UNIVERSITY OF MEDICINE AND PHARMACY "CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL MEDICINE

CLINICAL AND ELECTROPHYSIOLOGICAL CORRELATIONS IN CHILDREN WITH SPINAL AMYOTROPHY TREATED WITH NUSINERSEN

PHD THESIS SUMMARY

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2024

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List of published scientific papers

1. **Axente, M.**, Sporea, C., Grigoriu, A., Mirea, A., Leanca, M.C., Marinescu, G.I., et al. Clinical and Electrophysiological Aspects in Children with Spinal Muscular Atrophy Type 1, 2 and 3 before Treatment. J Neurophysiol Neurol Disord [Internet]. 2021; 9(1), https://www.jscholaronline.org/full-text/JNND/9_103/Clinical-and-Electrophysiological-Aspects.php
2. **Axente, M.**, Shelby, E-S., Mirea, A., Sporea, C., Badina, M., Padure, L., Ion, D.A. (2021). Clinical features and genetics in non-5q spinal muscular atrophy caused by acid ceramidase deficiency. Journal of Medicine and Life, 14(3), 424 – 428, https://medandlife.org/wp-content/uploads/20.-jml-2021-0147.pdf
3. Mirea, A.; Shelby, E.-S.; **Axente, M.**; Badina, M.; Padure, L.; Leanca, M.; Dima, V.; Sporea, C. Combination Therapy with Nusinersen and Onasemnogene Abeparvovec-xioi in Spinal Muscular Atrophy Type I. J. Clin. Med. 2021, 10, 5540. https://doi.org/10.3390/jcm10235540, Hatpas://vv.madp.com/2077-0383/10/23/5540/Hatma
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6. Badina, M.; Bejan, G.C.; Sporea, C.; Padure, L.; Mirea, A.; Leanca, M.-C.; **Axente, M.**; Grigoras, F.P.; Bejan, M.; Shelby, E.-S.; et al. Changes in pNFH Levels in Cerebrospinal Fluid and Motor Evolution after the Loading Dose with Nusinersen in Different Types of Spinal Muscular Atrophy. Medicina 2023, 59, 1244. https://doi.org/10.3390/medicina59071244, Hatps://vv.madp.com/1648-9144/59/7/1244

Spinal muscular atrophy (SMA) is a neuromuscular, degenerative, hereditary disease with an incidence in the pediatric population of 1:6,000- 1:11,000 [1], with different evolution depending on the age at which the first signs of disease begin, respectively from the extremely severe early form, with mortality up to 2 years, with bulbar involvement, to the mild clinical form, without respiratory involvement, with proximal motor deficit, but maintaining independent walking.

The natural course of the disease changed with the approval of innovative treatments (ASO/antisense oligonucleotide) that led to the change of the clinical picture, respectively to the emergence of new phenotypes. Depending on the moment of starting treatment, the results obtained are promising, so that in early forms children survive over 2 years in their vast majority, and in intermediate and late forms it was observed to stop motor regress with longer term preservation of autonomous walking.

Along with drug treatment, it is necessary to comply with the standards of care (general and respiratory physiotherapy, respiratory and nutritional management, orthosis, prevention of orthopedic complications: progressive scoliosis, hip dislocation, tendon retractions) [2,3].

The study of electrophysiological changes in this condition is a new topic, original both nationally and internationally, being little found in the specialized literature. In the era of genetic diagnosis, electrophysiological exploration brings additional elements about the degree of degeneration of motor neurons / axons but also about compensatory processes of chronic reinnervation / regeneration.

The purpose of this research was to identify predictability biomarkers for the evolution of spinal muscular atrophy and to monitor the clinical and electrophysiological parameters under disease-modifying treatment (nusinersen), for a duration of 3 years from initiation of treatment.

The research was conducted for the first time in Romania and included pediatric patients with SMA types 1, 2 and 3 undergoing treatment at the "Dr. Nicolae Robănescu" National Neuroreabilitation Center for Children (CNCRNC).

This *observational analytical* study is an original theme in our country because with the introduction of specific treatment (nusinersen) a new, previously unexplored clinical context was created. In the Romanian literature there are no data on the electrophysiological evolution under this treatment, and in international publications they are very few. The main objective was to research the electrophysiological and clinical evolution in a representative group of pediatric SMA 5q patients (types 1, 2 and 3) after 3 years of treatment with nusinersen by comparing baseline and final data.

As secondary objectives we followed the statistical correlations between electrophysiological and clinical data (motor scales) at initiation of treatment, comparing the evolution of patients with SMA treated versus those untreated (data from literature - natural evolution of the disease), the relationship between the clinical picture and the number of SMN2 copies. We also analyzed the influence that the age of initiation of treatment has on the results, both on the clinical and electrophysiological evolution, but also particular cases of non 5q SMA (clinical, laboratory and treatment aspects), particular types of genetic impairment (compound heterozygous) and the case of a presymptomatic patient from onset (clinical and electrophysiological evolution under treatment).

The limitations of the study were related to the relatively small number of patients, heterogeneity of the group (very different ages even within the same type, with onset of disease at variable intervals of time), pediatric age which punctually created small problems of cooperation during examinations.

The incidence of the disease is 1:6000-10000 live births, and the frequency of healthy carriers in the general population is 1:40-1:60 [1,4]. In 1990, the position of the gene responsible for SMA was located by linkage studies at the 5q11.2-13.3 locus, containing approximately 500 kb [5]. In this region, duplicate gene and nongenic sequences in variable numbers have been identified [6]. At the level of the human genome, unlike animals, this region contains at least four genes present in the centromeric and telomeric region. At the level of chromosome 5 we find 2 pairs of isoform genes - 2 SMN1 genes at telomeric level and 2 SMN 2 genes at the centromeric level that differ only by a few nucleotides at exon level 7, one being functionally important: the SMN1 gene has a cytosine (C) in the same position where the SMN2 gene contains a thiamine (T).

In 1995, Lefebvre et al. reported that the presence of mutations in both telomere alleles (SMN1) causes SMA. This gene has been called survival motor neuron (SMN) [7]. Coovert et al. later showed that the centromeric SMN2 gene has the same 99% nucleotide sequences as SMN1, but the disease is caused only by mutation in the SMN1 gene [8]. The typical mutation, found in 95% of SMA cases, is found in the SMN1 gene on the long arm of chromosome 5 (5q) through homozygous exon deletion 7.

SMN1 gene, the pathogenic gene in SMA is the telomeric copy of duplication (SMNtel). The number of copies varies as follows: one SMN1 copy on each chromosome in 82-96% of healthy subjects, 2 SMN1 copies on each chromosome in 4-18% of healthy subjects. The presence of at least one SMN1 copy is indispensable for the survival of

motoneurons. The SMN1 gene contains 20 Kb and 9 exons (1, 2a, 2b, 3-8) [9]. The SMN1 gene encodes the SMN protein containing 294 amino acids. [10]

SMN2 gene is located centromeric to the SMN1 gene (SMNcen), being responsible for the severity of the disease phenotype. The SMN1 and SMN2 genes differ by 5 nucleotides, only one being functionally important, namely the traclocation of cytosine (C) to thiamine (T) at the level of an exon 7 SMN2 splicing site [11]. This change alters the incorporation of exon sequence 7 into messenger ARM resulting in the synthesis of a shorter, rapidly degradable protein with reduced functional efficiency. However, the SMN2 gene can produce about 10% of the functional SMN protein. The number of SMN2 copies has been shown to vary from individual to individual and is responsible for the severity of the clinical picture.

Genotype-phenotype correlations

There are no correlations between pathogenic SMN1 type and disease severity: homozygous exon 7 deletion is observed in almost all patients (94%), only a small part of cases by gene conversion. The gene conversion mechanism consists in transforming the SMN1 gene into SMN2 and implicitly increasing the number of SMN2 copies [12,13].

SMN Protein

It is a large molecule, weighing 38 kDa , present in the cytoplasm and nucleus. In the nucleus it is concentrated in structures called "gems" (Cajal bodies). They contain increased levels of factors involved in transcription and process various types of nuclear RNA. The number of Cajal bodies in tissues or cell lines in patients with SMA correlates inversely with disease severity, cases with type 1 SMA have very few or none at all. SMN protein is also present in neuronal axons, transported birectionally. It is well represented at the level of growing endings and at the level of the postsynaptic membrane of the neuromuscular junction.

Clinical symptoms

The clinical signs characteristic of this condition are hypotonia/motor deficit and muscle atrophy due to loss/degeneration of spinal motoneurons +/- bulbar [14]. Motor deficit is proximal, pelvic girdles more affected than scapular, as well as axial. The severity of the disease can range from mild deficit on belts with preservation of autonomous gait to severe infantile forms with severe generalized hypotonia, respiratory failure and swallowing disorders. The onset and evolution of the disease are distinct and range from a presymptomatic period with unpredictable duration, from a few days/weeks in early forms with rapid motor regression, to years in late forms, then with stabilization of motor function

and eventually slow progression [14]. Under conditions of stress/acute infections, motor regression may increase [15].

Decimal classification (Dubowitz 1997) reflects the difference between the infant with type 1 SMA, the early form with severe motor deficit, muscle atrophy, early bulbar dysfunction and a serious prognosis for survival (type 1.1), then the infant with typical SMA evolution (1.5), and those with the onset of motor regression probably at 3 months of age, some achieving the ability to have head control and with better respiratory function, minimal dysfunction (type 1.9). For the intermediate group 2 (type 2), clinical variability between the severe "weak" form able to sit only with support, poor head control, severe axial hypotonia but also in the lower limbs (2.1) and the "strong" form (2.9) represented by patients who acquired the sitting position without support but do not stand upright, without significant bulbar dysfunction. In patients with type 3, a severe form is identified (3.1) in which the child is able to stand upright and take several steps unstable, and the late form (3.9) that walks autonomously stable, with minimal proximal deficit, without bulbar dysfunction [16].

Functional classification 2018

Since 2018, the classification of SMA according to acquired motor acquisitions has been redefined, patients being grouped into 3 categories: nonsitters, sitters and walkers. In the category of "nonsitters" are most type 1 patients, but there may also be those in type 2 who have lost the ability to sit (especially type 2a). "Sitters" are those in type 2, but they can also be those in type 1 or 3 who won or lost, respectively, the functional purchase. In the last category "walkers" are those of type 3 walkers and those of types 1 and 2 who have gained independent walking.

Complications of spinal amyotrophy are varied depending on the early or late type of disease. Thus, in type 1 and 2a, respiratory dysfunction is frequent, of restrictive type, due to damage to the accessory respiratory muscles (intercostal muscles). Damage to the respiratory muscles causes decreased clearance of secretions from the airways, making coughing ineffective, causing nocturnal hypoventilation and recurrent pneumonia. The application of non-invasive ventilation (BiPAP/ Bilevel Positive Airway Pressure) and the use of tracheobronchial clearance techniques ("cough assist") have brought a clear improvement in the quality of life, preventing life-threatening respiratory complications, and implicitly led to a longer survival time of these patients. Also in early forms of disease we frequently encounter swallowing and gastrointestinal disorders, patients presenting dysphagia and microaspirations.

Gastrointestinal signs encountered may be: constipation, delayed gastric evacuation and potentially life-threatening gastroesophageal reflux due to the risk of lung microaspirations. Individuals with 2nd and 3rd nonambulant forms are at risk of developing obesity.
Orthopedic complications encountered in SMA are: progressive scoliosis, hip dislocation and joint pain are the most common. They are more common in type 2 and severe forms of SMA type 3 (SMA type 3a).

Functional scales used in the evaluation of children with SMA are: CHOP INTEND (The Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders) for type 1 [17,18] , HFMSE (Hammersmith Functional Motor Scale Expanded) for types 2 and 3, and 6MWT (6 minute walking test) for cases that kept their gait autonomous. CHOP INTEND was developed for patients aged between 3 months and 4 years, contains 16 items, and the scale notes the degree of fulfillment of the motor requirements indicated by the physiotherapist, both by direct observation and by the response given by the mother. The possible total score is 64. HFMSE is a scale used to assess physical abilities in patients with SMA types 2 and 3. It contains 33 items that are rated on a scale of 0, 1, 2, with a total achievable score of 66. [19,20]. 6MWT [21–23] measures the distance in meters traveled by the patient in 6 minutes, during which he walks with normal step, without running, without stopping. This is the reference test of many studies, with the help of which the progression of the disease in type 3 SMA is assessed.

Diagnostic

Genetic testing is the gold standard in the diagnosis of MSDs. In practice, the clinical suspicion we have before a hypotonic infant ("floppy infant") with marked, generalized motor deficit, areflexia and bulbar involvement (respiratory and swallowing) must be supported by a genetic test (MLPA/qPCR). This test should be performed as soon as possible, because in the case of early forms of disease (SMA type 1) every day means significant motor regress (associated with the degenerative process of motoneurons) [24].

Number of SMN2 copies

Analysis of the number of SMN2 copies is not routinely performed as part of the diagnosis or testing of the carrier for SMA. Although the number of SMN2 copies influences disease severity, SMN2 sequence variants and other genes have also been implicated in influencing the SMA phenotype [25]. Therefore, SMN2 copies count results may provide probabilistic clinical severity information for an affected child or fetus, but are not definitive.

Non 5q spinal muscular atrophy

5% of SMA cases are not due to the typical mutation in the SMN1 gene on chromosome 5, but to other mutations, resulting in a clinical picture indicating motoneuron disease with specific peculiarities. So far, 16 genes associated with non-5q SMA have been described [47]. The most common mutations encountered non5q are in the ASAH1 gene (SMA with myoclonic epilepsy), IGHMBP2 (SMA with severe respiratory dysfunction), ATP7A (distal X-linked SMA), GARS and PLEKHG5 (sensory-motor neuropathy), EXOSC3 (pontocerebellar hypoplasia and SMA). The clinical aspects are similar to the typical form of SMA 5q, patients have predominantly proximal but also axial motor deficit, severe amyotrophies, progressive evolution, bulbar dysfunction, osteotendinous areflexia. In all these forms, motor deficiency is not due to a lack of SMN protein, therefore they have no therapeutic indication. In medical practice, additional investigations were needed to establish the form of SMA: electrophysiological tests, electroencephalography/EEG, magnetic resonance imaging/MRI, but also additional genetic tests (Sanger monogenic sequencing, whole exome sequentiong /WES).

Natural evolution of SMA

Regarding the natural evolution of SMA before the therapeutic era, literature data present studies conducted on populations of patients with types 1, 2 and 3 following their evolution over a variable period of time (12-24 months), monitoring motor function (CHOP INTEND scale), bulbar function (respiratory and swallowing), but also survival rate. The data were useful in clinical trials (ENDEAR, CHERISH) in response to nusinersen treatment, which were also controlled in SMA patients without treatment ("sham controlled"). Thus, patients with type 1 SMA without treatment were followed in 3 longitudinal studies of natural disease evolution using the CHOP INTEND scale for monitoring: two were performed in the United States by the Pediatric Neuromuscular Clinical Research (PNCR) [26] and the NeuroNEXT network [27], and the other in Italy in one center (Nemo Center of the Gemelli Hospital) [28].

Treatment

Nusinersen [29] is the first targeted SMN2 pre-messenger RNA therapy approved for SMA (December 2016 for the United States and May 2017 for Europe) [30,31].

Nusinersen is an antisense oligonucleotide (ASO) belonging to phosphorylated oligodeoxynucleotides . Nusinersen exerts its effect by modifying the genetic product, acting on the SMN2 gene [32]. The SMN2 gene encodes an SMNΔ7 protein, which differs from the SMN protein (produced by the SMN1 gene) by 11 nucleotides, is a truncated,

nonfunctional protein, resulting in the omission of exon 7 in 80-90% of the messenger RNA transcript [33]. Nusinersen targets heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) ('splicing zone') in SMN2 pre-messenger RNA, downstream of exon 7. This adjusts the merging process that increases the synthesis of transcripts containing exon 7 [34-36] and implicitly increasing the amount of functional protein (SMN). Nusinersen has a long half-life of about five months, so it is initially given as four loading doses over a period of 2 months, followed by maintenance doses every 4 months [37,38]. Nusinersen has proven highly effective in improving motor function [39,40] but also respiratory function and swallowing / nutritional status [41,42]. The dose is the same in all age groups. ASO toxicity, usually dose-dependent, includes systemic side effects such as fever and arthralgia, thrombocytopenia, proximal renal tubular toxicity, and hepatotoxicity [39-45].

In December 2016, the Food & Drug Administration (FDA) approved nusinersen under the brand name Spinraza as the® first drug for the treatment of SMA. The European Medicines Agency (EMA) approved it 6 months later. As of August 2021, nusinersen is available in 22 European countries [46]. According to Biogen, by March 2021, more than 11,000 patients worldwide had received nusinersen treatment [48].

In Romania, nusinersen is officially approved for use in May 2018 for all types of SMA, age groups and stages of the disease. The first patient starts treatment in October 2018, CNCRNC "Dr Nicolae Robanescu" being the first administration center.

Electrophysiological aspects

The compound motor action potential (CMAP) is the sum of individual motor potentials. These individual potentials are generated by muscle fibers that are depolarized by nerve fiber axons with similar conduction speeds. The amplitude depends on the integrity of the axons, the muscle fibers they depolarize, and the degree of variability in the conduction speed of individual fibers. In SMA, CMAP has reduced amplitude due to loss of the number of viable axons , but with distal latencies and normal conduction speeds, since the myelin is intact. Because of axonal loss, the amplitude of CMAPs can be significantly reduced. In SMA type 1, the amplitude of CMAP is extremely small, usually <0.5 mV, reaching a maximum of 1 mV pre-treatment, being correlated with the degree of motor impairment. In type 2, the amplitude of CMAP is on average between 1-2.5 mV, sometimes with a slight increase in area, this aspect being correlated with the chronic process of nervous reinnervation. In SMA type 3 , the amplitude of CMAP is usually normal, but the area and duration of CMAP are increased in the context of chronic reinnervation/sprouting. [49,50]. According to literature

data [27], CMAP was followed as a prognostic factor and biomarker for the evolution of the disease.

The research carried out within the doctoral studies analyzed the clinical and electrophysiological evolution under ASO treatment of a representative group of patients in our country with symptomatic SMA types 1, 2 and 3. Also, an important aspect was to compare the evolution of CMAP in our treated patients with that of untreated patients (literature data), in order to prove the importance of CMAP as a predictor of the evolution of SMA.

Studies carried out within doctoral research

During the doctoral research, ***5 studies*** were conducted:

First study revealed differences between clinical and electrophysiological parameters of patients with SMA types 1, 2 and 3 before initiation of treatment and of healthy populations of the same age [51].

Second study analyzed the clinical picture and electrophysiological data in atypical forms of SMA (non 5q) compared to the typical form, SMA 5q [52].

Third study identified correlations between electrophysiological parameters (CMAP amplitude) and functional scores on motor scales following periodic monitoring, for 2 years, every 4 months, of 34 patients with SMA types 1, 2 and 3 under nusinersen treatment [53].

Fourth study, conducted on an extended group of 60 patients with SMA types 1 and 2, analyzed the influence of time between symptomatology onset and treatment initiation on disease progression [54].

The fifth study included 71 patients with SMA types 1, 2 and 3 who were followed for 3 years.

A special place is occupied by the case of the presymptomatic patient (Nr 72) diagnosed antenatally and genetically confirmed perinatally, who under treatment with nusinersen and correct standards of care had a normal motor development at age stages.

Material and method

The research was carried out within CNCRNC "Dr. Nicolae Robănescu", with the recruitment of patients between October 2018 and May 2020, included 72 children diagnosed with SMA types 1, 2 and 3, whose evolution was monitored until May 2023. All patients were genetically confirmed by MLPA test, the vast majority having homozygous mutation of the SMN1 gene, with only 2 having the genetic appearance of compound

heterozygous, requiring gene sequencing. One of the patients was presymptomatic (diagnosed antenatally genetically and confirmed perinatally), the remaining 71 being symptomatic. The research was carried out in compliance with the legislation in force, after obtaining the approval from the CNCRNC Ethics Commission (no. 7465/01.10.2018) and the informed consent of the legal guardians of all patients enrolled in the study. Prior to obtaining informed consent, legal guardians were explained all the details related to how the research is carried out and in which the data are collected, analyzed and processed, as well as the form in which the research results are to be disseminated.

All patients followed a clinical evaluation protocol - neurological examination, functional testing on internationally approved scales (CHOP INTEND, HFMSE, 6MWT), subsequently they were electrophysiologically evaluated with the recording of compound motor action potential (CMAP) at the right distal ulnar nerve level. Data were collected at baseline, prior to introduction of nusinersen and prior to each dosing intrathecally every 4 months for 3 years from the initiation of treatment.

Electrophysiological evaluation

In order to obtain electrophysiological data, a Keypoint Electromyography (EMG) with 6 channels,classical/pediatric stimulator, adhesive surface electrodes were used.

In the office was created a pleasant, comfortable atmosphere to ensure the relaxation of the child and family for the best possible collaboration. General or local sedation was not administered. The distal skin temperature recorded was above 35.5°. Adhesive gel surface electrodes were placed at the level of the hypotenar eminence - the active (picking) electrode and 3 cm distal from it - the reference electrode. Electrical stimulation was performed at the level of the wrist joint, at the level of the right ulnar nerve (2-3 cm medial from the median nerve) using the pediatric stimulator, applying progressive intensities from 10-15 mA to 50 mA. The CMAP obtained was feasible, reproducible, obtained at supra-maximal intensities, electrical stimulation being repeated 3 times. The software of the device recorded the maximum amplitude (negative peak). All examinations went without major incidents, the children endured the working procedure well. There were no errors related to lack of collaboration or other reasons.

Functional assessment

CHOP-INTEND it was applied to patients with SMA type 1 by direct observation but also by mother’s responses (certain motor skills observed at home and which could not be replicated in the examination room). The scale measures improvement in motor function by assessing active movements, mobility and muscle strength following 16 motor functions

grouped into 3 categories: head and neck, hands; arms and shoulders; leg, lower limb and hip. Each of them is marked from 0 to 4, with 0 being no response/ability to perform movement and 4 being "complete response" (being able to perform the task). The possible total score is 64.

HFMSE is a scale used to assess physical abilities in patients with spinal muscular atrophy types 2 and 3. It contains 33 motor functions grouped into 7 categories: sitting, rolling, transition/rolling, orthostatism/step, transition/kneeling, jumping, climbing/descending the ladder, which are rated with a score of 0, 1, 2, with a total achievable score of 66.

6MWT used in children who have acquired independent walking. The test consists of asking to go in normal step, no running, no stopping for 6 minutes to measure the distance they can meet. This very simple test is the benchmark test of many studies. The result is in meters.

The functional evaluation of the SMA patient is performed with specific scales, depending on its functional status [55]. These are shown schematically in Table I.1.

Table I.1 Recommended motor scales according to the functional status of the patient

Functional state of the patient	**Recommended motor scales**
"Non-sitting"	CHOP INTEND
"Sitter"	HFMSE
"Walker"	6 MWT, HFMSE

In study 1 we looked at clinical and electrophysiological aspects in children with SMA types 1, 2 and 3 prior to initiation of nusinersen treatment. We studied a group of 36 patients admitted in CNCRNC "Dr Nicolae Robănescu" between September 2019 and January 2021, having the inclusion and exclusion criteria established in the research plan. The electro-physiological evaluation and motor scales were performed according to the methodology described in the research study, thus CMAP was recorded at the ulnar nerve level, by distal supramaximal stimulation. All of these patients were also functionally assessed using the CHOP INTEND (SA type 1) and HFMSE (SMA type 2 and 3) scales. CMAP values in SMA patients in the study group compared to healthy subjects of the same age were consistent with data mentioned in the literature. Compared to normal CMAP values in healthy subjects [56,57] which have an average ulnar nerve value of 4-11.5 mV (increasing with age), values below this curve [58] were observed in all forms of SMA in the analysed group. CMAP

levels raised suspicion of peripheral motoneuron disease, being significantly low in types 1 and 2 and decreasing moderately/normally in type 3 [59,60]. They were associated with the duration from the onset of the first signs of illness, being much shorter in children who already have motor regression and muscle atrophy [61]. In disease subgroups we observed similar CMAP amplitude amplitude values in types 1 and 2a, as well as for 2b and 3a, these were associated with severity of clinical outcome/motor regression. Similar to literature data [14,27,59,61], an inverse proportional relationship was observed in the study group between the clinical picture and the number of SMN2 copies, with patients with reduced numbers of children having a more severe disease phenotype. Within our group we have demonstrated statistically significant correlations between CMAP and functional scale score, i.e. in SMA type 2 on the HFMSE scale ($p < 0,01$, $r = 0,687$) and in SMA type 1 on the CHOP scale ($p < 0,05$, $r = 0,586$), they demonstrating synergy between the two parameters at a point in disease progression. In SMA type 3, the lack of statistical significance between CMAP and HFMSE was interpreted in the context of the heterogeneous group of patients, most of whom were non-ambulant patients with long-term chronic outcome.

In **study 2** we analyzed aspects of differential diagnosis in patients with non 5q SMA, presenting suggestive clinical cases encountered in medical practice.

The evaluation of cases of atypical SMA with non 5q mutations was an objective of the research, the identification in practice of these patients was an important process because these cases require a different therapeutic conduct and implicitly had a different evolution / prognosis. 5% of SMA cases are not due to the typical mutation in the SMN1 gene on chromosome 5, but to other mutations, resulting in a clinical picture indicating motoneuron disease but with specific peculiarities [52].

During 2018-2021 within CNCRNC "Dr. Nicolae Robănescu" we had 7 cases of SMA non 5q. The mutations encountered were in the gene ASAH1, GARS, PLEKHG5, EXOSC3. Similar clinical aspects were predominantly proximal and axial motor deficit, progressive evolution, bulbar dysfunction, osteotendinous areflexia. Initially, all patients were tested for MLPA for mutation in the SMN1 gene that ruled out the typical form of SMA.

We presented in detail the case of a patient with mutation in the ASAH1 gene who raised special difficulties of etiological classification, with the onset of symptoms at the age of 5-6 years with accentuated motor deficit on the girdles, severe generalized amyotrophies, progressive scoliosis, speech disorder (severe dysphonia), periarticular nodules in the fingers, epileptiform changes (electroencephalographic aspect) without clinical seizures,

clinical picture corresponding to an overlap phenotype between spinal amyotrophy - progressive myoclonic epilepsy/ SMA-PMS and Farber's disease /FD [52,62].

The ASAH1 gene, located at the 8p22 locus, encodes the protein ASAH (N-acylsphingosine amidohydrolase 1, N-acylsphingosine deacylase, or acid ceramidase), an enzyme involved in the cleavage, at the lysosomal level, of ceramide into sphingosine and a free fatty acid, as well as, under different pH conditions, in the synthesis of ceramide from the aforementioned components [63–69]. Biallelic pathogenic mutations in ASAH1 are involved in the production of two diseases with an autosomal recessive mode of transmission whose phenotype can sometimes overlap, namely Farber lipo-granulomatosis and spinal muscular atrophy with progressive myoclonic epilepsy [64–67,69,70]. To date, there are fewer than 200 cases of FD and SMA-PME reported in the literature [71].

The patient presented to our hospital at the age of 13 for severe respiratory distress, global motor deficit, predominantly lower limbs, motor regression, axial hypotonia with poor head control, muscle strength of 2-3/5 on the MRC (Medical Research Council) scale in the upper limbs, respectively 2/5 for the lower limbs, severe generalized muscle atrophy, retractions in the elbows and knees more than in the distal joints. Genetic testing for SMA 5q (MLPA and Sanger sequencing) was negative. Between 5 and 10 years the evolution was slowly progressive, the proximal motor deficit increased, and the patient developed severe generalized muscle atrophy, swallowing and breathing disorder, severe contractures at all levels, severe progressive scoliosis, lack of osteotendinous reflexes. Gastrostomy was performed in 2019. After the age of 10, his motor decline was dramatic, he lost his independent gait and was confined to a wheelchair. In March 2021, surgery was performed to correct thoracolumbar scoliosis, as a result of which the motor deficit increased, muscle atrophy became extremely severe, the patient still had minimal active movements only at the distal level of the upper limbs. He had paradoxical, difficult breathing without needing ventilatory support or oxygen. The presence of speech disorder with severe dysphonia. He also had retractions in the mandibular joint with very limited movements at this level, subcutaneous nodules around the interphalangeal joints in the upper limbs more than the lower limbs. He did not have paroxysmal events, such as myoclonus or other types of seizures. Electroneurography showed a slightly reduced amplitude of compound motor action potential , but with an increased area (due to the reinnervation process). The sensitivity action potential had normal amplitude and normal driving speeds. Needle electromyography showed chronic denervation changes with very broad, polyphasic motor unit potentials and incomplete recruitment. MLPA and Sanger mongenic sequencing ruled out a mutation in the

SMN1 gene, followed by whole exome sequencing with the identification of three pathogenic heterozygous mutations in the ASAH1 gene. Having two out of three clinical criteria of Farber's disease, but with motoneuron damage, we consider this case to be extremely rare, "overlapping" between the two conditions caused by mutation in the ASAH1 gene. The clinical appearance initially suggested a form of SMA type 3a , in which gait is acquired but is unstable, and regress sets in quickly with the loss of this acquisition. Because of the different etiopathogenesis in this type of SMA, this case has no indication of gene therapy or antisense oligonucleotide therapy like typical forms of SMA 5q. SMA is a heterogeneous disease. With a very similar phenotype, the etiology of 5q and non 5q SMA can be difficult to distinguish. Cases of non-5q SMA caused by ASAH1 mutations are very rare, with only 45 patients reported worldwide and the first in Romania so far.

Study 3 presented an analysis of clinical and electrographic changes in paediatric SMA after 2 years of treatment with nusinersen. We evaluated a group of 34 patients with SMA types 1, 2 and 3, aged 1-16 years, during 2 years of treatment, with initial clinical and electrophysiological evaluations (T0), and subsequently every 4 months, up to 26 months (T26). The motor scales CHOP for SMA type 1 and HFMSE for types 2 and 3 and 6MWT for ambulants were applied. At the same time, distal CMAP was recorded on the ulnar nerve. The study was conducted between October 2018 and October 2021. Patients with genetic confirmation of SMA with 2 or more copies of SMN2 were included in the study. Patients who were receiving another medicine (risdiplam or onasemnogene abeparvovec-xioi) at that time, or who were agitated or uncooperative, were excluded.

At the start of treatment (T0), the type 1 SMA group comprised 11 non-sitters. After 10 administrations of nusinersen (T26), 45.45% became sitters, with the amplitude of CMAP increasing from 0.53 ± 0.23 to 1.85 ± 1.05. The 54.55% of patients who remained "non-sitters" experienced an increase in CMAP from 0.26 ± 0.23 to 1.19 ± 0.66. At T0, the AMS Type 2 group consisted of 16 sitters. In T26, 4 patients (25%) became walkers, with CMAP amplitude increasing from 2.73 ± 2.08 to 3.65 ± 1.55. The other 12 patients (75%) who remained sitters experienced an increase in CMAP from 0.99 ± 0.91 to 1.73 ± 1.18. The SMA type 3 group consisted of 4 sitters and 3 walkers at both T0 and T26. The sitters' CMAP rise from 1.25 ± 0.55 to 2.05 ± 0.66, while the walkers CMAP rise from 2.73 ± 2.38 to 3.66 ± 2.85. There was a significant increase in CHOP consistent with CMAP in 5 patients for SMA type 1. In patients with SMA type 2, HFMSE was generally stationary, with only 2 of them having significant variations in CMAP above the group average. In patients with SMA

type 3, CMAP/HFMSE showed a stationary tendency between T0 and T26. The most significant increase in distal CMAP amplitude was recorded in patients with SMA type 1, statistically correlated with CHOP scale scores ($p<0.001$). Most patients with SMA type 2 started treatment long after symptom onset (13-96 months), who had CMAP amplitude and stationary HFMSE scores. The majority of patients with SMA type 3 (4 out of 7 patients) were non-ambulant at T0. They began treatment a few years after losing their autonomous gait, remaining non-ambulant at T26. Children with shapes the "borderline" passed into a superior level, some acquiring the sitting position, others assisted / independent gait. Patients who had a higher amplitude of CMAP (viable motoneurons/axons) at the start of treatment had a better clinical outcome, demonstrating that CMAP is a marker of the degenerative process. After 2 years of treatment with nusinersen, children with SMA types 1, 2 and 3 had a favourable clinical outcome, as shown by motor scale scores. Clinical data were correlated with electrophysiological data, with statistical significance in SMA type 1. However, it is important to monitor CMAP in dynamics as an electrophysiological marker in all types of SMA to capture motor regression [50] but also as a predictor of the evolution.

Study 4 analyzed time-effectiveness in patients with SMA types 1 and 2 on nusinersen treatment over 2 years. The main objective of this study was to analyze the correlation between the time of initiation of treatment versus the time of disease onset and the subsequent evolution of clinical and electrophysiological parameters in a group of patients with SMA types 1 and 2 monitored for 2 years [54]. We evaluated a group of 60 cases, 29 children with SMA type 1 and 31 with SMA type 2; 29 cases with 2 copies SMN2 , 29 cases with 3 copies SMN2 and 2 children with 4 copies SMN2 , aged between 3 weeks and 16 years 4 months. Treatment with nusinersen was started between 2018 and 2020 and patients were monitored until 2022. All patients in our study were genetically confirmed MLPA with homozygous mutation in the SMN1 gene (SMA 5q) and were symptomatic at the start of treatment.

The SMA group type 1 (N=29) was relatively homogeneous, characterized by "non-sitters", the onset of symptoms being at 1-3 months of age. The time to diagnosis ranged from 1 to 8 months. The average age at which he started treatment was between 5 and 8 months. Motor regression was observed rapidly, within days or weeks, and the first motor and electro-physiological assessments were performed at the beginning of treatment.

The SMA group type 2 (N=31) was more heterogeneous, characterized by "sitters", with the onset of symptoms between 8-12 months of age, but with different forms of the disease,

from "weak" – 2.1 (Dubowitz) with severe axial hypotonia, with minimal movements in the lower limbs, to "strong" – 2.9 (Dubowitz) who maintains the orthostatic position, but needs support to walk [72].

Looking at the length of time relationship between disease onset/initiation and motor progress, it has been shown that there is a significant correlation in patients with SMA types 1 and 2. The values of the negative correlation coefficient of -0.713 and -0.560 for SMA type 1 and type 2 indicate that as the time between symptom onset and treatment initiation increases, motor function of patients with SMA types 1 and 2 worsens.

In patients with SMA type 1, a statistically significant negative correlation was established between age of disease onset and treatment-related patient progress ($p<0.05$ and $r=-0.378$).

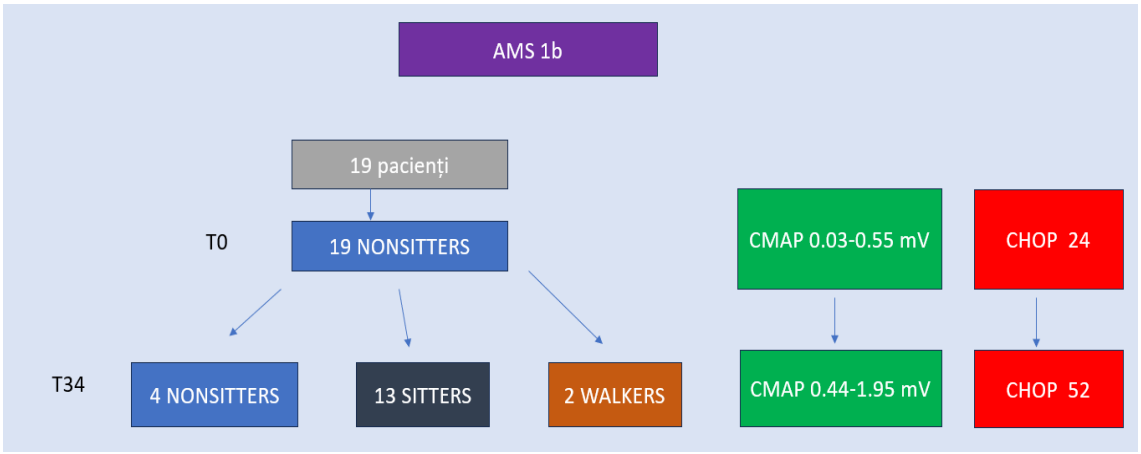
The early age of the patient at the initiation of treatment has a favorable influence on the course of the disease. Thus, there was a strong negative correlation between age at initiation of treatment and evolution on the motor scale, for both types of SMA (type 1: $p<0.0001$ and $r=-0.726$; type 2: $p<0.001$ and $r=-0.553$). The distal amplitude of CMAP at baseline assessment (T0) was found to be significantly correlated with the evolution of motor parameters ($p<0.001$ and $r=0.600$).

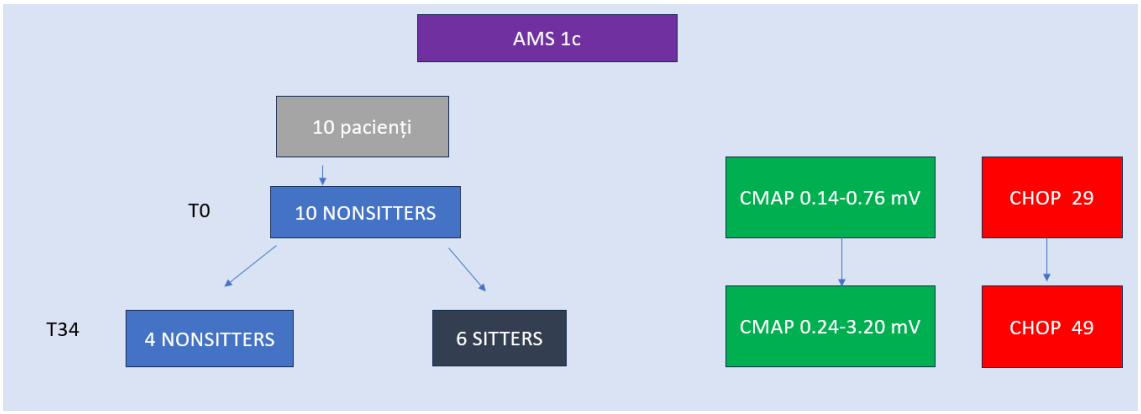
The results of the study showed the importance of early diagnosis and prompt initiation of treatment (ASO) for children with SMA types 1 and 2 in order to maximize clinical outcomes and recover as much as possible from the motor regression already established. It is important to highlight the role of physical therapy alongside drug treatment, which plays an essential role in the recovery and management of patients with SMA, should be considered as part of the overall treatment plan. It has also been shown that compound motor action potential is a predictive factor in disease progression, useful in medical practice to capture motor regression before the first clinical signs appear.

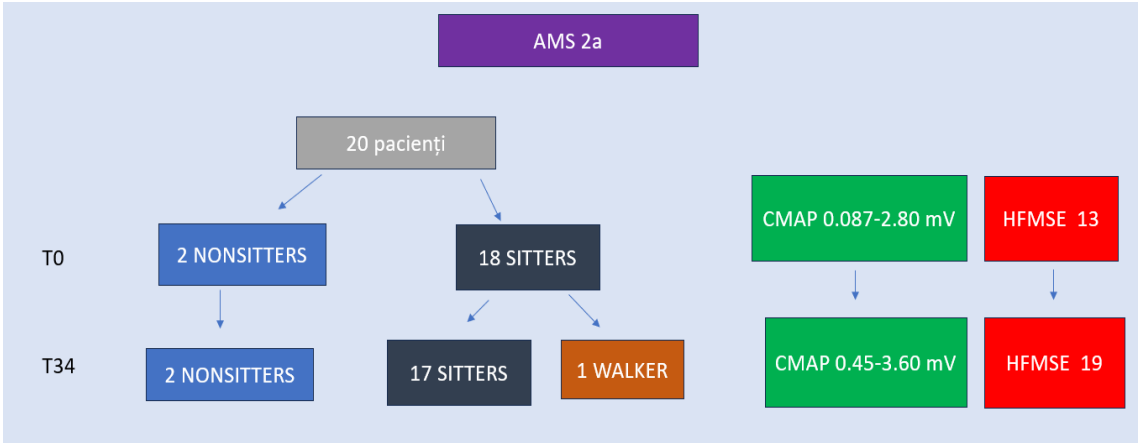
The final study presented final, electrophysiological and clinical data on the entire group of paediatric patients (N=72) with SMA types 1, 2 and 3 treated with nusinersen, after 3 years of follow-up. We analyzed the entire group of patients, all genetically confirmed with biallelic mutation in the SMN1 gene (69 with homozygous deletion and 2 compound heterozygous). 71 patients were symptomatic at baseline and one patient was presymptomatic (diagnosed antenatally and confirmed by perinatal MLPA test). The main objective was to research the electrophysiological and clinical evolution in a representative group of pediatric SMA 5q patients (types 1, 2 and 3) during 3 years of treatment with nusinersen (ASO) by *comparing initial and final data*. The entire group of patients was

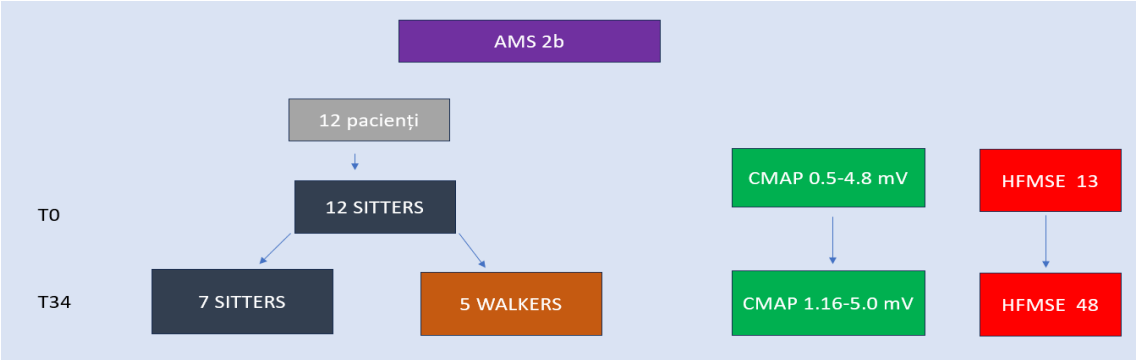
recruited between October 2018 and May 2020 and monitored for 3 years in terms of clinical and electrophysiological evolution under treatment with nusinersen.

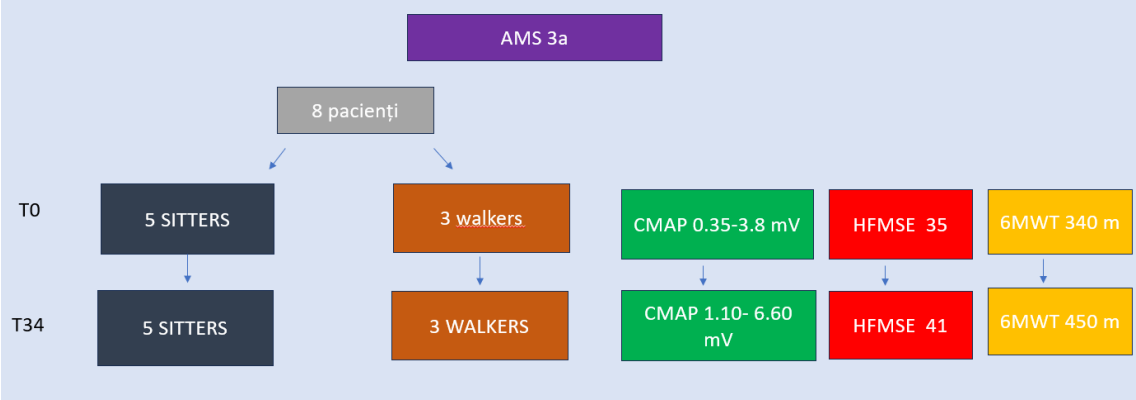
The results on the motor and electrophysiological scales according to the SMA subtype are shown in the figures below

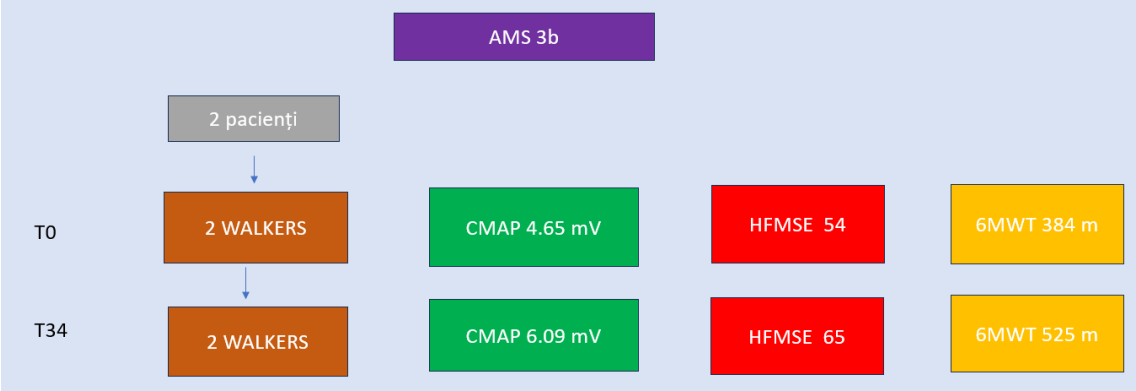












Electrophysiological evolution (distal amplitude of CMAP) from T0 to T34, depending on the number of SMN2 copies - in the group of patients with 2 copies SMN2 (N = 32) the average increase of CMAP was by 0.93 mV, in the group of patients with 3 copies SMN2 the increase in CMAP amplitude was by 0.77 mV, while in those with 4 copies SMN2 the level obtained at the end of the study was not significantly higher than the initiation of treatment 0.70 mV.

A national reference center in pediatric neuromotor rehabilitation, the National Neurorehabilitation Center for Children "Dr Nicolae Robanescu", where spinal muscular atrophy was well known and previously treated conventionally applying standards of care,

was the first hospital in Romania where nusinersen treatment was initiated in October 2018. The high addressability, the important number of patients recruited made it possible to initiate this study for the first time in Romania.

Patients were recruited between October 2018 and May 2020, and the last assessments in the study were conducted in May 2023. Preliminary data have been published through previously presented studies (studies 1-4).

Out of a total of ***39 patients*** with type 1 only 8 patients remained in the same motor level ("nonsitters"), the remaining **31** obtained and retained ***significant new motor acquisitions***, 2 reaching independent gait. All patients in this group survived, there was no motor regression. All improved bulbar function, with some requiring noninvasive ventilation on the mask only during the night. For the entire group of patients with SMA type 1 there were strongly positive correlations with statistical significance between the initial and final electrophysiological results CHOP (T34) and CMAP (T34), between the yield on the CHOP scale and CMAP (T0), but also strongly negative correlations between *the age of initiation of treatment* and *CHOP(T34)/CMAP(T34)*, these data demonstrating that motor and electrophysiological evolution parameters were consistent during the monitoring period, but also that motor response depends on early initiation of treatment.

The SMA type 2 group was the most heterogeneous with 32 patients, with the onset of symptoms in this form between 6-18 months after the child acquired the sitting position. In evolution, severe forms show relatively rapid motor regression, with the loss of this motor ability becoming "nonsitters" (subtype 2a). The more stable, slower-evolving form (subtype 2b) may preserve sitting for years, but due to axial hypotonia, the patient develops severe, progressive forms of scoliosis that require surgery before age 10. From subtype 2a of the 18 patients "sitters" 1 became „walker”, and in form 2b of the 12 patients "sitters" 7 became "walkers", with correlations with statistical significance between electrophysiological and motor parameters.

The 10 patients with SMA type 3, relatively homogeneous group, 8 patients in subtype 3a and 2 patients in subtype 3b, all keeps at the same motor level, did not regress, and those who were initially ambulant, improved their balance and endurance in walking (walking distance) having an increase on the 6MWT scale of 30-40%.

Regarding the reporting of the results obtained to the natural course of the disease, literature data show groups of patients restricted with SMA without treatment in clinical trials that preceded the approval of nusinersen (before 2018). Depending on the type of SMA, the evolution of these untreated patients was rapidly progressive, with significant

motor decline in early forms, to death (type 1) by the age of 18-24 months, or loss of motor skills previously gained in late forms. Compared to this evolution, the introduction of disease-modifying treatment has greatly changed the course of the disease for these patients, creating new phenotypes. Although it is not curative, however, early administration, before the onset of motor regress (regardless of the type of disease) created the premises for normal development and increased hope for autonomy.

The presymptomatic patient, diagnosed antenatally and confirmed immediately after birth (sister of a patient with type 2a), had normal CMAP and CHOP values at onset, without motor involvement, treatment with nusinersen was initiated at 2 weeks of life, and during the three years of monitoring all motor and electrophysiological parameters remained normal, without regression. Motor and cognitive development was within normal limits.

During the study we did not encounter any special incidents, all examinations went accordingly. The group of patients was heterogeneous, with very different age groups and motor evolution/regression prior to initiation of treatment, which ranged from a few weeks to years, this being reflected in the response to treatment.

Conclusions

The introduction of nusinersen treatment changed the paradigm on the evolution of this neurodegenerative condition. The monitoring of functional and electrophysiological parameters of a group of 72 patients over 3 years confirmed that the effectiveness of the treatment was undeniable. Although there were 45% SMA type 1 in the group, there was no death, 21/29 patients made motor progress, no significant regression. In type 2 - 6/32 patients became ambulants, and in type 3 the ambulants kept walking and increased endurance, without regression.

We demonstrated how important it is when the treatment is introduced, i.e. before the onset of motor regression, which is correlated with the baseline CMAP value and functional score. These 2 parameters correlated with each other have proven predictive factors in further evolution under treatment. Even if the diagnosis of SMA is genetic, electrophysiological evaluation has shown that CMAP can be a useful parameter, an important predictor in establishing the prognosis, more sensitive than functional scale evaluation or clinical evaluation. Thus, a slight decrease in CMAP may mean the onset of motor regression, and initiation of treatment starting from a low CMAP value may be a negative prognostic factor in terms of disease progression.

Following a presymptomatic patient and finding that his evolution remained within normal limits, compared to his brother diagnosed and treated late, who made minimal

progress that does not allow autonomy, opens new perspectives in creating the premises for the implementation of neonatal screening for spinal muscular atrophy in Romania.

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