Research article

The dynamic of changes of pNFH levels in the CSF compared with the motor scales’ scores during three years of nusinersen treatment in children with spinal muscular atrophy types 2 and 3

Mihaela Badina 1,2, Corina Sporea 1,2,* , Gabriel Cristian Bejan 1,2,*, Andrada Mirea 1,2,*, Daniela Adriana Ion 1

Abstract: Neurofilaments are crucial in neuronal cytoskeleton formation, influencing axonal growth and impulse modulation. This study focuses on understanding the dynamics of the phosphorylated neurofilament heavy subunit (pNFH) in pediatric spinal muscular atrophy (SMA) patients undergoing Nusinersen treatment. The presence of five neurofilament types, particularly pNFH, is explored as a potential biomarker. SMA, an autosomal recessive disease impacting motor neurons, is characterized by disease severity linked to the number of SMN2 gene copies. Approved drugs, including Nusinersen, have demonstrated efficacy in enhancing motor activity. Methods: A retrospective analysis was conducted on 18 pediatric SMA patients treated with Nusinersen from October 2018 to July 2023. Cerebrospinal fluid (CSF) samples were utilized to assess pNFH levels. Motor scales were employed to evaluate performance, focusing on patients with varying SMN2 gene copies. Results: Following the initiation of Nusinersen treatment, a substantial decrease in pNFH levels was observed in CSF samples. Motor scales indicated improved performance, particularly in patients with more SMN2 copies. However, the correlation between pNFH levels and motor improvement was not strongly evident, suggesting a limited role as a prognostic indicator within this timeframe. Conclusion: Nusinersen effectively reduced pNFH levels in pediatric SMA patients, showcasing promising outcomes in motor function. However, the predictive value of pNFH remains inconclusive, emphasizing the need for further research. Study limitations, including the rarity of SMA, the absence of a control group, and the disease’s dynamic nature over time, should be considered when interpreting these findings.

Keywords: Neurofilaments; phosphorylated neurofilament heavy subunit (pNFH); spinal muscular atrophy (SMA); SMN2 gene; Nusinersen; biomarkers; pediatric population; motor function; cerebrospinal fluid (CSF); treatment efficacy

Introduction

Neurofilaments are specific neuronal protein heteropolymers that, in addition to participating in the formation of the structure of the neuronal cytoskeleton, also contribute to the growth and maintenance of the axonal diameter and have a role in the modulation of nerve impulses [1]. From a structural point of view, neurofilament structure has a constant central unit of approximately 310 amino acids, a short, variable, amino-terminal end, and a carboxy-terminal end whose length determines the size of the molecule and at
which the chemical processes of glycosylation and phosphorylation take place. Of the five types of neurofilaments, heavy neurofilaments (NFH), medium neurofilaments (NFM), light neurofilaments (NFL) that are found both in the central and peripheral nervous system, internexin exist only in the central nervous system, and peripherin only in the peripheral nervous system, pNFH represents the most stable phosphorylated subunit, with the largest size and found in the most significant quantity [2,3].

In various clinical studies, pNFH and other parameters affected by the disease have been correlated with diagnosis, evolution, prognosis, and the effectiveness of disease-modifying treatment, even if pNFH is a non-specific marker for neuronal damage [4,5].

The amount of neurofilaments present in the CSF and blood is directly related to the degree of damage to the neurons and depends on the disease that caused the damage, mainly since the results of treatment for many progressive neurological diseases are correlated with the time of starting the treatment [4–7].

Neurofilaments have been used lately as biomarkers to increase the accuracy of diagnosis, prognosis, and evolution under treatment for neurodegenerative diseases, being exclusive structural elements at the neuronal level, which appear in small amounts in CSF as a result of normal neuronal metabolism but are excessively released in case of neuronal injury or death [6,7].

Spinal muscular atrophy, although a rare disease with an incidence of 1:10,000, is one of the most common causes of mortality and morbidity among the pediatric population [8]. SMA belongs to neurodegenerative diseases that evolve with the progressive degradation and death of motor neurons and the release of their components into the CSF, including neurofilaments [9].

The cause of this rare autosomal recessive disease is the insufficient synthesis of the protein necessary for the survival of motor neurons (SMN) due to a deletion or mutation in the SMN1 gene, a genetic defect that cancels the production of the protein by the SMN1 gene that under normal conditions produces 80-90% of the amount of the SMN protein [8,10].

The remaining 10-20% of the functional SMN protein is produced by the SMN2 gene, which differs from the SMN1 gene because of a single nucleotide at position 840 (C → T), thus creating a shorter protein component that is rapidly degraded in cells [11].

The amount of SMN produced also depends on the number of copies of the SMN2 gene, which influences clinical manifestations, the age of symptoms onset, evolution, prognosis, and treatment possibilities [12,13].

Depending on the age of clinical manifestations, spinal amyotrophy cases were classified into five types before the appearance of specific therapy. In type 0 - the most severe form, patients have symptoms since birth or even from the prenatal period; they usually survive for less than a month and have only one copy of the SMN2 gene. In type 1 SMA, symptoms appear under six months of life, life expectancy is under 24 months, and they generally have two copies of the SMN2 gene [9].

Type 2 patients frequently have three copies of the SMN2 gene, with the onset of symptoms under 18 months, and live for over 24 months. Those with type 3 have 3-4 copies of the SMN2 gene, with clinical manifestations occurring over 18 months and reaching adult age but with a period of gradual loss of physical acquisitions. The fourth type of SMA is unique to adults, with symptoms emerging at over 20 years of age and characterized by having 4-8 copies of the SMN2 gene [13].

Currently, three drugs are approved for treating SMA by mutation or deleting the SMN1 gene in the q11.2-q13.3 region of chromosome 5. Nusinersen (Spinraza) – approved in December 2016 by the FDA and in 2018 in Romania, is an antisense nucleotide for the SMN2 gene that prevents premature splicing, thus forcing the gene to produce more functional protein [14]. The treatment is administered intrathecally, frequently through lumbar puncture, according to a particular therapeutic scheme [15]. Onasemnogene abeparvovec-xioi (Zolgensma), the second drug approved in 2019 for the treatment of SMA, is part of the category of gene therapies based on an adenovirus as a vector for providing a functional copy of the SMN1 gene for the synthesis of the SMN protein, and it is administered intravenously, in a unique dose. The third drug, approved in 2020, is
Risdiplam (Evrysdy), with the exact mechanism of action as Nusinersen; it has a much smaller molecule, crosses the blood-brain barrier, and is administered orally daily [13].

Studies on the effectiveness of the treatment showed significant improvements in the motor activity of patients treated with any of the three types of approved drugs compared to the control groups, as proven by the results obtained on the functional scales specific to patients with spinal amyotrophy [14]. Thus, while there were visible improvements in the group of treated patients, in the control group, the disease followed its natural evolution, and the condition of the patients gradually deteriorated [13].

The evolution of the motor activity of patients with spinal amyotrophy is evaluated on functional motor scales, with different degrees of complexity, created especially for patients with this diagnosis, compared to the physical abilities of healthy children. Various aspects are observed, ranging from posture and walking, with or without support, to voluntary movements. The Children’s Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) was created for the evaluation of patients with spinal amyotrophy type 1 due to their limited motor possibilities, with a maximum of 64 points obtained by evaluating 16 parameters with scored results from 0 to 4 and is currently used for patients under two years of age or with very advanced motor deficits [16].

The Hammersmith Infant Neurological Examination (HINE-2) is used to evaluate patients between two months and two years old. It examines neurological aspects, including cranial nerve activity, posture, reflexes, tone, and movements, with a maximum of 26 points for motor evaluation [17].

Hammersmith Functional Motor Scale Expanded (HFMSE) is a scale with 33 parameters, each evaluated with a maximum of 2 points, and it is used mainly for patients with SMA type 2 and 3 with limited ambulation [18].

The Revised Upper Limb Module (RULM) is a scale used to evaluate the strength and motor function of the arm, forearm, and hand for patients over 30 months old [19].

The 6-minute walk test (6MWT) measures the distance traveled by the patient in 6 minutes on a flat plane: the distance traveled in the first minute is compared to that traveled in the last minute to assess the degree of fatigue. It is used for ambulatory patients over one year old [20].

2. Materials and Methods

Our study followed the changes in the level of pNF-H in the CSF during three years of maintenance treatment with nusinersen and the comparative analysis of this level with the values from the beginning of the treatment and the beginning of the maintenance period for different ages and types of SMA, and the results expressed through evolution on the functional motor scales.

Nusinersen is administered to influence the synthesis of the SMN protein necessary for the survival of motor neurons in the appropriate quantity and quality. This stops the degradation of motor neurons and the natural evolution of the disease, as clinically objectively demonstrated by the results obtained on the functional motor scales.

The administered treatment dose is calculated to cover the physiological requirements, and the level of neurofilaments released in the CSF would be similar to that of healthy people, given by normal neuronal metabolic processes, and to that of other neurodegenerative diseases with disease-modifying treatment.

This monocentric retrospective study encompassed 18 patients diagnosed with spinal muscular atrophy (SMA) who received Nusinersen treatment between October 2018 and July 2023 through the national program within The National Teaching Center for Children’s Neuromuscular Rehabilitation “Dr. Nicolae Robanescu”.

The study assessed the phosphorylated neurofilament heavy chain (pNF-H) level in cerebrospinal fluid (CSF) three years after initiating Nusinersen treatment, specifically at 38 months. Age, sex, SMA type, SMN2 gene copy number, and functional motor scale evolution were analyzed at various time points, including before treatment initiation, six months (when maintenance treatment started), and ten months post-treatment.
Participants under 18 years with SMA types 2 or 3, confirmed SMN1 gene mutation/deletion, at least two SMN2 gene copies, and a history of at least 13 administered treatment doses were included.

Exclusion criteria encompassed the administration of other disease-modifying treatments, Nusinersen treatment outside the center, non-adherence to the treatment regimen, and conditions influencing neurofilament levels, motor performance, or CSF circulation.

A multidisciplinary team conducted evaluations. Pediatricians and neurologists assessed clinical parameters, physiotherapists evaluated functional aspects, and laboratory doctors validated biological parameters.

Physiotherapists evaluated mobility and physical ability using recommended scales. Motor assessments were conducted by the same team administering treatment doses to minimize subjectivity.

CSF samples were collected before each Nusinersen administration and analyzed for macroscopic appearance, chlorine content, leukocyte content, and pNFH levels. Visual scales and laboratory instruments were employed for assessment and analysis.

The sample size for our study was not determined through a formal power analysis; it comprised all eligible patients from our hospital who met the predefined inclusion and exclusion criteria. Due to the rare nature of the disease and the limited number of patients meeting the specific requirements, the study aimed to include the entire available population.

Given the inherent challenges associated with recruiting patients for rare diseases, particularly when applying strict inclusion criteria, our study design prioritized the inclusion of all eligible individuals to maximize the available data. Although a formal power analysis was not conducted, the study’s focus on the entire accessible patient population aims to provide a comprehensive exploration of the treatment’s effects within the constraints of the available sample.

Our study did not have a separate control group. We leveraged the treatment evolution itself as a form of control by analyzing data collected before the initiation of treatment. Specifically, our ‘Control Group’ consisted of measurements taken before administering the first nusinersen dose. This approach allowed us to assess the natural progression of the condition before intervention, providing a baseline for comparison with subsequent treatment outcomes.

All patients have consented to participate through their authorized legal guardians – by local regulations and the World Medical Association Declaration of Helsinki, revised in 2000 in Edinburgh.

Informed consent was obtained in writing after prior information of the patients and their families. The information was provided in Romanian writing using an accessible language and was complemented by face-to-face discussions with the study investigator. Patients and their relatives were informed in advance, at the first admission, before the commencement of the study. They received comprehensive information about the study objectives, samples to be taken, data processing, and the anonymization of final results to prevent patient identification. Participants were assured that participation is voluntary, unpaid, and has no impact on their hospital treatment. Informed consent can be withdrawn at any time without repercussions. Contact details of the study investigator were provided for inquiries.

Our study received prior approval from the Ethics Committee of the National University Center for Children Neurorehabilitation “Dr. Nicolae Robanescu” (protocol code 7464, approved on October 1st, 2018).

Data confidentiality was ensured through secure storage and anonymization, preventing patient identification.

Participants were informed of their right to withdraw from the study by notifying the investigator with no consequences for their continued treatment.

For these patients, the level of pNFH neurofilaments in the cerebrospinal fluid was determined three years after the Nusinersen treatment initiation, more precisely at 38 months. The results were analyzed both according to age, sex, type of SMA, and the
number of copies of the SMN2 gene, as well as from the point of view of the evolution of the functional motor scales compared to the values obtained before the initiation of the treatment, at six months - when the maintenance treatment was started, and at ten months after the initiation of Nusinersen administration.

Patients under the age of 18, diagnosed with spinal amyotrophy type 2 or 3, a disease due to a mutation/deletion of the SMN1 gene in the long arm of chromosome 5, with at least two copies of the SMN2 gene, were accepted in the study, with evaluations and treatment performed in our center, by the same staff, with at least 13 treatment doses administered.

Administration of another disease-modifying treatment, administration of Nusinersen and evaluation in another center, failure to adhere to the treatment regimen, or conditions that could increase neurofilament levels, influence motor performance, or affect CSF circulation were exclusion criteria.

Pediatricians and neurologists evaluated the clinical parameters, physiotherapists established the functional ones, and laboratory doctors checked and validated the biological parameters. All the staff qualified for the particularities of these activities.

After the neurological and pediatric consultations, the patients were evaluated by physiotherapists on the mobility and physical ability assessment scales for patients with SMA, recommended according to age and locomotor status, by qualified staff specialized in the assessment of children with SMA within The National Teaching Center for Children's Neurorehabilitation "Dr. Nicolae Robanescu". Motor assessments were performed just before the same team administered the treatment dose to eliminate the risk of subjectivity.

Approximately 5 ml of cerebrospinal fluid was collected steriley during lumbar puncture just before each drug administration to administer Nusinersen treatment. It was aliquoted into two samples, one for immediate pre-storage analysis and the other for determination of the level of pNFH, preserved by freezing at a minimum temperature of -20°C, according to the instructions of the analysis kit manufacturer.

The CSF samples were studied after collection from the point of view of the macroscopic aspect - by visual qualitative assessment of the chlorine content - by the potentiometric method with the ionometer I Smart-30 Pro (i-Sens, Inc., Seoul, Republic of Korea) and of leukocyte content - by optical microscopy, with a Nikon Eclipse E 100/300 microscope (Nikon, Minato City, Tokyo, Japan), in the Burker-Türk counting chamber (Fischer Scientific Company L.l.c., P.O. Box 1768, Pittsburgh, PA 15275, US).

The visual assessment of the macroscopic appearance of CSF was made using the following visual scale with four steps for color: 0 – clear, colorless liquid; 1 – clear liquid, with a slight shade of pink; 2- clear liquid with an intense shade of pink; 3 – liquid with hemorrhagic appearance, and two situations were considered for turbidity: clear appearance – 0 – McFarland standard; cloudy - any other value.

The pNF-H level was determined by the ELISA technique, according to the instructions of the manufacturer of the reagent kit - EUROIMMUN Medizinische Labordiagnostika AG (Seekamp 31, 23560 Lübeck, Germany), using the ELISA BIORAD 3100 PSC microplate reader (Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547, USA).

Participants’ demographic, anthropometric, and clinical data were retrieved from patient observation sheets and data routinely recorded in the hospital computer program.

Statistical analyses were conducted using IBM SPSS Statistics 24 and Microsoft Excel 2021, ensuring a robust examination of our data. Before analysis, we employed the D’Agostino-Pearson test to assess the normality of the distributions. For data displaying uniform distribution, means ± standard deviations (SD) were calculated, and the paired t-test was applied. In cases of abnormal distribution, medians and interquartile ranges (IQR) were computed, and the Wilcoxon test was utilized.

The choice of statistical tests was deliberate and aimed at ensuring the validity of our analysis. Two-tailed statistical tests, including the Mann-Whitney test for group comparisons and the Spearman coefficient for assessing associations between variables with non-normal distributions, were selected based on the characteristics of our data.
The Mann-Whitney test was chosen for comparing groups. It provides a robust non-parametric alternative to the t-test, particularly suited for variables with skewed distributions or outliers. The Spearman coefficient, a non-parametric measure of correlation, was employed to assess associations between variables that did not conform to a normal distribution.

A significance level of \( p < 0.05 \) was established to determine statistical significance, guiding the interpretation of our results. These statistical methods were selected to suit the nature of our data and ensure the validity and reliability of our findings.

3. Results

The prevalence of spinal amyotrophy types in the patients included in the study was 61.11% (11 patients – 8 male and three female) in the case of type 2 SMA and 38.89% (7 patients – 3 male and four female) in the case of SMA type 3. Depending on gender, type 3 SMA was more frequent in females (57.14%, four patients), while in males, most patients had type 2 SMA (11 patients).

In male patients, 2 copies of SMN2 genes predominated with 90.9% (10 patients), while in female patients, carriers of 2 SMN2 genes 42.9% (3 patients) were in equal number with carriers of 3 SMN2 genes, with 42.9% (3 patients).

Regarding the SMN2 copy number, 13 patients had 3 SMN2 copies, 4 had 2 copies, and only one had 4 copies of the SMN2 gene. Among the 18 patients, 10 male patients had 3 SMN2 copies, and the only patient with 4 SMN2 copies was female.

The youngest patient was 13 months old at treatment initiation, diagnosed with type 2 SMA and with 2 copies of the SMN2 gene. The mean age for patients with SMA type 2 ranged from 13 months to 177 months, with a mean of 85.8 months, and those with SMA type 3 ranged from 50 to 185 months, with a mean of 108.1 months. Depending on the number of SMN2 copies, patients with 2 copies were between 46 and 76 months, with a mean of 61.5 months, and those with 3 SMN2 copies were between 13 and 185 months, with a mean of 103.1 months. The characteristics of the study group are shown in Table 1.

Table 1. Characteristics of the study group participants

<table>
<thead>
<tr>
<th>No of SMN2 copies</th>
<th>SMA All types</th>
<th>Type 2</th>
<th>Type 3</th>
<th>2 copies</th>
<th>&gt; 2 copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Sex – Male</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Age – month Min + Max</td>
<td>13.00 ÷ 185.00</td>
<td>13.00 ÷ 177.00</td>
<td>50.00 ÷ 185.00</td>
<td>46.00 ÷ 76.00</td>
<td>13.00 ÷ 185.00</td>
</tr>
<tr>
<td>(Mean)</td>
<td>(94.50)</td>
<td>(85.82)</td>
<td>(108.14)</td>
<td>(61.50)</td>
<td>(103.93)</td>
</tr>
<tr>
<td>SMN Copies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(No of cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 copies</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 copies</td>
<td>13</td>
<td>8</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 copies</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The time points of data analysis for the present study were: T0 (before administration of the first loading dose), T1 (before administration of the first maintenance dose – approximately six months (± 2 weeks) after initiation of treatment - dose 5), T2 (before the administration of the second maintenance dose – roughly ten months ± 2 weeks after initiation of therapy – the sixth dose) and T3 (before the administration of the thirteenth dose of treatment – approximately three years after initiation).

Evaluation of motor performance - on the scales for evaluating mobility and physical abilities specific to patients with SMA recommended according to age and locomotor status, and for the present study, two scales were taken into account: CHOP INTEND (specially created for children with SMA between 1.4 and 38 months, with a score between 0 and 64 points for a scale of 16 requirements with 4 degrees of response complexity each) and HFMSE (for patients over 24 months, with 33 operations scored between 0 and 2 points, with a maximum of 66 points). To compare the data, percentages relative to the
maximum possible value of the scores obtained on the two scales were used. From the point of view of the evolution of the functional motor scales, a performance improvement was observed for each patient and moment. The evolution of the patients on the motor scales was also analyzed from the point of view of the performance against the maximum value available for different time intervals, as shown in Figure 1.

![Figure 1](image1)

**Figure 1.** Percentage of the maximum possible score of the motor scale per patient (left) and per group (right) at the analyzed milestones

After analyzing the results, we observed that the level of pNFH neurofilaments at the time of treatment initiation had the highest level for most patients, gradually decreasing with each time studied, with a normal distribution for I1, I5, and I13 on the Shapiro-Wilk Test. Table 2 presents the level of pNFH according to SMA type, number of SMN2 copies, and analyzed moment.

**Table 2.** pNFH level according to SMA type, number of SMN2 copies, and analyzed moment

<table>
<thead>
<tr>
<th></th>
<th>SMA Type 2</th>
<th>SMA Type 3</th>
<th>No of SMN2 copies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 copies</td>
</tr>
<tr>
<td><strong>pNFH at I1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min ± Max</td>
<td>0.035 ± 0.817</td>
<td>0.040 ± 0.180</td>
<td>0.110 ± 0.191</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.259 ± 0.072</td>
<td>0.137 ± 0.019</td>
<td>0.167 ± 0.019</td>
</tr>
<tr>
<td><strong>pNFH at I5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min ± Max</td>
<td>0.014 ± 0.772</td>
<td>0.026 ± 0.058</td>
<td>0.026 ± 0.062</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.113 ± 0.066</td>
<td>0.039 ± 0.004</td>
<td>0.052 ± 0.009</td>
</tr>
<tr>
<td><strong>pNFH at I6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min ± Max</td>
<td>0.005 ± 0.088</td>
<td>0.015 ± 0.112</td>
<td>0.018 ± 0.078</td>
</tr>
<tr>
<td>Median*</td>
<td>0.043</td>
<td>0.036</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>pNFH at I13</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min ± Max</td>
<td>0.016 ± 0.115</td>
<td>0.026 ± 0.062</td>
<td>0.020 ± 0.062</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.045 ± 0.010</td>
<td>0.036 ± 0.005</td>
<td>0.033 ± 0.010</td>
</tr>
</tbody>
</table>

Figure 2 shows the evolution of pNFH level according to patients’ age for each treatment administration moment.

![Figure 2](image2)

**Figure 2.** pNFH level according to patients’ age
The only exception, patient 16, had an increase in the level of neurofilaments before the start of the maintenance period due to an acute event before that time. However, the evolution over time was similar to that of the other patients.

Three years after initiation of treatment, pNFH levels in CSF ranged from a maximum of 0.115 ng/ml for a female patient with SMA type 2 and 4 copies of the SMN2 gene to a minimum of 0.016 ng/ml in a male patient, with SMA type 2 and 3 copies of the SMN2 gene, the youngest patient in the study group, therefore the patient who started the earliest Nusinersen therapy (13 weeks of life).

Compared to the pNFH level at the 13th drug dose, at the initiation of treatment, both the lowest value and the highest value of the neurofilament level in the cerebrospinal fluid were observed for one patient with SMA type 2 with 3 SMN2 copies, which suggests that the level of neurofilaments is not only related to the type of SMA and the number of SMN2 copies but also the age of the patients at initiation and the time of initiation of treatment depending on the time elapsed since the onset of symptoms.

At the fifth dose of treatment, neurofilament values dropped dramatically, on average, to one-tenth of the initial values, below 0.1 ng/ml, for most patients except for one patient with type 2 SMA and 3 copies with SMN2, whose value was 0.772 ng/ml. Approximately one year after the initiation of treatment, a decrease in the pNFH level of all patients to values below 0.112 ng/ml was observed (Figure 3).

![Figure 3. Evolution of pNFH level per patient (left) and per group (right)](image)

The macroscopic appearance of the samples (from the point of view of turbidity and color), the concentration of chlorine ions, and the number of nucleated cells per cubic millimeter did not significantly correlate with any of the situations (administration of maintenance doses from 3 years, one year, six months and the initiation of treatment with Nusinersen) with the level of pNFH in CSF.

![Figure 4. The percentage evolution of the pNFH level compared to II per patient (left) and per group (right)](image)
From the analysis of the obtained data, there are no statistically significant correlations between the level of pNFH in the CSF at the 13th injection and the time/moment of treatment initiation, the type of spinal amyotrophy, the number of copies of the SMN2 gene, or the patients’ sex.

The dynamics of the motor evolution according to the yield obtained on the motor evaluation scales after three years of treatment compared to the moment of its initiation does not significantly depend on the sex, age, or type of SMA of the patients, as shown in Figures 5 and 6.

Instead, there is a strong negative correlation with the number of copies of the SMN2 gene ($r=-0.527$, $p=0.024$) and a positive correlation with the motor scales from the initiation of the treatment, both in absolute value and in percentage obtained compared to the maximum possible score of the scales ($r=0.688$ and 0.689, respectively). There are also positive and robust correlations between this performance and the score obtained on the motor scales both from the moment of starting the maintenance treatment (upon the administration of the fifth dose of Nusinersen) with $r=0.807$ (both in relative value and absolute value), approximately one year after the initiation of treatment (for both situations, $r=0.777$) and after three years of treatment ($r=0.902$ in relative value and $r=0.899$ in absolute value), with the performance obtained on the motor scales in the first six months of treatment ($r=0.912$) or up to the sixth dose of the drug ($r=0.846$) and with the yield obtained during the maintenance treatment until the time of the last evaluation taken in the study (I13-I5 with $r=0.975$, $p =0.0001$).

Regarding the level of pNFH neurofilaments three years after the treatment initiation, there are strong correlations in the studied group only with the evolution of the level of
pNFH in the CSF during the maintenance treatment, i.e., before the administration of the 5th and 13th doses of medicine ($p=-0.676$, $r=0.002$). Thus, after three years of treatment, the level of neurofilaments in the CSF does not correlate with the age of the patient at the start of the treatment or with sex, nor with the type of SMA in which it was categorized based on the symptoms at the beginning or with the number of copies of the SMN2 gene. Also, with a $p>0.05$, there are correlations of this level of pNFH with that of all three previous moments followed in the study, as well as with the motor performances in absolute values of the scores on the functional motor scales and relative to the maximum possible of the respective scales, as well as motor yields between all previous periods.

Moreover, the relative change in the pNFH level calculated as a percentage of the value obtained after three years of treatment compared to the initial value before the initiation of therapy does not significantly correlate with any studied aspect (age at initiation of treatment, gender of the patient, type of SMA, the number of SMN2 copies, the scores obtained at different moments on the motor evaluation scales, the yield obtained between different moments evaluated or the level of pNFH in the CSF before the other administrations).

4. Discussion

pNF-H neurofilaments, a specific neuronal structure, reach the CSF as a result of the normal metabolism of motor neurons in the central or peripheral nervous system, and from here they are released into the blood [21]. The level of neurofilaments in blood for healthy individuals is 40 times lower than that in CSF [6]. In case of injury or degradation of these neurons, larger amounts of neurofilaments reach the CSF and blood, proportional to the size of the neuronal injury.

During treatment with Nusinersen, an improvement in motor performance was observed in most patients, while the level of neurofilaments in the cerebrospinal fluid decreased significantly compared to the initial value before the start of treatment, the results being similar to those published in other studies for different periods and different groups of patients [22–24].

The fastest decrease in the pNFH level was obtained in the first six months of treatment with Nusinersen, as was also observed in the studies for adult patients, with forms of SMA with late-onset (type 3 and 4) as proof of the effectiveness of the treatment in slowing down or even stopping the neuronal degradation due to the existence of an insufficient amount of protein necessary for the survival of motor neurons [23].

The obtained scores on the motor evaluation scales suggest a stop in the degradation of motor functions or even a gradual improvement in performance with the passage of time and the continuation of the treatment administration.

The rate of decrease in pNFH does not correlate with a faster improvement of motor performance in any of the studied moments (at 6, 8, or 38 months after the initiation of treatment), with pNFH level not being able to be taken into account as a predictive factor of the patients’ evolution, but only as a diagnostic criterion, compared to the studies carried out on patients with ALS where the level of pNFH had both diagnostic and prognostic value [25].

The insignificant correlation of the decrease in the level of pNF-H in the CSF with the increase in motor scores may be due to the varied time interval between the onset of symptoms and the initiation of therapy, the different degrees of motor impairment at the first injection, and the intensity of individual physical therapy, which plays a significant role in recovering the functionality of these patients [24].

The results obtained are similar to those described in the analyses on the level of pNF-H in CSF from the SHINE, NURTURE, EMBRACE, ENDEAR, and CHERISH studies on the control groups (the healthy population aged between 0 and 20 years) and the population diagnosed with SMA (before treatment and with or without treatment) [21].

During the initiation period in SMA types 2 and 3, the evolution of the level of neurofilaments is similar to that described in studies on adult patients, totally different from the results obtained for patients with SMA type 1, which show a more pronounced
rate of decrease in the level of pNFH neurofilaments from the CSF under treatment with Nusinersen relative to the value from the moment of the initiation of therapy. The amount of existing pNFH depends on the number of viable neurons, the rate of degradation, and the elimination of pNFH from the CSF [21,23,26].

The highest values of pNF-H neurofilaments were observed before the initiation of treatment and the average per subgroup of patients was higher for patients with SMA type 2 compared to those with type 3, similar to the data from the studies on the effects of nusinersen on the pediatric population with SMA [21,23]. On the other hand, compared to the results from other studies, patients with 2 copies of the SMN2 gene had lower average values of pNF-H in CSF compared to those with more than 2 copies of the SMN2 gene, a fact probably also influenced by the almost double number of patients with more than 2 copies of the SMN2 gene, but belonging to type 2 of SMA, according to the age of onset of the symptoms, an aspect that underlines the fact that the SMN2 gene cannot provide the necessary SMN regardless of the number of existing copies.

The fact that the youngest patient had the highest pNF-H value before initiation of treatment suggests a more accelerated rate of neuronal destruction in the first months of life, underscoring the importance of neonatal screening for SMA with the advent of disease-modifying therapy.

The increase in the level of pNFH was observed both in patients with multiple sclerosis during periods of disease activity and correlated with the progression of the disease in amyotrophic lateral sclerosis. However, it could be helpful in the differential diagnosis of these diseases for patients with late-onset forms of SMA [27,28].

Monitoring the level of pNF-H in SMA brings information about the effectiveness of the treatment in stopping neuronal degradation due to the deficiency of the SMN protein, but it can also signal the appearance of an associated condition on the motor neurons before other clinical manifestations.

5. Conclusions

During treatment with Nusinersen in the pediatric population diagnosed with less severe forms of SMA (types 2 and 3), the level of pNFH in the CSF decreased significantly, especially during the loading period. After that, it remained at a low level compared to the initial values in conditions in which no external events would produce damage or degradation of the motor neurons from other causes.

The decrease in pNFH is accompanied by improvement in the scores on the motor evaluation scales. However, the two parameters do not correlate significantly, at least for the group studied during three years of treatment with Nusinersen.

The increase in the level of pNFH in the CSF in conditions of some clinical types with late onset of spinal amyotrophy in pediatric patients may be part of the diagnostic criteria. Still, at least in the first three years of treatment with Nusinersen, it has no proven predictive value for these patients.

6. Limitation

One notable limitation of our study is the relatively small number of patients enrolled. This constraint arises from the rarity of spinal muscular atrophy (SMA), coupled with the recent introduction of the modifying disease treatment in Romania, which commenced in October 2018. The limited pool of eligible participants affects the generalizability of our findings and necessitates cautious interpretation.

Being an evolutionary disease with serious sequelae and due to the appearance of specific disease-modifying treatments, all diagnosed patients are included in different treatment schemes so that a control group is not available. While we acknowledge the absence of a traditional control group, this methodological choice enabled us to draw insights into the treatment effects by contrasting post-treatment measurements with the pre-treatment baseline. Given the inherent challenges in recruiting and establishing control groups for rare diseases, we believe this design offers valuable perspectives on the impact of nusinersen on the studied parameters.
Furthermore, the duration of our study may not capture long-term outcomes and variations in treatment responses over an extended period. As modifying disease treatments evolve, future studies with extended follow-up periods could offer valuable insights into the sustained effects and potential variations in patient outcomes.

By openly acknowledging these limitations, we aim to provide a transparent assessment of our study’s boundaries and encourage researchers to consider these factors in the context of their interpretations and applications of our findings.

7. Future Research Direction

Our current study represents a foundational step in understanding changes in pNFH levels in Spinal Muscular Atrophy. Moving forward, we plan to expand the study group to enhance the generalizability of our findings. Additionally, we aim to compare our results with those from diverse treatment modalities for this pathology, fostering a more comprehensive understanding.

Furthermore, future research could delve into the mechanism of changes in pNFH levels in the CSF to deepen our insights. Exploring these changes and their impact on outcomes may contribute valuable information to guide treatment strategies. We also see potential for conducting longitudinal studies to assess the long-term effects of the interventions explored in our current research.

By undertaking these initiatives, we aim to contribute to the broader body of knowledge on SMA and its treatment, ultimately informing clinical practices and potential avenues for therapeutic interventions. We appreciate the importance of building on the foundation laid by this study and look forward to the ongoing advancement of research in this field.


Funding: This research received no external funding.

Institutional Review Board Statement: The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the National University Center for Children Neurorehabilitation “Dr. Nicolae Robanescu” (protocol code 7464, approved on October 1st, 2010).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The patient(s) also gave written informed consent to publish this paper.

Data Availability Statement: The data published in this research are available upon request from the first author and corresponding authors. Interested parties may contact them directly for access.

Acknowledgments: We thank the families and relatives for granting consent to participate in the study, ensuring the best possible outcomes for those undergoing diagnosis and treatment for SMA. We also appreciate the direct and indirect support from the National Teaching Center for Children’s Neurorehabilitation “Dr. Nicolae Robanescu” staff in obtaining data for this study.

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

References