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# Evaluating next-generation sequencing in neuromuscular diseases with neonatal respiratory distress

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## ABSTRACT

With the exception of infantile spinal muscular atrophy (SMA) and congenital myotonic dystrophy 1 (DM1), congenital myopathies and muscular dystrophies with neonatal respiratory distress pose diagnostic challenges. Next-generation sequencing (NGS) provides hope for the diagnosis of these rare diseases.

We evaluated the efficiency of next-generation sequencing (NGS) in ventilated newborns with peripheral hypotonia. We compared the results of our previous study in a cohort of 19 patients analysed by Sanger sequencing from 2007 to 2012, with a diagnostic yield of 26% (5/19), and those of a new retrospective study in 28 patients from 2007 to 2018 diagnosed using MyoPanel, a neuromuscular disease panel, with a diagnostic yield of 43% (12/28 patients). Pathogenic variants were found in five genes: *ACTA1* (n = 4 patients), *RYR1* (n = 2), *CACNA1S* (n = 1), *NEB* (n = 3), and *MTM1* (n = 2).

MyoPanel increased the diagnosis of congenital neuromuscular diseases, but more than half the patients remained undiagnosed. Whole exome sequencing did not seem to fully respond to this diagnostic limitation. Therefore, explorations with whole genome sequencing will be the next step.

## Keywords:

Congenital myopathy  
Congenital muscular dystrophy  
Neuromuscular diseases  
Neonatal respiratory distress  
Next-generation sequencing

## 1. Introduction

In the neonatal period, respiratory distress associated with peripheral hypotonia may be an early sign of neuromuscular disease [1]. With the exception of infantile spinal muscular atrophy (SMA) and congenital myotonic dystrophy 1 (DM1), congenital myopathies and congenital muscular dystrophies constitute the majority

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<sup>4</sup> These authors contributed to data collection and scientific expertise.

of these diseases, with the involvement of more than 100 known mutated genes [1–7], some of which are common to both groups, such as the *RYR1* and *SEPN1* genes [5,8,9]. This clinical presentation may also be indicative of early onset neuromuscular junction pathologies or congenital myasthenic syndromes [10–12]. Differential diagnoses predominantly rely on the exclusion of metabolic myopathies, congenital myotonic dystrophies, congenital neuropathies, SMA and Prader-Willi syndrome (PWS) [13,14].

Currently, the correlation between the severity of the neonatal impairment and the subsequent functional prognoses or predictors of survival [15], cardiorespiratory injury, or risk of developing malignant hyperthermia during anaesthesia, is not already obvious. An accurate early diagnostic tool is thus needed [16,17] to establish the prognosis, implement preventive therapeutic strategies [18], and initiate the commensurate genetic counselling and support.

The clinical phenotype of neonatal neuromuscular diseases is variable and nonspecific [16]. Until the advent of high-throughput gene sequencing techniques, the diagnostic approach consisted of collecting a set of clinical, biochemical, electrophysiological, anatomopathological, cerebral, medullary and muscular iconographic data and performing molecular analyses of individual candidate genes by Sanger sequencing [1,19,20]. However, this approach was often arduous, incomplete, time and cost consuming, except in cases of SMA and DM1.

In recent years, the development of high-throughput sequencing techniques has improved diagnostic efficiency and cost-effectiveness. Next-generation sequencing (NGS) can examine several genes or genomic regions simultaneously. This process is faster, less expensive, and more sensitive and specific than conventional gene-by-gene Sanger sequencing. It encompasses several different approaches depending on the size of the genome to be sequenced, with gene panel sequencing (GPS) for specific genes known to be implicated in a given type of pathology or symptoms, whole exome sequencing (WES) for all coding regions (exons), and whole genome sequencing (WGS) for the entire genome, which includes all coding and non-coding regions. RNA sequencing (RNAseq) is the study of the transcriptome to detect promoter or splicing errors [21,22]. Until recently, WGS and RNAseq were deemed too expensive and were therefore rarely used in routine clinical practice, so there is precious little available data concerning molecular diagnosis using these techniques [16]. According to the literature, GPS [23–40] and WES [36,41,42] offer a diagnostic yield of between 15 and 83% and between 29 and 67% respectively, for neuromuscular diseases. For WES this increases to 73% in consanguineous cases [43]. GPS, with a sequencing coverage of 94–99% vs. 80–85% for WES, presents several advantages: greater diagnostic efficiency, faster results and lower cost [30]. In their studies, Biancalana and Laporte reported that the cost for GPS of about 150 genes is usually equivalent to the cost for analysing 3–4 genes of medium size using Sanger sequencing [44]. Schofield et al., after an economic analysis on 56 patients suspected of having nemaline myopathy or congenital muscular dystrophy, concluded that their neuromuscular disease panel (464 genes) cost less than a quarter of the cost of the traditional diagnostic investigations pathway [30]. In addition, a trio analysis of the patient and both parents increases GPS efficiency by 10–20% compared to an isolated analysis of the patient alone. Analysing both parents helps to differentiate *de novo* from inherited pathogenic variants and also eliminates non-pathogenic genetic polymorphisms [9,42].

We previously carried out a retrospective study of diagnostic strategy by Sanger sequencing in 19 children with neuromuscular disease with neonatal respiratory involvement (excluding DM1 and SMA) who were treated in the neonatal intensive care units of the France Southwest Reference Centres for Neuromuscular Diseases (Bordeaux-Montpellier-Toulouse) between 2007 and 2012 [1].

Diagnoses were made in five out of 19 cases, for a diagnostic yield of 26% (95% CI [13;57]). The diagnoses were: distal spinal muscular atrophy type 1 with respiratory distress (*SMARD1*) (n = 1 patient), congenital myotubular myopathy (*MTM*) (n = 1), congenital myasthenic syndromes (*CHAT*, *RAPSN*) (n = 2), and nemaline myopathy NEM3 (*ACTA1*) (n = 1).

The main objective of the current study was to evaluate the contribution of NGS to the diagnosis of congenital neuromuscular diseases with neonatal respiratory failure, using GPS targeted on the genes known to be responsible for congenital myopathies, congenital muscular dystrophies and several congenital myasthenic syndromes (MyoPanel; [2]).

## 2. Patients and methods

### 2.1. Patients

We carried out a retrospective multicentre diagnostic study on children hospitalised during the first month of life in the Neuro-paediatric, Neonatology and Resuscitation Departments of the three paediatric facilities of the University Hospital Reference Centres in Bordeaux, Montpellier and Toulouse, between January 2007 and December 2018.

Inclusion criteria were the presence of peripheral hypotonia and/or arthrogryposis and the absence of neurological central signs in children who experienced respiratory distress during the first month of life. Exclusion criteria were the detection of *SMA*, *DM1* and *PWS* variants and no performance of congenital myopathy or muscular dystrophy panels.

### 2.2. Clinical assessment

Extensive patient details were collected during the first examination by a neuropaediatrician or a paediatric intensivist; this included the patient's age, reasons for hospitalisation and neurologic, muscular, respiratory, nutritional and orthopaedic features. Early disease onset was defined by the onset of symptoms of respiratory distress or muscular weakness before one month of age.

For children who died during the course of the study, we reported the date of death and specified whether a multidisciplinary palliative care meeting had been held prior to death.

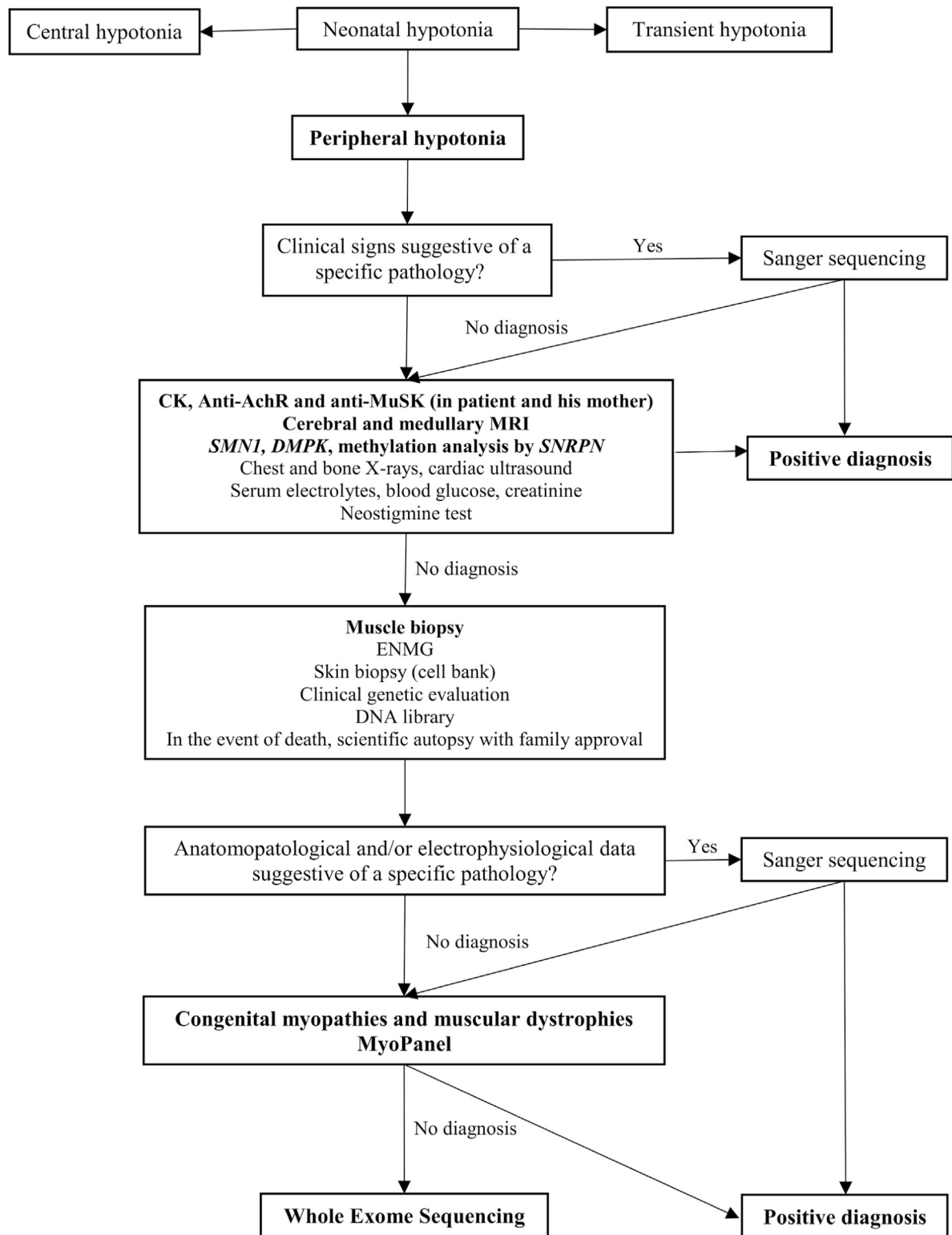
### 2.3. Paraclinical investigations

A unique paraclinical examination protocol was implemented to investigate the aetiology of neonatal hypotonia according to previously reported recommendations [1] presented in Fig. 1. This comprised, as a bare minimum, creatine kinase (CK) assay, anti-acetylcholine receptor antibody and anti-MuSK antibody studies coupled with a pyridostigmine assay, and electrophysiology studies that included electroencephalography (EEG) and electro-neuromyography (ENMG), cerebral and medullary MRI, muscle biopsy, pangenomic CGH array, echocardiography, and chest and whole-skeleton X-rays.

Nerve, muscle and skin biopsies were fixed, paraffin-embedded, and sectioned with a microtome. Samples were analysed with an optical microscope and depicted by the local pathologist, sometimes after histoenzymological, immunohistochemical and ultra-structural study. Several patient biopsies required analysis by electron microscopy.

### 2.4. Genetics

The patients benefited from several myopathy and muscular dystrophy gene panels. One panel was composed of 18 genes



**Fig. 1.** Investigation protocol for patients with neonatal hypotonia suspected of being of neuromuscular origin.

CK: creatine kinase; anti-AchR: antibodies against acetylcholine receptor; anti-MuSK: antibodies against muscular tyrosine kinase receptor; MRI: magnetic resonance imaging; SMN1: survival motoneuron; DMPK: myotonic dystrophy protein kinase; DNA: deoxyribonucleic acid library; ENMG: electroneuromyogram.

(n = 1) (*ACTA1*, *BIN1*, *CACNA1S*, *CCDC78*, *CFL2*, *DNM2*, *KLHL40*, *KLHL41*, *KBTBD13*, *MTM1*, *MYH7*, *NEB*, *RYR1*, *SEPN1*, *STAC3*, *TNNT1*, *TPM2*, *TPM3*). Another panel, previously reported [2], was composed of 135 genes (n = 12) and was secondarily increased to 185 genes (n = 15) including 95 genes in which pathogenic variants

have been reported in neonatal hypotonia (Neonatal hypotonia *in silico* panel, supplementary data panel NGS). Parents were also analysed by GPS when samples were available.

Coverage at a sequencing depth of  $\geq 50X$  was more than 98.5%. Bioinformatic analyses of the fastq files generated by Illumina

sequencing, for single nucleotide variant (SNV) and copy number variant (CNV) detection, were performed using two independent pipelines, as previously described [2]. Variant pathogenicity was evaluated using criteria commonly used according to the American College of Medical Genetics and Genomics (ACMG) guidelines [45], as reported in Zenagui et al. in 2018 [2]. Variants were expected to be present in less than 1% of general population databases such as the Exome Aggregation Consortium (EXAC) and the Genome Aggregation Database (GnomAD). The prioritisation score using the MoBiDiC prioritisation algorithm (MPA; a 0-to-10 prioritisation score algorithm based on 10 pathogenicity prediction tools) was used to estimate the probability of pathogenicity [46].

Conventional Sanger sequencing was used to confirm candidate variants in patients and for familial segregation analyses [2].

### 2.5. Evaluated parameters

The primary endpoint was the MyoPanel genetic diagnostic yield for the total 2007–2018 cohort and for each of the two cohorts (i.e. 2007–2012 and 2013–2018). Secondary endpoints were the correlation between the clinical data and genotype, the muscle biopsy result and a positive genetic diagnosis, and the gene-phenotype profile of the identified genes. Diagnostic yield was the proportion of patients diagnosed by the tested technique among all cohort patients. Diagnostic gain was the proportion of additional patients diagnosed by the NGS technique compared to the Sanger technique in the same cohort.

### 2.6. Statistical analysis

Qualitative variables were expressed as percentages with 95% confidence interval (95% CI). Data analysis was performed using Stata 14.2 software (Stata Corp., College Station, TX).

### 2.7. Ethics approval

The study was approved by the institutional ethics committee according to the guidelines issued by our institutions for clinical studies in compliance with the Helsinki Declaration (MR004: 2206723 v 0). Parents or legal guardians gave informed consent for the genetic analysis in accordance with the appropriate French legislation.

## 3. Results

### 3.1. Population

A total of 28 children from 26 different families were included in the current study, eight from the initial 2007–2012 cohort and 20 from the 2013–2018 cohort. The patients included in this study were extracted from the first cohort initially analysed between 2007 and 2012 ( $n = 19$ ) completed with a new cohort of patients analysed between 2013 and 2018 ( $n = 22$ ). From the initial 2007–2012 cohort of 19 eligible patients, six were diagnosed by Sanger sequencing, five patients died before NGS sampling, and the remaining eight patients (from 8 families) without molecular diagnosis were included in the present study. From the 2013–2018 cohort of 21 eligible patients, two patients were excluded: one patient had a muscle biopsy that oriented the diagnosis towards merosin deficiency and led to the confirmation of a *LAMA2* pathogenic variant by Sanger sequencing, and the other patient, with neuropathic symptoms, was not screened against a MyoPanel but a neuropathic gene panel. Thus, 20 patients from 19 families were included in the study. One patient from the 2007–2012 cohort and one patient from the 2013–2018 cohort belong to the same family (Fig. 2).

Clinical features of the total cohort are summarised in Table 1. One third of the patients had a family history of neuromuscular disease. One half exhibited antenatal symptoms such as polyhydramnios and/or decreased foetal movements. Baseline clinical data indicated that 43% exhibited areflexia or arousal described as anomalous; joint retractions or arthrogryposis were present in 79% of the patients, associated with amimia in 76%; and facial dysmorphism was identified in 36%.

In terms of disease progression, 16 patients, i.e. 57% of the cohort, died half within the first month of life, 31% between 1 and 3 months and 18% between 3 months and 3 years. Of the 11 survivors (39% of the cohort), 63% learned to walk between 18 months and 3 years of age, although only two patients were younger than 1 year at the time of the first paediatric consultation. Two patients were lost to follow-up.

### 3.2. Further examinations

Molecular screens for pathogenic variants in *SMN1* (SMA) and *DMPK* (DM1) and methylation analysis of *SNRPN* (PWS) were performed for all patients and yielded negative results (this was part of the inclusion criteria).

The muscle biopsy proved to be informative in 21 out of the 22 tested patients. In one patient, the analysis revealed a pattern of nonspecific congenital muscular dystrophy, whereas the remaining 20 patients displayed a pattern of congenital myopathy: nemaline myopathy ( $n = 5$  patients), centronuclear myopathy ( $n = 2$ ), central core disease ( $n = 2$ ), congenital fibre type disproportion ( $n = 4$ ), myofibrillar myopathy with glycogen overload ( $n = 1$ ), size irregularity of the fibres with lipid and mitochondrial overload ( $n = 1$ ), and nonspecific myopathic biopsy ( $n = 5$ ).

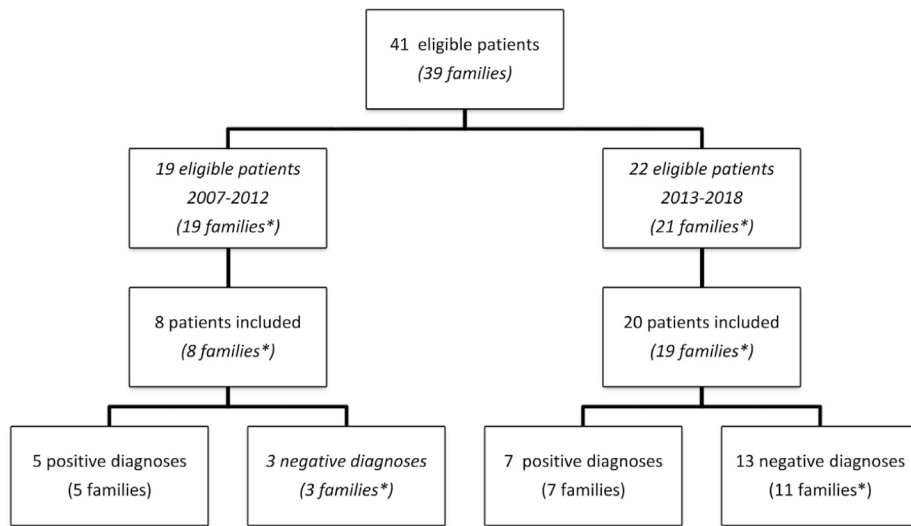
Among the 26 patients who performed a cerebral MRI, abnormalities were present in half of them ( $n = 13$ ): dystrophy or agenesis of the corpus callosum ( $n = 3$ ), periventricular leukomalacia or periventricular white matter abnormalities ( $n = 5$ ), a punctiform haemorrhage ( $n = 1$ ), minor ventriculomegaly or enlargement of the subarachnoid spaces ( $n = 3$ ), and anoxo-ischaemic lesions ( $n = 1$ ).

The electrophysiology studies revealed that six of the 14 examined patients exhibited EEG abnormalities. ENMG identified myogenic alterations in four patients and neurogenic alterations in one patient out of a total of 20 examined patients.

The remaining tests demonstrated poor cost-effectiveness: 32% of the 28 patients tested ( $n = 9$ ) had an abnormal CK level of  $>500$  UI/L. All results came back negative for the CGH array ( $n = 11$ ), karyotype ( $n = 10$ ) and anti-acetylcholine and anti-MuSK antibodies for myasthenia gravis diagnosis ( $n = 19$ ). Nonspecific whole-body radiological abnormalities were observed in 46% of 13 patients tested ( $n = 6$ ) and thoracic abnormalities in 56% of 25 patients tested ( $n = 14$ ). Cardiac ultrasound was anomalous in 27% of 22 patients tested ( $n = 6$ ).

### 3.3. Diagnostic yield of the MyoPanel

fdb 9.1.450/W UnicodeOut of the 28 cohort patients, MyoPanel NGS identified causal molecular anomalies in 12 patients from 12 different families. This represented a total diagnostic yield of 43% (95% CI [24;63]) of the study population. The diagnostic yield was 63% (95% CI [24;91]) ( $n = 5/8$ ) for the 2007–2012 cohort and 35% (95% CI [15;59]) ( $n = 7/20$ ) for the 2013–2018 cohort. The diagnostic yield of the MyoPanel was 50% (95% CI [28;72]) in patients who had previously had a muscle biopsy (11/22) and only 17% (95% CI [0;64]) in patients without pathological analysis (1/6). Pathogenic variants were identified in the following genes: *RYR1* ( $n = 2$ ),



\*1 patient from the 2007-2012 cohort and 1 patient from the 2013-2018 cohort belong to the same family

**Fig. 2.** Study diagram

Distribution of patients according to the two study cohorts and the results of the various genetic diagnostic techniques. \*1 patient from the 2007–2012 cohort and 1 patient from the 2013–2018 cohort belong to the same family.

*ACTA1* (n = 4), *NEB* (n = 3), *MTM1* (n = 2), and *CACNA1S* (n = 1) (Table 2). The clinical, paraclinical and genetic characteristics of these patients (patients 1 to 12) are summarised in Table 3. Others patients characteristics (patients 13 to 28) are summarised in Supplementary Table 1.

### 3.4. Genotype-phenotype correlation

#### 3.4.1. *ACTA1* gene

*ACTA1* gene pathogenic variants were detected in patients 1 to 4 (n = 4/28). Three of these patients had an antenatal history of

**Table 1**  
Clinical features of the cohort.

	Number of patients	Percentage (%) of patients
<b>Genders</b>		
Female	14	50%
Male	14	50%
<b>Consanguinity</b>	2	7%
<b>Spontaneous miscarriage</b>	5	18%
<b>Family history of neuromuscular disease</b>	9	32%
<b>Antenatal signs</b>	14	50%
Polyhydramnios and decreased perceived foetal movements	6	21%
Polyhydramnios only	4	14%
Decreased perceived foetal movements only	4	14%
<b>Perinatal anoxia</b>	7	25%
<b>Prematurity</b>	5	18%
<b>Neonatal respiratory distress</b>	28	100%
Intubation	15	54%
CPAP	13	46%
<b>Ophthalmoplegia</b>	7	25%
<b>Orality disorders</b>	20/25	80%
Gastrostomy	8/25	32%
<b>Hypotonia</b>	28	100%
<b>Arthrogryposis</b>	22	79%
<b>Loss of tendon reflexes</b>	12	43%
<b>Amimia</b>	19/25	76%
<b>Abnormal cognitive development</b>	12	43%
<b>Facial dysmorphism</b>	10	36%
<b>Death</b>	16 <sup>a</sup>	57%
0–1 month	8/16	50%
1–3 months	5/16	31%
3–months-3 years	3/16	18%
<b>Alive</b>	11	44%
<b>Started walking<sup>a</sup></b> (18 months-3 years)	7/11	63%

CPAP: continuous positive airway pressure.

<sup>a</sup> Two patients still alive were between 6 and 12 months at the time of investigation. Two patients were lost to follow-up.

**Table 2**  
Description of genetic pathogenic variants.

Patients	Gene	Localisation	Pathogenic variants	Mutation effect	Familial segregation	ExAC/ gnomAD	SIFT/ Polyphen2	MPA score	reference
1	<i>ACTA1</i> NM_001100.3	Exon 3	c.449C>T p.(Thr150Ile)	Missense	Not tested	Absent	S: - Po: B (0.38)	9	Ref Zenagui et Jutas-Morales et al., submitted
2	<i>ACTA1</i> NM_001100.3	Exon 7	c.1057A>G p.(Thr353Ala)	Missense	<i>de novo</i>	Absent	S: - Po: D (0.91)	9	Ref Zenagui et Jutas-Morales et al., submitted
3	<i>ACTA1</i> NM_001100.3	Exon 4	c.493G>C p.(Val165Leu)	Missense	<i>de novo</i>	Absent	S: - Po: D (0.93)	7.7	Ref Zenagui et Jutas-Morales et al., submitted
4	<i>ACTA1</i> NM_001100.3	Exon 4	c.553C>T p.(Arg185Cyr)	Missense	<i>de novo</i>	Absent	S: - Po: D (0.93)	7.7	Ref Zenagui et Jutas-Morales et al., submitted
5	<i>RYR1</i> NM_000540.2	Exon 94	c.13740T>G p.(Phe4580Leu)	Missense	<i>de novo</i>	Absent	S: De (0.014) Po: D (0.99)	9	This report
6	<i>RYR1</i> NM_000540.2	Exon 76 Exon 77	c.11122A>C c.11186T>C p.(Thr3708Pro) p.(Met3729Thr)	Missense Missense	Parents not tested	Absent Absent	S: T (0.157) Po: P (0.876) S: De (0.021) Po: D (0.946)	7 9	Ref Zenagui et Jutas-Morales et al., submitted
7	<i>MTM1</i> NM_000252	Exon 8	c.589A>C p.(Thr197Pro)	Missense	Present in mother	Absent	S: De (0.001) Po: D (0.997)	10	This study
8	<i>MTM1</i> NM_000252.2	Exon 5	c.290G>A p.(Gly97Glu)	Missense	Present in mother and maternal uncles	Absent	S: De (0.001) Po: D (0.999)	10	Ref Zenagui et Jutas-Morales et al., submitted
9	<i>NEB</i> NM_001271208 (compound heterozygote))	Exon 58 Intron 62	c.8038C>T c.8889+1G>A	Nonsense Splice-site	Inherited from the father Inherited from the mother	Absent Absent		10 10	Ref Zenagui et Jutas-Morales et al., submitted
10	<i>NEB</i> (NM_001271208.1 (compound heterozygote))	Exon 89 Intron 47	c.13661_13666delinsA c.6075+5G>A	p.(Ser4554Asnfs*10) Frameshift Splice-site	Inherited from the mother Inherited from the father	Absent Absent		10 10	Ref Zenagui et Jutas-Morales et al., submitted
11	<i>NEB</i> NM_001271208 (NM_001271208.1 (compound heterozygote))	Exon 8 Exon 168	c.518delA c.23998dupG	p.(Lys173Serfs*55) p.(Glu8000Glyfs*11) Frameshift Frameshift	Inherited from the mother Inherited from the father	Absent Absent		10 10	Ref Zenagui et Jutas-Morales et al., submitted
12	<i>CACNA1S</i> NM_000069 (compound heterozygote)	Exon 20 Exon 41	c.2618del c.5104C>T	p.(Leu873Argfs*21) p.(Arg1702*) Frameshift Nonsense	Inherited from the father Inherited from the mother	0.009 0.0000916		10 10	This report

*CACNA1S*:  $\alpha 1s$  sub-unit of the dihydropyridine receptor; *MTM1*: myotubularin 1; *NEB*: nebulin; *RYR1*: ryanodine receptor 1; *ACTA1*: actin alpha 1; VOUS: variant of unknown significance; ExAC: Exome Aggregation Consortium; gnomAD: Genome Aggregation Database; MPA score: MoBiDiC prioritising algorithm. S: Sift; Po: Polyphen2; B: benign; T: tolerated; De: deleterious; D: damaging; P: Probably damaging.

polyhydramnios or decreased foetal movement. One half of these patients suffered from arthrogryposis and areflexia. All presented with amimia and severe respiratory distress requiring invasive ventilatory support, but cognitive status was unaffected. Their anatomopathological profiles were extremely variable: an irregularity of the size of the fibres with lipid and mitochondrial overload (patient 1), myofibrillar disorganisation with glycogen deposition (patient 2), isolated perimysial and endomysial fibrosis (patient 3), and nemaline myopathy (patient 4). All pathogenic variants detected in our patients were *de novo* dominant missense variants.

#### 3.4.2. *RYR1* gene

The *RYR1* gene was mutated in patients 5 and 6 ( $n = 2/28$ ). Both patients had antenatal symptoms (polyhydramnios and decreased foetal movements), postpartum symptoms (respiratory distress

requiring invasive ventilatory support, arthrogryposis, anomalous cognitive development, amimia, facial dysmorphism, ophthalmoplegia) and CK >1000 mmol/l; both also died prematurely before one month. Patient 5 had a *de novo* *RYR1* pathogenic variant, NM\_000540.2: c.13740T>G; p.(Phe4580Leu), even though there was familial evidence of dominant transmission (family history of neuromuscular disease from the father and paternal uncles). The pathogenic nature of this variant is well established: the amino acid involved is highly conserved evolutionarily, is characterised by loss of an aromatic ring, and is not included in the polymorphism databases ExAC; bioinformatic analysis predicts a deleterious effect on protein function caused by damage to the TM1 transmembrane domain of the calcium channel. Its *de novo* aspect in the context of the family history of neuromuscular disease suggests the presence of an additional familial pathogenic variant. Patient 6 had two

**Table 3**  
Clinical phenotypes of genotyped patients.

	ACTA1			RYR1			MTM1		NEB		CACNA1S	
Patients	1	2	3	4	5	6	7	8	9	10	11	12
Cohort	A	A	A	B	A	B	B	B	B	A	B	B
Gender	F	M	M	M	F	M	M	M	M	M	F	M
Consanguinity	—	—	—	—	—	—	—	—	—	—	—	—
Spontaneous miscarriage	—	—	—	—	—	+	+	—	+	—	—	—
Family history	—	—	—	—	AD	—	—	XL	—	ID	—	—
Polyhydramnios	+	—	—	—	+	+	+	—	+	—	—	—
Fetus with immobilism	—	+	—	+	+	+	+	—	+	—	+	—
Prenatal anoxia	+	+	—	—	—	—	—	—	+	+	—	—
Age at birth (weeks of pregnancy)	40	38	41	39	31	38	41	39	38	34	38	38
Ventilatory support	TI	TI	TI	TI	TI	TI	CPAP	CPAP	TI	CPAP	TI	CPAP
Ophthalmoplegia	—	—	—	—	+	+	+	—	—	—	—	+
Orality disorders	+	Ga	Ga	+	—	+	+	+	—	+	—	Ga
Arthrogryposis	+	—	+	+	+	+	+	—	—	+	+	—
Loss of tendon reflexes	+	—	—	+	+	—	+	+	—	—	+	—
Amimia	+	+	+	+	+	+	+	+	+	+	+	+
Abnormal cognitive development	—	—	—	—	+	+	+	—	+	+	+	—
Facial dysmorphism	—	—	—	—	+	+	—	—	+	+	+	—
Started walking (months)	—	—	—	—	—	—	—	20	—	—	—	—
Death (d: days, m: months)	62(d)	35(m)	—	20(d)	5(d)	27(d)	12(m)	—	5(d)	3(d)	50(d)	—
MRI	N	N	N	N	LM	VM	N	N	N	N	N	N
EMG	MS	N	—	—	—	MS	N	N	MS	—	N	MS
CK (U/l)	524	—	470	300	1063	4291	75	185	258	271	N	38
Muscular biopsy	I	MFM	Fibrosis	NM	NMB	CCD	—	CNM	NM	NM	NM	CNM
Genes previously analysed by Sanger sequencing	—	CHRN	—	—	CHRN	—	—	SMARD1	—	ACTA1	—	—
	—	RAPSN	—	—	RAPSN	—	—	FMR1	—	TPM2	—	—
	—	DOK7	—	—	DOK7	—	—	—	—	TPM3	—	—
	—	CHAT	—	—	CHAT	—	—	—	—	TNNT1	—	—
NGS technology	DNAmP											

A: 2007–2012 cohort ; B : 2013–2018 cohort.

ACTA1: actin alpha 1; RYR1: ryanodine receptor 1; MTM1: myotubularin 1; NEB: nebulin; CACNA1S:  $\alpha$ 1s sub-unit of the dihydropyridine receptor; F: female; M: male; AD: familial disease suspected autosomal dominant disease. Father and paternal uncles present neuromuscular symptoms; XL: familial disease suspected X-linked disease. Mother and maternal uncles present neuromuscular symptoms; ID: intellectual disability in a maternal uncle and a paternal cousin; TI: tracheal intubation; CPAP: continuous positive airway pressure; Ga: gastrostomy; N: normal; LM: leukomalacia; VM: ventriculomegaly; AIL: anoxic ischaemic lesions; MS: myogenic syndrome; NM: nemaline myopathy; I: size irregularity of fibres with lipid and mitochondrial overload; MFM: myofibrillar myopathy; CNM: centronuclear myopathy; NMB: nonspecific myopathic biopsy; CCD: central core disease; CFTD: congenital fibre type disproportion ; DNAmP : mitochondrial DNA panel.

missense variants, one of them (c.11186T>C; p.(Met3729Thr)) being predicted as deleterious (absent in the general population ExAC, MPA score at 9, sift: 0.021, predicted deleterious, polyphen: 0.946 , predicted damaging), the other (c.11122A>C; p.(Thr3708Pro)), predicted as probably pathogenic (absent in the general population, MPA score at 7, sift: 0.157, predicted tolerated, polyphen:0.876 , probably damaging). Sift and polyphen give low pathogenicity prediction scores because the amino acid is in a poorly conserved domain in birds (but conserved up to the lamprey) and the physico-chemical change is moderate. However, there is a loss of polarity and a rigidification of the domain caused by the substitution of a threonine by proline. The absence of available parents sample for segregation studies does not allow to conclude about a recessive effect of the variants or a dominant effect of the c.11186T>C; p.(Met3729Thr) variant alone.

#### 3.4.3. MTM1 gene

Patients 7 and 8 carried an X-linked pathogenic variant in the MTM1 gene (n = 2/28). Both these patients were boys and experienced distal arthrogryposis, and one exhibited antenatal polyhydramnios with a decrease in foetal movements. Family segregation analysis of patient 8 relatives revealed a symptomatic involvement of the patient's maternal uncles, suggestive of an X-linked recessive inheritance pattern. The presence of the MTM1 variant was confirmed in one maternal uncle. The patient, who is currently 4 years old, displayed normal or slightly delayed psychomotor development. On the other hand, patient 7 had a more unfavourable evolution and died at 12 months.

#### 3.4.4. Nebulin gene

The three patients with NEB gene pathogenic variants (patients 9 to 11, n = 3/28) presented with facial dysmorphism, amimia, abnormal cognitive development, and premature death (prior to 3 months of age), and two presented with antenatal symptoms. Patient 9 was diagnosed with hypospadias. From an anatomopathological perspective, these three patients systematically presented nemaline myopathy. Identified pathogenic variants included recessive splice-site, nonsense, or frameshift pathogenic variants. Each patient's pathogenic variant was inherited from one of the parents.

#### 3.4.5. CACNA1S gene

Patient 12 was found to have a frameshift and a nonsense pathogenic variant in the CACNA1S gene. There was evidence of neonatal hypotonia with respiratory distress associated with ophthalmoplegia and amimia, with no other antenatal or postpartum symptoms. The muscle biopsy identified a centronuclear myopathy. Its progression at 12 months was encouraging and motor function showed gradual improvement.

### 4. Discussion

Neuromuscular diseases with neonatal respiratory involvement make up a group of heterogeneous pathologies with variable prognosis. They therefore present major challenges for the investigation of disease aetiology and therapeutic management. Despite an adapted protocol, their nonspecific semiological features make aetiological diagnosis difficult, except in cases with SMA and DM1

pathogenic variants. We previously reported a diagnostic yield of 26% ( $n = 5/19$ ) by Sanger sequencing of single candidate genes based on phenotypic orientation in a cohort of 19 patients [1]. Compared to this time consuming approach, the one-step gene panel analysis reported in our study is faster and has a higher diagnostic yield, 43% ( $n = 12/28$ ). Originality of our study is the analysis of a cohort exclusively composed of newborns with hypotonia and neonatal respiratory distress, since such cohorts have not been reported in the literature. Among published data on the diagnostic effectiveness by GPS or WES of “early onset congenital neuromuscular disease”, some studies include patients with neonatal onset. Wang et al. [47] conducted in 2016 a study on 186 hypotonic newborns with 22.5% of efficiency of GPS with 35 neonatal hypotonia associated genes. Oliveira et al. [37] detected a diagnostic yield of 70% in a small sample of 12 patients, with five patients developing symptoms in the antenatal or neonatal period and two patients developing symptoms in childhood. Schofield et al. [30] evaluated the advantages of WES and GPS (464 neuromuscular genes panel) over conventional techniques in 56 patients suspected of having nemaline myopathy or congenital muscular dystrophy, with neonatal symptoms for 60% and early onset before one year for 20% of patients. All age, diagnostic yields were found to be 46% (26/56) for Sanger gene-by-gene approach, 75% (42/56) for GPS and 79% (44/56) for WES, that is comparable with GPS results. Vill et al. [36] conducted a study in 2017 on 98 patients under one year old and suspected of having myopathy; they used panels of between 99 and 152 genes and reported a diagnostic yield of 52% (23/44 patients); WES revealed genetic diagnosis in 67% (8/12 patients). Todd et al. [41] investigated the effectiveness of NGS for diagnosing neuromuscular diseases in the antenatal and neonatal periods and found a diagnostic yield of 47% (18/38 families). Of the 18 diagnosed families, 12/23 were identified with WES alone, 5/7 by GPS alone (336 genes panel) and 1/8 using both techniques. Finally, in studies including patients with neonatal onset, WES is slightly more efficient than GPS with diagnostic yield between 52 and 79% for WES and between 22.5 and 75% for GPS ([30,36,37,41,47]).

Our study identified pathogenic variants in the *ACTA1*, *RYR1*, *MTM1*, *NEB* and *CACNA1S* genes, the first four genes being known in neonatal hypotonia. Our patients phenotypes are similar to those in the literature [15,47–71]. Pathogenic variants in the *CACNA1S* gene, which were initially identified in dominant malignant hyperthermia [71], may also be implicated in dominant and recessive congenital myopathies. A study of 11 patients from seven families with *CACNA1S* congenital myopathies was conducted in 2016 [72]. Loss of function pathogenic variants was identified in the recessive forms and missense pathogenic variants in the dominant cases. Patients with recessive forms presented one frameshift variant with one frameshift, nonsense or missense variant. Our patient showed compound heterozygosity for two loss of function pathogenic variants. In terms of phenotype, neonatal hypotonia was reported in almost all patients and was associated with ophthalmoplegia in four out of 11 patients [72]. The pathological analysis was consistent with the patterns of centronuclear myopathies present in our patient.

The MyoPanel enabled a molecular diagnosis to be established for only five genes from a panel of 95 genes known to be involved in neonatal hypotonia, i.e. with only 5% of the panel used. This can be attributed in part to the very restrictive selection criteria of our population. Indeed, in a paediatric study described by Colombo et al. [15] in 2015, only 30% of patients with myopathies met the criteria for “neonatal ventilatory support”. This selection criterion for severe patients therefore excludes approximately two thirds of patients suspected of displaying neuromuscular disease symptoms during the neonatal period. Moreover, the lack of specific causes for neuromuscular pathologies manifesting during the neonatal period

hinders the establishment of a short list of candidate genes for further analysis, thus justifying the use of a larger panel of genes that may be implicated in later manifestations of neuromuscular disease.

Half of our cohort was left with an equivocal molecular aetiology. There are several potential explanations for this poor result. The MyoPanel only investigates nuclear congenital myopathy, congenital muscular dystrophy and specific congenital myasthenic syndrome gene candidates, and therefore does not cover all aetiologies, notably all the genes associated with rare congenital myasthenic syndromes, mitochondrial myopathies, metabolic myopathies, specific anterior spinal cord syndromes and neuropathies. As the phenotype of hypotonia patients who are younger than one month old is quite subtle, the MyoPanel is sometimes erroneously performed, for lack of precise clinical orientation, in children who may actually present with a neurological impairment. In these cases, anatomopathological data should enable a more accurate selection of patients who would benefit from a MyoPanel analysis. This would explain the higher diagnostic yields in the 2007–2012 cohort (63%) compared to the 2013–2018 cohort (35%). Indeed, muscle biopsy was less systematic in the 2013–2018 cohort ( $n = 15/20$ ) than in the 2007–2012 cohort ( $n = 7/8$ ), since anatomopathological analysis can identify features indicating a muscular and not central origin of the disorders. Muscle MRIs, which are very informative in older children, remain difficult to perform and interpret during the neonatal period.

We found no pathogenic variant in approximately two thirds of our patients, despite the presence of anatomopathological phenotypes supporting a myogenic process. This is quite a high rate, which raises the fundamental question of how many causative gene pathogenic variants still remain to be identified in these pathologies. This alone justifies the use of alternative methods such as WES, WGS and RNAseq. In our study, only five patients (from 4 families) benefited from a WES, but only one of these was decisive [73], a rather low degree of efficiency compared to data presented in the literature [36,41–43]. This alone justifies the use of alternative methods such as WES, WGS and RNAseq. In our study, five patients (from 4 families) benefited from a WES, and one of these was decisive [73]. The fact that WES is only slightly more efficient than GPS in the literature [30,36,37,74] could be related to the lack of investigation of intron regions, promoter regions, and regions that are prone to epigenetic modifications. WGS and RNA sequencing would therefore present significant advantages in the aetiological diagnosis of these pathologies. Results obtained from our cohort and evidence from the literature prompted us to modify the protocol for investigating neonatal hypotonia that is suspected of being of neuromuscular origin. We thus included a second-line WGS protocol to replace the GPS and WES approaches, as part of the “Plan France médecine génomique 2025”. As part of the first-line assessment, we included an analysis of the *MTM1* gene because a gene therapy protocol is available for patients with an early form of myotubular myopathy.

## 5. Conclusion

The congenital muscular myopathy and dystrophy panels are not enough efficiency in the diagnosis of peripheral neonatal hypotonia without suggestive clinical signs. Indeed, over half of the neonatal peripheral hypotonia cases with early respiratory failure are refractory to diagnosis using this type of approach. We hope that systematic second-line screening by WGS of patients with neonatal hypotonia suspected of being of neuromuscular origin will lead to a major improvement in diagnostic performance in these pathologies.

Declaration of competing interest

The authors have no conflict of interest to declare.

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