscular disorders encompass all childhood onset diseases affecting the muscle, the neuromuscular junction, peripheral nerve, and the anterior horn cell. The multitude of sites as well as the heterogeneity and rarity of these disorders, make their diagnosis and treatment challenging. Therefore, joint efforts of researchers and clinicians are mandatory to diagnose and treat patients more accurately using integrative systems biology tools. Such appealing approach uses high-throughput technologies to comprehensively study multilayered molecular changes. It is valuable in the neuromuscular diseases field where innovative treatments are being produced. The objectives of this thesis were to apply omics techniques to study the two most common neuromuscular disorders (Spinal muscular atrophy and Duchenne muscular dystrophy) and explore their pathophysiological mechanisms, identify potential diagnosis biomarkers, and better stratify patients. The second objective was to find biomarkers for response to treatment in SMA. Metabolomics study on muscle biopsies of Duchenne patients revealed significant changes in energy and phospholipid metabolisms. SMA metabolic profiling investigations in plasma and CSF confirmed the presence of profound metabolic remodeling in SMA patients. Moreover, we demonstrated that these alterations are more pronounced in the severe phenotype. SMA proteomics studies are ongoing. In conclusion, systems biology tools are powerful for the study of neuromuscular diseases as they allow the discovery of biomarkers, the understanding of the physiopathology and the identification of new therapeutic targets.

**Key Words:** Neuromuscular disorders, omics, precision medicine, systems biology, metabolomics, proteomics, classification, biomarkers, spinal muscular atrophy, SMA, Duchenne muscular dystrophy, DMD.