BALNEO RESEARCH Balneo and PRM Research Journal
English Edition
e ISSN 2734 - 8458 p ISSN 2734 - 844X ROMANIAN ASSOCIATION OF BALNEOLOGY

Research article

Biomarker Evolution in Pediatric SMA: Insights from CSF pNF-H Dynamics and SMN2 Copy Number During Nusinersen Therapy

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Abstract: Spinal muscular atrophy (SMA) is a severe neurodegenerative disorder caused by insufficient survival motor neuron (SMN) protein synthesis, leading to progressive motor neuron loss and debilitating symptoms. This study evaluates cerebrospinal fluid (CSF) phosphorylated neurofilament-heavy chain (pNF-H) levels as predictive markers of motor function in 73 pediatric SMA patients undergoing nusinersen treatment. pNF-H, a structural component of neurons, is released into the CSF and serum during neuronal damage or degeneration. This study aims to address this gap by assessing pNF-H dynamics in relation to motor function changes over the course of treatment. It examines motor function evolution over different time periods in relation to initial clinical and biological parameters and their progression at the start of treatment or as a response to therapy. Patients were stratified by SMN2 gene copy number, which modulates disease severity and response to therapy. pNF-H levels inversely correlated with SMN2 copy number, with higher levels indicating more severe neurodegeneration. pNF-H levels also correlated with motor function, with higher baseline levels linked to lower scores. During nusinersen treatment, pNF-H declined alongside motor improvements, supporting its role as a longitudinal biomarker. In patients with 2 SMN2 copies, larger early pNF-H variations predicted better motor gains at 1 and 2 years, while smaller changes during maintenance correlated with lower improvement. In patients with 3 copies, larger early fluctuations were associated with higher motor scores, along with higher serum creatinine at 2 years. Longitudinal analyses revealed that early and sustained decreases in CSF pNF-H were associated with enhanced motor outcomes. The study highlights CSF pNF-H level variations as a robust predictor of treatment efficacy, offering insights into disease progression and therapeutic impact. These findings underscore the critical role of early intervention and personalized biomarker monitoring in optimizing quality of life for SMA patients.

Keywords: spinal muscular atrophy; cerebrospinal fluid; pNF-H neurofilaments; creatinine; biomarkers; motor evolution; neurodegenerative disease; Spinraza nusinersen; SMN2 copy; predictive factor marker;

Citation: Badina M., Bejan G.C., Mirea A., Sporea C., Leanca M.C., Nicolae G., Cioca I.E., Morcov M.V., Pellegrini A. a Bordea E.N. - Biomarker Evolution in Pediatric SMA: Insights from CSF pNF-H Dynamics and SMN2 Copy Number During Nusinersen Therapy
Balneo and PRM Research Journal **2025**, *16(1): 777*

Academic Editor(s):
Constantin Munteanu

Reviewer Officer:
Viorela Bembea

Production Officer:
Camil Filimon

Received: 03.02.2025
Published: 31.03.2025

Reviewers:
Mihai Hoteteu
Mariana Rotariu

Publisher's Note: Balneo and PRM Research Journal stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Spinal amyotrophy (SMA) is a rare neurodegenerative disease with an incidence of 1 in 10,000 live newborns, caused by insufficient synthesis of the survival motor neuron (SMN) protein necessary for the survival of motor neurons.

The symptoms, due to the progressive degradation of the motor neurons in the anterior medullary horns, cause progressive muscle weakness and atrophy of the skeletal muscles, along with respiratory and digestive manifestations with varying degrees

of severity which, in the absence of adequate treatment, can lead to respiratory failure, paralysis and even death in severe forms [1,2].

Depending on the age of onset of symptoms, SMA was classified into 5 types, type 0 being the most severe, with manifestations from intrauterine life, a single copy of the SMN2 gene and a maximum life expectancy of 6 months. In type 1, with symptoms in the first 6 months of life and survival around 2 years, there are usually 2 copies of the SMN2 gene, children will not acquire the ability to walk, will not be able to maintain a sitting position without support and sometimes they will also have no head position control [3,4]. Patients with SMA type 2 have 2 or 3 copies, symptoms appear between 6 and 18 months, they can sit but cannot walk without support and often reach adulthood [5,6]. In type 3 symptoms appear after the age of 18 months, and in type 4 after 30 years, patients frequently have 4 or more copies of the SMN2 gene, show muscle weakness, gait disturbances and lose some motor acquisitions, but the hope of life is not influenced.

The SMN protein deficiency is due in 95% of cases to the existence of a genetic defect with autosomal recessive transmission (often mutation or deletion) at the level of the 5q13 region of the SMN1 gene which under normal conditions produces 80-90% of the required amount of protein. The rest of 10-20% of the amount is produced by the SMN2 gene, which appeared as a result of inversion and duplication processes of the SMN1 gene during evolution [7,8]. The two genes are almost identical except for a few base pairs (7 in intron 6, 2 in intron 7, one in exon 7 and one in exon 8 – where thymine is replaced by cytosine at position 820) [9]. This modification leads to the alteration of the splicing of the messenger RNA, by activating it in another place and removing exon 7 from the SMN2 gene structure by some splicing initiator proteins [10]. As a result of missing of exon 7, which occurs in approximately 90% of SMN2 transcripts, a structurally and functionally altered protein is synthesized, shorter and rapidly degraded in cells by the ubiquitin-proteasome system [7–9].

The SMN2 gene is usually present in multiple copies located in the same chromosomal region as the SMN1 gene, and the amount of SMN protein produced depends on the number of copies of the SMN2 gene present and influences the age of onset of symptoms, the severity of clinical manifestations, and the rapidity of progression to paralysis, atrophy and organ failure [3,11,12].

The SMN1 gene was identified as the cause of SMA manifestations in 95% of cases in 1995 by the French geneticist Judith Melki, and then the role of the presence of several copies of the SMN2 gene - homologous to the SMN1 gene - in determining the severity of the disease was also confirmed. With the new technological breakthroughs of the gene era have come new approaches for disease-modifying treatments, starting in 2016 with Nusinersen, followed by Zolgensma in 2019 and Risdiplam in 2021 [13–15].

The number of copies of the SMN2 gene is a decisive aspect of the severity of the disease type, prognosis and response to treatment. It has recently been established that each copy of the SMN2 gene can produce approximately 10% of the amount of SMN protein required for normal function, but this amount varies from cell to cell and tissue to tissue, resulting in phenotypic variation [16–18].

Nusinersen (Spinraza), the first drug approved for the treatment of SMA, is an antisense oligonucleotide for the SMN2 gene with the role of preventing premature splicing of the messenger RNA, causing the synthesis of an SMN protein of normal length and functionality [19–22]. Administration is intrathecal, by lumbar puncture, with a loading period on days 0, 14, 28 and 60 of treatment, followed 6 months after initiation by a maintenance period with doses administered once every 4 months for life [20].

Motor evaluation of patients with SMA is done on motor scales adapted to the particular motor deficits of these patients, depending on age, type of SMA, degree of severity and stage of the disease. Tests assess voluntary or reflex movements, posture, or resistance to exercise (CHOP-Intend, HINE, HFMSE, RULM, 6MWT). An improvement in the condition of patients with SMA, reflected by increased scores on motor scales, will lead to enhanced functional independence for these patients. This, in turn,

will reduce the burden and stress experienced by both the patients and their families, significantly improving their quality of life. Additionally, caregivers will be better equipped to cope with the stress caused by the children's illness, enabling them to support the children more effectively in adhering to treatment regimens [23–27].

Neurofilaments are neuron-specific protein heteropolymers that appear as a result of neuronal damage in the cerebrospinal fluid (CSF) and from there in the peripheral blood in various acute or chronic conditions (Parkinson's disease, Alzheimer's disease, Charcot-Marie-Tooth disease, frontotemporal dementia, vascular dementia, multiple sclerosis, toxic neuropathy or in severe burns [28–31]. Of the 5 existing types, pNF-H is the largest, most stable and it is in the highest concentration, and the amount released is directly proportional to the severity of neuronal damage [32,33]. pNF-H was selected as the primary biomarker in this study due to its specificity and sensitivity in detecting neuronal damage and degeneration, making it particularly relevant for monitoring disease progression and treatment response in neurodegenerative disorders such as SMA. Unlike other potential biomarkers, pNF-H is a structural component of neurofilaments, and its release into cerebrospinal fluid (CSF) and serum occurs directly in response to axonal injury, providing a reliable indicator of neurodegeneration dynamics. Additionally, pNF-H has been extensively studied in other neurodegenerative diseases, such as ALS and multiple sclerosis, where its levels have been shown to correlate with disease severity and clinical outcomes. In SMA, previous studies have indicated that pNF-H levels are elevated in severely affected patients, suggesting its potential as a quantifiable and objective biomarker [34,35]. Furthermore, compared to other candidate biomarkers, such as neurofilament light chain (NfL) or pro-inflammatory cytokines, pNF-H has demonstrated greater stability in CSF, making it a more reliable and practically applicable marker for longitudinal monitoring. The ability of pNF-H to reflect early neurodegenerative changes and its dynamic response to treatment makes it a valuable tool for evaluating the efficacy of disease-modifying therapies like nusinersen in SMA patients. Despite significant advances in biomarker research for SMA, several gaps in knowledge remain, particularly regarding their longitudinal utility, specificity, and correlation with clinical outcomes. While neurofilament proteins, such as pNF-H and NfL, have shown promise as biomarkers of neuronal injury, their precise role in tracking disease progression and treatment response over extended periods is not fully established.

Creatinine is an indicator of muscle activity in various neuromuscular diseases, its level being in direct correlation with the mass of muscle tissue existing in the body and with muscle metabolism (for example in Duchenne or Becker muscular dystrophy, in Kennedy's disease or in other diseases that evolve with muscle atrophy regardless of etiology). Decreased serum creatinine levels in neurodegenerative diseases suggest decreased muscle mass and metabolism through the progression of the muscle denervation process, correlated with the severity of the condition [36–40].

Prior to the initiation of specific treatment, the highest values of pNF-H due to neuronal degradation, the lowest values of serum creatinine and motor rating scale scores due to motor deficit, and during treatment with nusinersen the values of the monitored parameters should improve.

In this study we analyzed the evolution under nusinersen treatment of pNF-H level in CSF related to the changes in serum and serum creatinine, scores and yield on motor scales, to evaluate pNF-H as a predictive marker for motor evolution in function of the number of copies of the SMN2 gene.

2. Materials and Methods

73 pediatric patients diagnosed with SMA participated in this study, evaluated and treated with nusinersen in the National Teaching Center for Children's Neurorehabilitation “Dr. Nicolae Robanescu”. The study had approval from the Center´s Ethic Committee (protocol code 7464, approved on October 1st, 2018) and the participants had

patient's legal relatives following local regulations and the Declaration of the World Medical Association of Helsinki, revised in 2000 in Edinburgh.

The inclusion criteria in the study were: pediatric patient diagnosed with SMA included in the national treatment program with nusinersen, with 2 or 3 copies of the SMN2 gene, as determined by genetic testing. The exclusion criteria included the use of other disease-modifying drugs and conditions that could elevate pNF-H levels in CSF and serum. Patients with acute medical events or treatment interruptions were also excluded, ensuring that the analyzed cohort consisted exclusively of those who completed nusinersen treatment per protocol, without significant acute complications.

Biological samples were collected just before drug doses were administered. The analysis of pNF-H in CSF and serum was performed by the ELISA method according to the working instructions in the leaflet issued by the manufacturer of the reagent kit - EUROIMMUN Medizinische Labordiagnostika AG (Seekamp 31, 23560 Lübeck, Germany), using the ELISA plate reader BIORAD 3100 PSC (Bio-Rad Laboratories, 1000 Alfred Nobel Drive, Hercules, CA 94547, USA). The kit is intended for the in vitro quantitative determination of pNF-H neurofilaments in CSF or serum samples of human origin and belongs to the category of in vitro diagnostic medical devices.

Serum creatinine level was determined by the improved Jaffe method, based on the reaction of creatinine with sodium picrate, on the Arhitect c4100 analyzer (Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60064, USA).

The performances on the motor scales were evaluated by the same team of physiotherapists, specialized in the evaluation of patients with SMA, in order to minimize the degree of subjectivity. The data obtained on the CHOP-Intend scale (which evaluates the strength and mobility of axial and peripheral muscles with the help of 16 requirements in different degrees of difficulty with a possible maximum of 64 points) were used for patients diagnosed with SMA type 1, and the HFMSE for those with types 2 and 3, with a maximum score of 66 points that can be obtained by correctly completing 33 requirements. Both the absolute values recorded and those relative to the maximum score possible on the motor scales (yield) were followed in order to be able to compare the performances achieved by the patients [20,41–45].

In this study, we took into account the results obtained at the following time points: T0 – before the initiation of treatment (first dose I1), T1 – before the start of the maintenance period (fifth dose I5, 6 months after the initiation of treatment), T2 – sixth treatment dose I6, 8 months after initiation, respectively T3, T4 and T5 – one, two or three years after initiation of therapy – doses 7 (I7), 10 (I10) and 13 (I13) of the drug respectively.

3. Results

Of the 73 participants, aged between 0.67 and 215 months, female patients slightly predominated (56%) and patients with 3 copies of the SMN2 gene slightly predominated (52%). The characteristics of the studied group such as age, sex, type of SMA and number of copies of the SMN2 gene are presented in Table 1.

Table 1. Characteristics of study group participants according to SMA type.

		No of SMN2 copies	
	All	**2 copies**	**3 copies**
Nr.	73	35	38
Sex (M)	33	12	21
Age (months)			
Min ÷ Max	0.67÷215	0.67÷176	5÷215
SMA type			
type 1	34	26	8
type 2	28	5	23
type 3	11	4	7

The youngest patient was male, aged 0.67 months at the start of treatment and was diagnosed with SMA type 1 with 2 copies of the SMN2 gene. The oldest patient was 215 months at baseline, diagnosed with SMA type 2 and with 3 copies of the SMN2 gene.

The values of the parameters analyzed at the initiation of treatment according to the type of SMA and the number of copies of the SMN2 gene are shown in Table 2.

Table 2. Biomarker Levels and Functional Scale Scores at Treatment Initiation by SMN2 Copy Number.

		No of SMN2 copies	
	All	**2 copies**	**3 copies**
CSF pNF-H (ng/ml)			
Min ÷ Max	0.035÷3.321	0.039÷3.321	0.035÷0.817
Serum pNF-H (ng/ml)			
Min ÷ Max	0.032÷5.268	0.05÷4.595	0.032÷5.268
Creatinine (mg/dl)			
Min ÷ Max	0.20÷0.62	0.20÷0.62	0.24÷0.52
CHOP-Intend (points)			
Min ÷ Max	7÷57	7÷50	24÷57
HFMSE (points)			
Min ÷ Max	0÷54	0÷54	0÷53

Before initiation of treatment, the highest CSF pNF-H neurofilament level of 3.321 ng/ml was obtained for a 3.63-month-old female patient with SMA type 1 and 2 SMN2 copies, and the lowest value, 0.035 ng/ml in a 104-month-old male patient with SMA type 2 and 3 copies of the SMN2 gene.

In the serum, the highest value of pNF-H neurofilaments was recorded for a male patient with SMA type 2 and 3 copies of the SMN2 gene, aged 17 months – 5.268 ng/ml, and the lowest value, of 0.032 ng/ml, for a female patient with 3 copies of the SMN2 gene, with SMA type 1, aged 10 months.

For creatinine, the lowest value was 0.20 mg/dl in a 24-month-old female patient with SMA type 1 and 2 copies of the SMN2 gene, and the highest value was 0.62 mg/dl, observed in a 176-month-old female patient with SMA type 3 and 2 SMN2 copies.

The highest values of pNF-H neurofilaments in the CSF were obtained at the initiation of treatment, for all patients, regardless of the type of SMA and the number of SMN2 copies, after which, during the maintenance period of the next three years, the levels of neurofilaments in the CSF maintained below a level of 0.401 ng/ml except for three patients, two with SMA type 1 and 2 SMN2 copies at one and two years of treatment and the third with SMA type 2 and 3 SMN2 copies at 6 months of treatment, probably due to acute events between injections, but at the next assessments the values returned to the limits of the groups in which they belonged.

The lowest values of pNF-H levels in CSF were observed at three years of treatment for all patients included in the study (Figure 1).

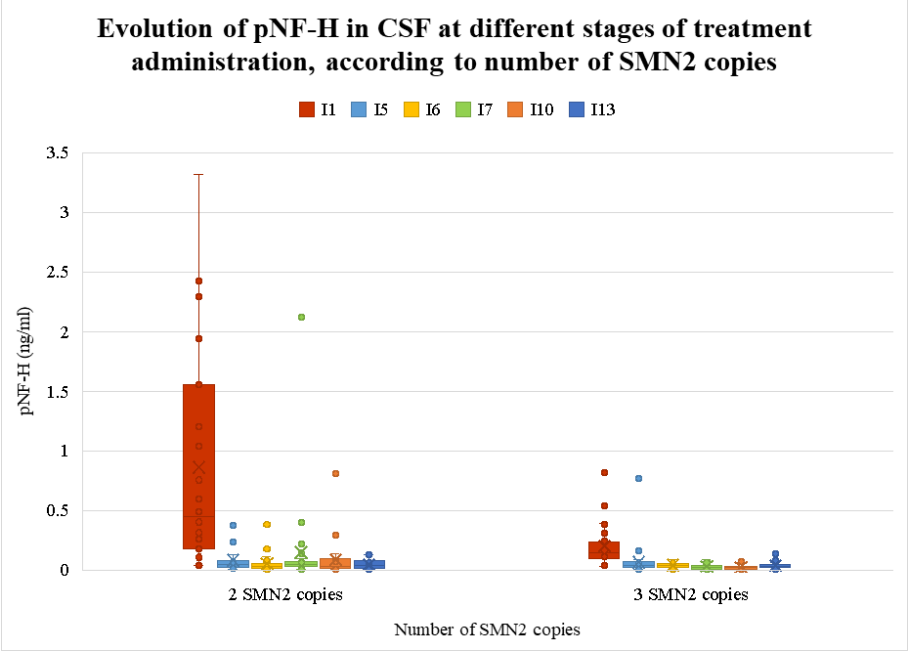

Figure 1. Longitudinal Analysis of pNF-H Levels in CSF Across Treatment Stages Stratified by SMN2 Copy Number.

The same trend was observed for pNF-H values in serum as in CSF, with the highest values at baseline for patients with SMA type 1 and 2 copies of the SMN2 gene, values that decreased during treatment with nusinersen below the value of 0.448 ng/ml, except for two patients with SMA type 1 and 2 SMN2 copies, with severe clinical forms of the disease, whose level of pNF-H in CSF decreased more slowly, at the evaluation from 6 months of treatment being 7.280 and 0.825ng/ml respectively (Figure 2).

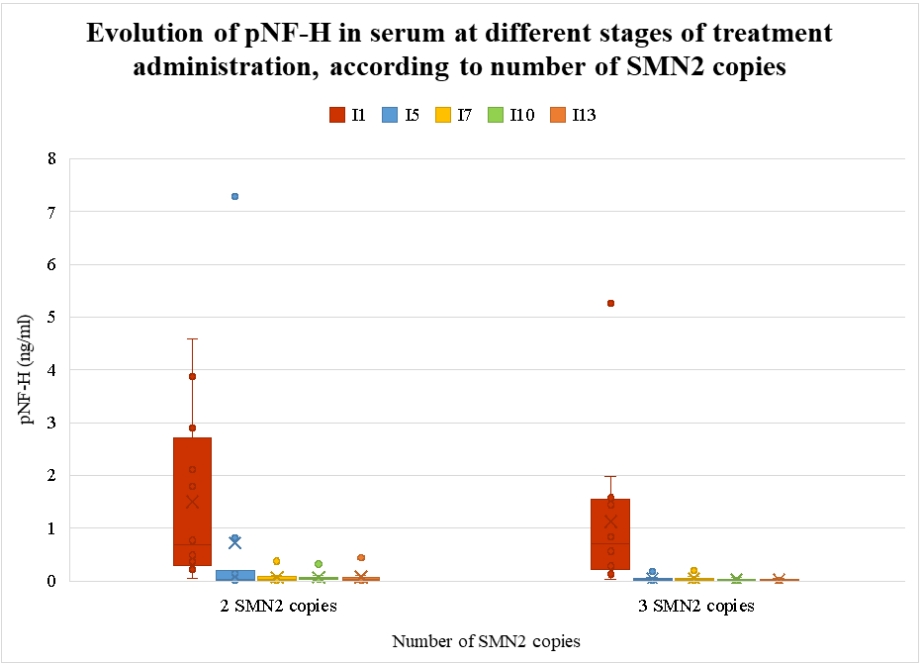

Figure 2. Longitudinal Evolution of pNF-H in Serum Across Treatment Stages Stratified by SMN2 Copy Number.

The level of serum creatinine increased during the three years of treatment compared to the initial values for all groups of patients, but for types 2 and 3 of SMA and in patients with 3 copies of SMN2 an initial decrease in the level was noted at 6 months of treatment (Figure 3).

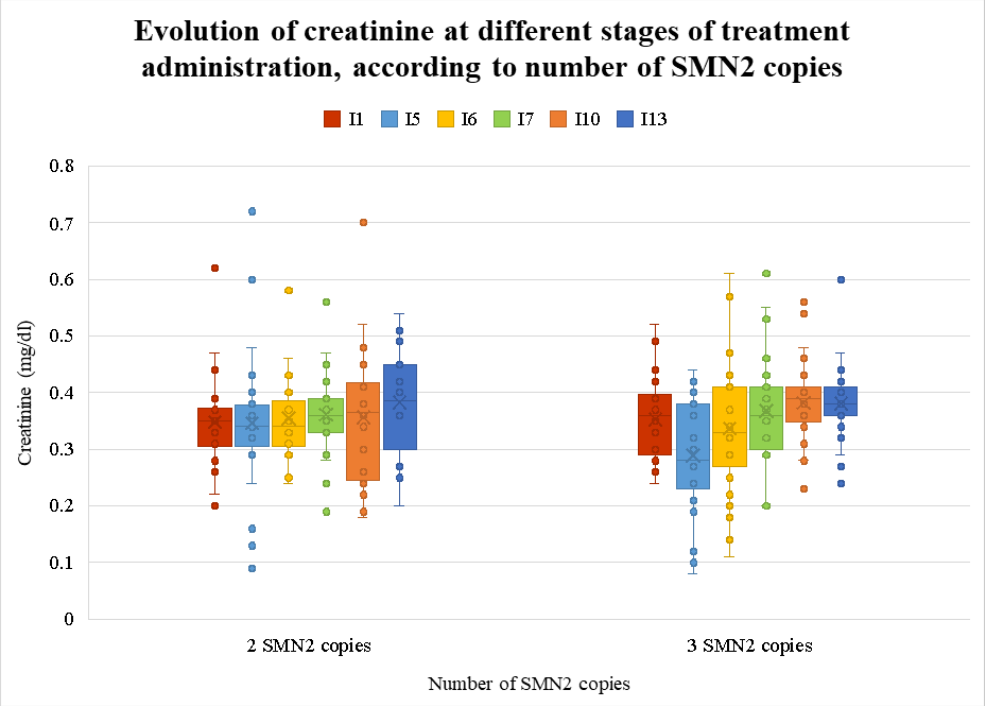

Figure 3. Longitudinal Evolution of Creatinine Across Treatment Stages Stratified by SMN2 Copy Number.

In terms of performance over three years of treatment, all groups of patients achieved better values compared to the initial value (Figure 4).

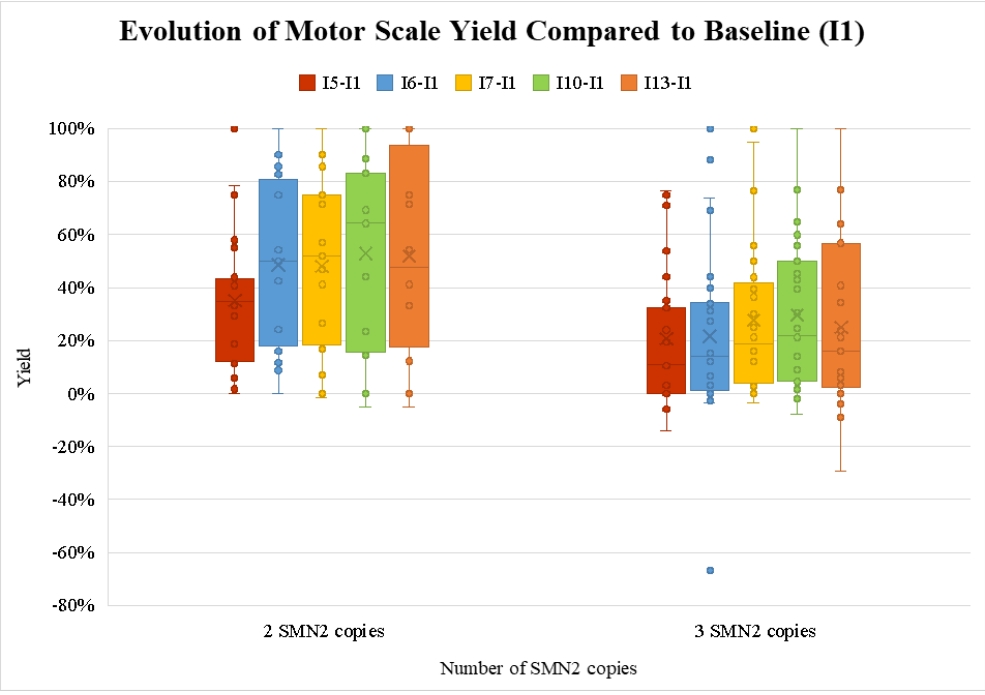

Figure 4. Evolution of Motor Scale Yield from Baseline (I1) Stratified by SMN2 Copy Number

3.1. SMA with 2 copies of SMN2 gene

3.1.1. Treatment initiation

Depending on the number of SMN2 gene copies, the ***CSF pNF-H level*** at treatment initiation for patients with 2 SMN2 copies ranged from a low of 0.039 ng/ml for a 9-

month-old patient to 3.321 ng/ml for a 3.63-month-old patient, both with SMA type 1, with mean values of 0.862 ng/ml and weakly negatively correlated with age at initiation ($r = -0.403$, $p < 0.05$) and type of SMA ($r = -0.474$, $p < 0.05$), very strongly positive correlated with variation in serum pNF-H level from the maintenance period in the first year of treatment ($r = 0.968$, $p < 0.01$), moderately or strongly positive with serum pNF-H level at two and three years of treatment ($r = 0.608$, $p < 0.05$ and $r = 0.940$, $p < 0.01$, respectively) and strongly negative with creatinine level at three years of treatment ($r = -0.714$, $p < 0.05$).

Age at onset for patients with 2 SMN2 copies, ranging from a minimum of 0.67 months in a patient with SMA type 1 to 176 months in a patient with SMA type 3, with a mean of 26.7 months, was strongly positive correlated with the type of SMA ($r = 0.800$, $p < 0.01$), moderately or weakly positive with the serum creatinine level from the first two years of treatment for the moments analyzed ($r = 0.617$, $p < 0.01$ at I1, $r = 0.472$, $p < 0.05$ at I6, $r = 0.496$, $p < 0.05$ at I7, and $r = 0.532$, $p < 0.01$ at I10), weakly or moderately negative with change in CSF pNF-H level in the first year of treatment relative to baseline ($r = -0.471$, $p < 0.05$ for I5 , $r = -0.503$, $p < 0.05$ for I6, and $r = -0.528$, $p < 0.05$ for I7) and strongly negative in three years of treatment ($r = -0.818$, $p < 0.01$) and moderately negative with the performance on the motor scales obtained in three years of treatment ($r = -0.692$, $p < 0.05$).

The ***type of SMA*** correlated positively very strongly or strongly with the score obtained on the HFMSE scale ($r = 0.949$, $p < 0.01$ at I1, $r = 0.922$, $p < 0.01$ at I5, $r = 0.917$, $p < 0.01$ at I6, $r = 0.937$, $p < 0.01$ at I7, $r = 0.946$, $p < 0.01$ at I10 and $r = 0.875$, $p < 0.01$ at I13), weakly or moderately positively with serum creatinine level ($r = 0.390$, $p < 0.05$ at I1, $r = 0.409$, $p < 0.05$ at I6, $r = 0.488$, $p < 0.05$ at I7, $r = 0.631$, $p < 0.01$ at I10 and $r = 0.632$, , $p < 0.01$ at I13) and weakly, moderately or strongly negative with the variation of the CSF pNF-H level at the studied time points compared to the initial value ($r = -0.442$, $p < 0.05$ at I5, $r = -0.444$, $p < 0.05$ at I6 , $r = -0.467$, $p < 0.05$ at I7, $r = -0.521$, $p < 0.05$ at I10 and $-r = 0.720$, $p < 0.05$ at I13).

Creatinine level at the start of treatment correlated moderately positively with that at I6 ($r = 0.518$, $p < 0.05$) and I7 – at one year of treatment ($r = 0.540$, $p < 0.05$) and strongly negatively with the variation of pNF-H neurofilament levels in CSF over the three years of treatment in absolute value ($r = -0.733$, $p < 0.05$) and in relative value to the initial one ($r = -0.779$, $p < 0.05$).

The ***CHOP-Intend score*** at baseline correlated strongly positively with that obtained at the beginning of maintenance treatment ($r = 0.788$, $p < 0.01$) and with that obtained at one year of treatment ($r = 0.713$, $p < 0.05$), strongly positively with the variation in CSF pNF-H level and strongly negative with the variation of serum creatinine in the first year of treatment ($r = 0.796$, $p < 0.01$ and $r = -0.743$, $p < 0.05$ respectively). The HFMSE score at baseline correlated very strongly positively with the other scores obtained at the studied time points in the three years of treatment ($r = 0.994$, $p < 0.01$ at I5, $r = 0.993$, $p < 0.01$ at I6, $r = 0.997$, $p < 0.01$ at I7, $r = 0.990$, $p < 0.01$ at I10 and $r = 0.978$, $p < 0.01$ at I13) and strongly or very strongly positive with the returns obtained in the studied moments of time ($r = 0.820$, $p < 0.05$ at I5, $r = 0.934$, $p < 0.01$ at I6, $r = 0.971$, $p < 0.01$ at I7, $r = 0.975$, $p < 0.01$ at I10 and $r = 0.972$, $p < 0.01$ at I13), strongly negative with the variation of the creatinine level in the first two years of treatment ($r = -0.732$, $p < 0.05$) and with the variation of the pNF-H level in the CSF during the maintenance of the three years of treatment relative to the value at the beginning of this period ($r = -0.842$, $p < 0.05$).

3.1.2. The start of the maintenance treatment

The ***CSF pNF-H level*** at the start of maintenance treatment ranged from 0.009 ng/ml for a 6.5-month-old patient at baseline to 0.389 ng/ml for a 7-month-old patient at baseline, both with SMA type 1, it correlated moderately positively with the level after three years of treatment ($r = 0.617$, $p < 0.05$) and strongly positively with the variation of pNF-H serum level in the first year of treatment ($r = 0.848$, $p < 0.05$).

3.1.3. At one year of treatment

At one year of treatment, the ***CSF pNF-H level*** ranged from a low of 0.01 ng/ml for three patients aged 2, 6 and 7 months at the start of treatment to 2.122 ng/ml in the 6.5 months at baseline, all with SMA type 1, and was strongly positively correlated with the change in serum pNF-H level during the loading period of the first year of treatment ($r = 0.998$, $p < 0.01$).

The level of ***serum creatinine*** at one year of treatment was moderately positively correlated with the variation of pNF-H level in CSF during the maintenance period of the first three years of treatment, relative to the value at the beginning of this period ($r = 0.667$, $p < 0.05$).

3.1.4. At two years of treatment

The ***serum creatinine level*** at two years of treatment negatively moderated correlated with the change in CSF pNF-H level during the maintenance period of the first year of treatment compared to baseline ($r = -0.669$, $p < 0.01$).

The ***CHOP-Intend score*** at two years of treatment was strongly negatively correlated with the change in CSF pNF-H level at I6 ($r = -0.795$, $p < 0.05$), I7 ($r = 0.785$, $p < 0.05$) and I10 ($r = -0.758$, $p < 0.05$).

The ***HFMSE score*** at two years of treatment was strongly negatively correlated with the change in serum creatinine level in the first two years of treatment ($r = -0.794$, $p < 0.05$) and with the change in CSF pNF-H level during maintenance treatment in the first two years of treatment ($r = -0.718$, $p < 0.05$) and from the three years, relative to the value at the beginning of this period ($r = -0.845$, $p < 0.05$).

3.1.5. At three years of treatment

At three years of treatment, the ***CSF pNF-H level*** ranged between 0.009 ng/ml for two patients with SMA type 1 or 2, aged 46 and 37 months at treatment initiation, respectively, and 0.131 ng/ml for one patient with SMA type 2 initiated at 131 months of age and strongly positively correlated with serum pNF-H level at three years of treatment ($r = 0.751$, $p < 0.05$), level lying between a low of 0.009 ng/ml for a 6.3-month-old patient at baseline and a high of 0.448 ng/ml for a 6.17-month-old patient at baseline, both with SMA type 1 and very strongly positively correlated with variations pNF-H level in the CSF compared to the initial value ($r = 0.942$, $p < 0.01$ at I5, $r = 0.948$, $p < 0.01$ at I6, $r = 0.943$, $p < 0.01$ at I7, $r = 0.942$, $p < 0.01$ at I10 and $r = 0.940$, $p < 0.01$ at I13).

The ***serum creatinine level*** at three years of treatment was moderately or strongly negatively correlated with the change in CSF pNF-H level from baseline in the first two years of treatment ($r = -0.675$, $p < 0.05$ at I5, $r = -0.678$, $p < 0.05$ at I6, $r = -0.681$, $p < 0.05$ at I7 and $r = -0.716$, $p < 0.05$ at I10).

The ***CHOP-Intend score*** at three years of treatment was strongly negatively correlated with the change in CSF pNF-H level at one year of treatment from baseline ($r = -0.888$, $p < 0.05$).

The procentual variation in CSF pNF-H level during the loading period was weakly negatively correlated with the variation in serum creatinine level during the same period ($r = -0.493$, $p < 0.05$), weakly positively correlated with performance on motor scales at one year ($r = 0.449$, $p < 0.05$) and moderately positively with the one from two years of treatment ($r = 0.525$, $p < 0.05$).

3.2. SMA with 3 copies of SMN2 gene

3.2.1. Treatment initiation

In patients with 3 copies of the SMN2 gene, the level of neurofilament ***pNF-H in CSF*** at treatment initiation was between 0.035 ng/ml for a 104-month-old patient and 0.817 ng/ml for a 16-month-old patient, both with SMA type 2 , and was moderately positively correlated with the level of pNF-H neurofilaments in the serum at the initiation of treatment ($r = 0.652$, $p < 0.05$), with the from the beginning of the maintenance treatment ($r = 0.695$, $p < 0.05$) and with the variation of the serum pNF-H level during the loading period ($r = 0.634$, $p < 0.05$), strongly positive with the variation of the serum

pNF-H level for one and two years of treatment (r = 0.733, p < 0.05 respectively r = 0.828, p < 0.05) and for the variation of the pNF-H level during the loading period of the first two years of treatment relative to the value at the beginning of this period (r = 0.820, p < 0.05) and very strongly positive for the absolute value (r = 0.951, p < 0.01).

In patients with 3 SMN2 copies, the ***type of SMA*** correlated weakly or moderately positively with the scores obtained on the motor scales (r = 0.529, p < 0.01 for I1, r = 0.461, p < 0.05 for I5, r = 0.398, p < 0.05 for I7, r = 0.367, p < 0.05 for I10 and r = 0.411, p < 0.05 for I13), weakly or moderately negatively with the returns on the scales obtained during the maintenance period of the first year of treatment (r = -0.502, p < 0.01 at I5, r = -0.545, p < 0.01 at I6 and r = -0.410, p < 0.05 at I7), weakly negative with the variation of the serum creatinine level during the maintenance period of the first year of treatment (r = -0.423, p < 0.05), weakly positive with the creatinine level at one year of treatment (r = 0.371, p < 0.05) and moderately positive with creatinine level after two years of treatment (r = 0.471, p < 0.01).

The ***age*** at initiation of treatment, for patients with 3 SMN2 copies between 5 months for a patient with SMA type 1 and 215 months for a patient with SMA type 2, correlated weakly positively with the type of SMA (r = 0.490, p < 0.01), weakly negatively with creatinine level and HFMSE score at the start of maintenance treatment (r = -0.464, p < 0.01 and r = -0.465, p < 0.05 respectively), strongly positive with the CHOP-Intend score at start of maintenance treatment (r = 0.862, p < 0.05), weakly or moderately negative with HFMSE scores during maintenance treatment (r = -0.513, p < 0.01 at I6, r= -0.512, p < 0.01 at I7, r = -0519, p < 0.01 at I10 and r = -0.481, p < 0.05 at I13) and moderately negative with the motor yields obtained throughout the treatment (r = -0.544, p < 0.01 at I5, r = -0.537, p < 0.01 at I6, r = -0.515, p < 0.01 at I7, r = -0.582, p < 0.01 at I10 and r = -0.586, p < 0.01 at I13), weakly positive with the variation of creatinine level during the loading period (r = 0.417, p < 0.05) and weakly or moderately negative with the variation of the serum creatinine level during the loading period from the first year and from the three years of treatment (r = -0.395, p < 0.05 and r = -0.527, p < 0.01 respectively), strongly negative with the variation of serum pNF-H level in the first two years of treatment relative to the initial level (r = -0.901, p < 0.01) and weakly negative with the variation of pNF-H level in CSF during the loading period and during the three years of treatment (r = -0.413, p < 0.05 and r = -0.446, p < 0.05 respectively).

The baseline ***serum pNF-H level***, ranging from 0.032 ng/ml for a 10-month-onset SMA type 1 patient to 5.268 ng/ml for a 17-month-onset SMA type 2 patient, was strongly correlated positively with the HFMSE scale score at the beginning of the maintenance period, at one year and at two years of treatment (r = 0.798, p < 0.05 at I5, r = 0.775, p < 0.05 at I7 and r = 0.755, p < 0.05 at I10), moderately and strongly positive with the variation of pNF-H level in CSF during the maintenance period of the first two years of treatment compared to the initial value (r = 0.638, p < 0.05 for I5, r = 0.637, p < 0.05 for I6 r = 0.699, p < 0.05 for I7 and r = 0.801, p < 0.01 for I10) and strongly positive with the variation of serum pNF-H level during the maintenance period of the first and second year of treatment (r = 0.789, p < 0.05 and r = 0.842, p < 0.05 respectively).

Serum creatinine level at baseline was weakly positively correlated with HFMSE score at baseline (r = 0.431, p < 0.05) and scale performance obtained in the first year of treatment (r = 0.410, p < 0.05 at I1, r = 0.371, p < 0.05 at I6 and r = 0.453, p < 0.05 at I7) and with creatinine level at one year of treatment (r = 0.451, p < 0.05) and weakly and moderately positive with the variation of pNF-H level in CSF at two and three years of treatment compared to the value at the beginning of the maintenance period (r = 0.420, p < 0.05 and r = 0.578, p < 0.01 respectively).

The ***HFMSE score*** at baseline correlated weakly or moderately positively with performance at the studied time points in the three years of treatment (r = 0.628, p < 0.01 at I5, r = 0.411, p < 0.05 at I6, r = 0.675, p < 0.01 at I7, r = 0.635, p < 0.01 at I10 and r = 0.564, p < 0.01 at I13), strongly or very strongly positive with the HFMSE scores obtained in the analyzed moments (r = 0.958, p < 0.01 to I5, r= 0.906, p < 0.01 to I6, r= 0.944, p < 0.01 to I7, r = 0.895, p < 0.01 to I10 and r = 0.865, p < 0.01 to I13), weak or moderately positive

with maintenance serum creatinine ($r = 0.572$, $p < 0.01$ to I6, $r = 0.530$, $p < 0.01$ to I7, $r = 0.654$, $p < 0.01$ to I10 and $r = 0.469$, $p < 0.05$ to I13), moderate positive with pNF-H level in CSF after one year of treatment ($r = 0.552$, $p < 0.01$), weakly negative with the change in CSF pNF-H in the first year of treatment relative to baseline ($r = -0.475$, $p < 0.05$) and moderately negative with the change in CSF pNF-H in the maintenance period from the first year of treatment to of the value at the beginning of this period ($r = -0.516$, $p < 0.01$).

3.2.2. The start of the maintenance treatment

CSF pNF-H level at the start of the maintenance period ranging from a low of 0.009 ng/ml in a 10-month-old patient with SMA type 1 at baseline to 0.772 ng/ml in a 172-month-old SMA type 2 patient at baseline, it was moderately negatively correlated with change in serum pNF-H level during the loading period relative to baseline ($r = -0.659$, $p < 0.05$) and weakly or moderately positive with changes in serum creatinine level at the time points analyzed from baseline ($r = 0.467$, $p < 0.01$ at I5, $r = 0.539$, $p < 0.01$ at I6, $r = 0.384$, $p < 0.05$ at I7, $r = 0.523$, $p < 0.01$ at I10 and $r = 0.448$, $p < 0.05$ at I13).

3.2.3. At one year of treatment

CSF pNF-H level at one year of treatment, for patients with 3 SMN2 copies, ranging from a low of 0.009 ng/ml for 7 patients with SMA type 2 aged 16 to 172 months at onset and one with SMA type 1 of 7 months at baseline and a maximum of 0.091 ng/ml for a patient with SMA type 3 and 185 months at baseline, correlated weakly or moderately positive with motor scale scores ($r = 0.457$, $p < 0.05$ at I7, $r = 0.404$, $p < 0.05$ at I10 and $r = 0.502$, $p < 0.05$ at I13) and weakly positive with creatinine at I7 ($r = 0.452$, $p < 0.05$).

The ***HFMSE score*** at one year of treatment correlated weakly positively with the variation of the pNF-H level in the CSF during the loading period in absolute value and in relative value to the initial value ($r = 0.431$, $p < 0.05$ and $r = 0.410$, $p < 0.05$ respectively).

The ***serum creatinine level*** at two years of treatment correlated weakly positively with the change in CSF pNF-H level during the loading period ($r = 0.403$, $p < 0.05$).

3.2.4. At two years of treatment

The ***HFMSE score*** at two years of treatment was weakly positively correlated with the change in CSF pNF-H level during the loading period, both in absolute value ($r = 0.476$, $p < 0.05$) and in value relative to the initial value ($r = 0.483$, $p < 0.05$).

The ***serum creatinine level*** at three years of treatment was moderately positively correlated with the HFMSE score at three years of treatment ($r = 0.521$, $p < 0.05$) and moderately negatively correlated with the change in CSF pNF-H level in the first year of treatment relative to the baseline value ($r = -0.508$, $p < 0.05$).

3.2.5. At three years of treatment

The ***HFMSE score*** at three years of treatment was weakly positively correlated with the change in CSF pNF-H level during the loading period, both in absolute value ($r = 0.472$, $p < 0.05$) and in value relative to the initial value ($r = 0.447$, $p < 0.05$).

The change in CSF pNF-H level during the loading period for patients with 3 copies of the SMN2 gene was moderately negatively correlated with the change in serum creatinine during the first two years of treatment ($r = -0.616$, $p < 0.05$).

4. Discussion

SMA with 2 copies of the SMN2 gene

In patients with two copies of the SMN2 gene, a younger age at treatment initiation was associated with a more severe type of SMA, with lower serum creatinine and CHOP-Intend scores, with a higher pNF-H neurofilament level in CSF, with a greater change in neurofilament level at 6 months, one year and three years relative to baseline and with a better yield at three years of treatment [46–48].

A more severe type of SMA in patients with two copies of the SMN2 gene correlated with a higher baseline level of neurofilament pNF-H in CSF, a lower level of serum creatinine and HFMSE score at baseline and throughout treatment, and with greater changes in CSF pNF-H level from baseline [48–51].

The low pNF-H neurofilament level at baseline was followed by low pNF-H levels at two and three years of treatment and small changes in serum pNF-H level during the loading period of the first year of treatment but with values high levels of serum creatinine after three years of treatment [52].

A low baseline serum creatinine value was associated with large variations in CSF pNF-H level over three years of treatment, both absolute and relative to baseline.

The score on the motor function scales correlated better on the HFMSE scale than on the CHOP-Intend at different times during treatment and with the yields achieved at different stages.

At a smaller variation in CSF pNF-H level during the loading period relative to the initial value, a lower yield was observed at one year and at two years of treatment, and at a smaller variation in the pNF-H level during the maintenance from the first two years of treatment, a better performance on the motor scales was observed in three years of treatment.

A greater change in serum creatinine level during the loading period was associated with smaller changes in creatinine during the maintenance period compared to the level at the beginning of this period. The large variation in serum creatinine levels during the first two years of treatment evolved with lower scores on motor function scales during the three years of treatment.

For large variations in the level of pNF-H in the CSF during the maintenance period of the three years of treatment relative to the value at the beginning of this period, lower scores were obtained on the functional scales throughout the treatment.

Large variations in CSF pNF-H levels during the first 6 months, first and second year of treatment correlated with higher CSF pNF-H levels and lower serum creatinine values at three years of treatment. As the CSF pNF-H level decreased more in the earlier periods, higher levels of motor scale scores and lower serum creatinine values were obtained later at three years of treatment, probably because of the large initial degradation of motor neurons, followed by a more pronounced loss of muscle activity, more difficult to recover.

SMA with 3 copies of the SMN2 gene

The patients with 3 copies of the SMN2 gene, a younger age at onset was associated with a more severe type of SMA and a lower CHOP-Intend score, but with a higher HFMSE score of along treatment and greater variation in CSF pNF-H level at baseline and over the three years of treatment, with less variation in serum creatinine level during the loading period and with higher yields good in the analyzed moments of the three years of treatment [46,47,50,51].

A more severe type of SMA was associated with a lower HFMSE score during treatment and with lower serum creatinine values at one and two years of treatment and better outcomes in the moments analyzed from the first year of treatment.

Higher baseline CSF pNF-H was associated with greater variation in serum pNF-H neurofilament levels during the first two years of treatment, and higher baseline serum pNF-H was associated with greater high scores on the HFMSE scale at 6 months, one year and two years of treatment and with a greater variation in CSF pNF-H level in the first two years of treatment compared to baseline and serum pNF-H level in the maintenance period at one year and two years of treatment compared to the beginning of this period [52,53].

Higher baseline serum creatinine was associated with larger changes in CSF pNF-H level at two and three years of treatment compared to the level at the beginning of the maintenance period and with better returns in the first year of treatment.

Better performance was correlated with higher scores at each of the time points analyzed throughout treatment and with a higher serum creatinine level at the start of maintenance treatment.

A larger change in serum creatinine during the loading period was associated with smaller changes in serum creatinine at different times in the maintenance period compared to the value at the beginning of this period.

Large variations in serum creatinine level during the loading period and those during the first two years of treatment were observed with small variations in the CSF pNF-H level during the loading period, both in absolute value and relative value, and large variations of pNF-H levels in the maintenance period compared to the values at the beginning of this period.

Greater changes from baseline in serum pNF-H at 6 months, one year, and two years of treatment were associated with greater baseline CSF pNF-H and greater changes in pNF-H CSF pNF-H versus baseline at each of the analyzed time points in the first two years of treatment.

A greater change in CSF pNF-H level during the loading period relative to baseline correlated with a better HFMSE score at one, two, and three years of treatment, and with smaller changes in pNF-H level of the CSF during the maintenance period compared to the level at the beginning of this period.

For large variations in the serum pNF-H level during the maintenance period of the first two years of treatment, in absolute value and relative to the value at the beginning of this period, higher baseline values of pNF-H in CSF and variations were observed higher CSF pNF-H levels compared to baseline at each of the time points studied in the first two years of treatment. Greater variation in CSF pNF-H levels during the loading period was associated with smaller variations in CSF pNF-H levels during the maintenance period from baseline, with a better HFMSE score of -throughout treatment and with a higher value of serum creatinine at two years of treatment, and a higher value of the pNF-H level at the beginning of the maintenance period was associated with greater variations of the pNF-H level from CSF over treatment versus baseline.

A higher score on the HFMSE scale at three years of treatment was associated with higher serum creatinine values and better scores on motor scales during the nusinersen maintenance period.

Clinical Implications of pNF-H as a Biomarker in SMA

The clinical translation of these findings suggests that pNF-H could become an integral biomarker in SMA treatment protocols, helping to personalize therapy, monitor response, and identify patients at risk of suboptimal outcomes. Future research should focus on validating these results in larger cohorts and exploring their application across different SMA phenotypes and therapeutic modalities.

Further Research Directions

Future research should focus on expanding the biomarker portfolio beyond pNF-H, exploring multi-biomarker approaches, non-invasive testing methods, and predictive models for individualized treatment adaptation. By integrating molecular, imaging, and functional biomarkers, SMA research can move toward a precision medicine framework, ultimately improving long-term outcomes for patients.

Limitations

The main limitations of the present study were determined by the phenotypic variation of disease forms and implicit limitations of motor functions due to the varied age of the participants and the different time intervals between the onset of symptoms and the initiation of treatment.

5. Conclusions

Patients with a higher number of SMN2 copies produced more SMN protein and thus motor neurons were less damaged, and lower amounts of pNF-H appeared in the

CSF and scores on motor rating scales were better. The variations of the pNF-H level in the CSF between different periods over time, in absolute or relative value, represented a more important predictive marker regarding the evolution of motor functions over time for these patients compared to the absolute values obtained at different moments of the treatment.

For patients with 2 copies of the SMN2 gene, larger variations in CSF pNF-H level during the loading period suggested better motor performance evolution at one and two years of nusinersen treatment, and for smaller variations in pN -H from CSF at different maintenance periods lower yields were obtained.

Large variations in the pNF-H level in the CSF of patients with 3 copies of the SMN2 gene during the loading period, both in absolute value and in relative value to the initial level, were associated with higher HFMSE scores at 1, 2 and 3 years of treatment and a higher serum creatinine level at 2 years of treatment.

Depending on the patients' phenotype and the initial clinical-biological data, the variations of the pNF-H level in the CSF during the loading period could represent a marker for predicting the evolution of the performance or the scores on the motor scales at different time intervals.

Incorporating pNF-H into routine SMA monitoring represents a significant step toward precision medicine, allowing for early risk stratification, objective treatment response evaluation, and optimized therapy adjustments. By providing real-time insights into neurodegeneration dynamics, pNF-H has the potential to enhance clinical decision-making, improve patient outcomes, and shape future SMA treatment paradigms. Future research should focus on standardizing pNF-H measurement protocols and validating its role in multi-therapy SMA management to fully unlock its clinical utility.

Over the past decade, the treatment landscape for SMA has undergone a paradigm shift, transitioning from supportive care to disease-modifying therapies (DMTs). With the approval of nusinersen, onasemnogene abeparvovec (gene therapy), and risdiplam (oral SMN2 splicing modifier), SMA management now prioritizes early intervention, personalized treatment approaches, and long-term therapeutic monitoring. Our findings contribute to this evolving landscape by highlighting the potential of pNF-H as a biomarker for treatment response and disease progression, aligning with the growing emphasis on biomarker-driven precision medicine in SMA.

Author Contributions: Conceptualization, M.B.; methodology, M.B., G.C.B. and C.S.; validation, M.B.; formal analysis, M.B., C.S., M.V.M. and A.P.; investigation, M.B., A.M. M.C.L. and G.N.; resources, M.B., G.C.B., C.S. and M.V.M.; data curation M.B. and C.S.; writing—original draft preparation, M.B. and C.S.; writing—review and editing, M.B., C.S., G.N., I.E.C., A.P. and E.N.B; visualization, M.B., G.C.B., A.M., M.C.L., G.N., I.E.C., M.V.M., A.P. and E.N.B.; supervision, M.B. and A.M.; project administration, M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the National Teaching Center for Children's Neurorehabilitation "Dr. Nicolae Robanescu" (protocol code 7464/01.10.2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) 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.

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

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