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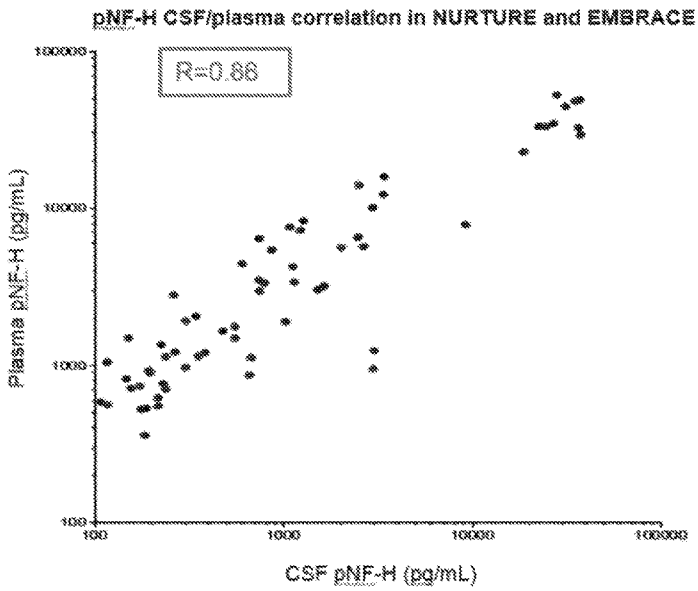

FIG. 1

(57) Abstract: Featured are biomarkers for, e.g., diagnosis and prognosis of spinal muscular atrophy (SMA) as well as identification of responders to treatment of SMA. Also provided are methods of treating subjects with SMA.

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METHODS OF TREATING SPINAL MUSCULAR ATROPHY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Appl. No. 62/622,027, filed January 25, 2018, U.S. Provisional Appl. No. 62/684,507, filed June 13, 2018, and U.S. Provisional Appl. No. 62/738,134, filed September 28, 2018. The content of the prior applications are incorporated by reference herein in their entirety.

TECHNICAL FIELD

This disclosure relates generally to biomarkers of spinal muscular atrophy.

BACKGROUND

Spinal Muscular Atrophy (SMA) is an autosomal recessive genetic disorder resulting in a deficiency in the SMN protein, which in turn leads to the loss of anterior horn motor neurons, axonal degradation, and a phenotype of progressive muscle wasting, mobility impairment, and respiratory failure. Disease severity is categorized into five types (0-4) with type 0 patients having the most severe (neonatal lethal) phenotype and type 4 patients having only the mildest symptoms and normal lifespan.

Timely and proper treatment of subjects with SMA requires the ability to select pre-symptomatic subjects in need of treatment with a therapy for treating SMA and determine whether the SMA therapy is effective. Thus, there is a need for biomarkers of SMA.

SUMMARY

Neurofilaments are a major component of the neuronal cytoskeleton, particularly in axons where they are essential for growth and maintenance. Structurally, they consist of three intertwined core subunits: neurofilament heavy (NF-H), medium/intermediate (NF-M) and light (NF-L) polypeptides that form the “neurofilament triplet.” This disclosure is based, at least in part, on the finding that neurofilament levels serve as effective biomarkers for spinal muscular atrophy (SMA).

In one aspect, the disclosure features a method of treating SMA in a human subject in need thereof. The method involves administering to the human subject a therapeutically effective amount of an SMA therapy, wherein the human subject has been previously

determined to have, in a biological sample obtained from the human subject, a neurofilament level prior to initiation of the SMA therapy that is higher than a control. This method can be used, for example, in treating a subject who is presymptomatic.

In another aspect, the disclosure provides a method of treating SMA in a human subject in need thereof. The method involves measuring a neurofilament level in a biological sample obtained from the human subject before initiation of an SMA therapy and administering a therapeutically effective amount of the SMA therapy to the human subject.

In some embodiments of the above two aspects, the neurofilament level (*e.g.*, phosphorylated neurofilament heavy (pNF-H) level) in the biological sample is above a control level. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 300 pg/mL. In some embodiments the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 400 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 500 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 600 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 700 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 800 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 900 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,000 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,100 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,200 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,300 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,400 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 1,500 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 2,000 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 3,000 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 4,000 pg/mL. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) in the biological sample is above 5,000 pg/mL. These neurofilament levels can be obtained using assays described in the examples section of this application. It should be understood that if a

different neurofilament assay is used that provides a different read out (e.g., O.D. or International Units), the values for neurofilament levels could be different.

In another aspect, the disclosure features a method of treating SMA in a human subject in need thereof. The method involves: measuring a neurofilament level (*e.g.*, pNF-H level) in a first biological sample obtained from the human subject before initiation of an SMA therapy; administering an SMA therapy (e.g., a therapeutically effective amount of the SMA therapy) to the human subject; and measuring a neurofilament level (*e.g.*, pNF-H level) in a second biological sample obtained from the human subject after initiation of the SMA therapy.

In another aspect, the disclosure features a method of treating SMA in a human subject in need thereof. The method involves: measuring a neurofilament level (*e.g.*, pNF-H level) in a first biological sample obtained from the human subject before administration of a candidate amount of an SMA therapy; measuring a neurofilament level (*e.g.*, pNF-H level) in a second biological sample obtained from the human subject after administration of the candidate amount of the SMA therapy, wherein the neurofilament level in the second biological sample is lower than the neurofilament level in the first biological sample, thereby indicating that the candidate amount of the SMA therapy is a therapeutically effective amount; and administering the therapeutically effective amount of the SMA therapy to the human subject after having measured the lowered neurofilament level in the second biological sample.

In certain embodiments, neurofilament level (*e.g.*, pNF-H level) in a first biological sample is above 300 pg/mL, above 400 pg/mL, above 500 pg/mL, above 600 pg/mL, above 700 pg/mL, above 800 pg/mL, above 900 pg/mL, above 1,000 pg/mL, above 1,500 pg/mL, above 2,000 pg/mL, above 3,000 pg/mL, above 4,000 pg/mL, or above 5,000 pg/mL. In certain embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is lower than the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample shows a greater than 30% decline relative to the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is between 10% to 80% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is between

20% to 95% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is between 30% to 90% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is between 30% to 95% of the neurofilament level measured in the first biological sample. In such instances, administration of the SMA therapy is continued. In certain embodiments, the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample is higher than the neurofilament level measured in the first biological sample. In such instances, administration of the SMA therapy is discontinued.

In some embodiments, the second biological sample is obtained from the human subject 40-90 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject 50-80 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject 60-70 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject about 64 days after initiation of the SMA therapy.

In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 50% compared to the neurofilament level measured in the first biological sample. In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 60% compared to the neurofilament level measured in the first biological sample. In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 70% compared to the neurofilament level measured in the first biological sample.

In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by less than 50% compared to the neurofilament level measured in the first biological sample. In

some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by less than 40% compared to the neurofilament level measured in the first biological sample.

In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the dose of the SMA therapy is changed for a subsequent administration to the human subject based upon the percent reduction in neurofilament level measured in the second biological sample as compared to the neurofilament level measured in the first biological sample.

In another aspect, the disclosure relates to a method of predicting the prognosis of SMA. The method involves measuring a neurofilament level (*e.g.*, pNF-H level) in a biological sample obtained from a human subject having mutations in both copies of the *SMN1* gene (the mutations can be homozygous or heterozygous) that lead to functional SMN protein deficiency. The method further involves comparing the neurofilament level (*e.g.*, pNF-H level) measured in the biological sample to a control. The neurofilament level (*e.g.*, pNF-H level) measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the subject will develop.

In certain embodiments, the biological sample is obtained from the human subject before initiation of an SMA therapy, and the neurofilament level (*e.g.*, pNF-H level) measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the human subject will develop in the absence of treatment.

In some embodiments, the biological sample is obtained from the human subject after initiation of an SMA therapy, and the neurofilament level (*e.g.*, pNF-H level) measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the subject will develop while receiving the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least two weeks after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least four weeks after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least six weeks after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least eight weeks after initiation of the SMA therapy. In certain instances, the biological

sample is obtained from the human subject at least ten weeks after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least twelve weeks after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least two months after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least three months after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least four months after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least five months after initiation of the SMA therapy. In certain instances, the biological sample is obtained from the human subject at least six months after initiation of the SMA therapy.

In another aspect, the disclosure relates to a method of predicting the prognosis of SMA. The method involves measuring, before initiation of an SMA therapy, a neurofilament level (*e.g.*, pNF-H level) in a first biological sample obtained from a human subject having mutations in both copies of the *SMN1* gene (the mutations can be homozygous or heterozygous) that lead to functional SMN protein deficiency. The method further involves measuring a neurofilament level (*e.g.*, pNF-H level) in a second biological sample obtained from the human subject after initiation of the SMA therapy. The method further involves comparing the neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample to the neurofilament level (*e.g.*, pNF-H level) measured in the first biological sample. The neurofilament level (*e.g.*, pNF-H level) measured in the second biological sample, as compared to the neurofilament level (*e.g.*, pNF-H level) measured in the first biological sample, is predictive of the severity or type of SMA that the subject will develop. In general, the greater the percent reduction in neurofilament level (*e.g.*, pNF-H level) in the second biological sample, as compared to the neurofilament level (*e.g.*, pNF-H level) measured in the first biological sample, the better the prognosis for the human subject's future motor function.

In some embodiments, the second biological sample is obtained from the human subject 40-90 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject 50-80 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject 60-70 days after initiation of the SMA therapy. In some embodiments, the second biological sample is obtained from the human subject about 64 days after initiation of the SMA therapy.

In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 50% compared to the neurofilament level measured in the first biological sample. In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 60% compared to the neurofilament level measured in the first biological sample. In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by at least 70% compared to the neurofilament level measured in the first biological sample.

In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by less than 50% compared to the neurofilament level measured in the first biological sample. In some embodiments where the second biological sample is obtained from the human subject 40-90 days, 50-80 days, 60-70 days, or about 64 days after initiation of the SMA therapy, the neurofilament level measured in the second biological sample is reduced by less than 40% compared to the neurofilament level measured in the first biological sample.

The following embodiments apply to any of the above aspects. In certain instances, the SMA therapy comprises nusinersen or a nusinersen salt. In some instances, the SMA therapy comprises nusinersen sodium. In certain instances, nusinersen sodium is administered by intrathecal injection of 5 mL of a 2.4 mg/mL solution. In certain instances, the SMA therapy comprises one or more of SPINRAZA®, olesoxime, AVX-101, CK-2127107, RG7916, RG7800, RO7034067, LMI070, or SRK-015. In certain instances, the SMA therapy comprises a small molecule. In certain instances, the SMA therapy comprises gene therapy. In certain instances, the SMA therapy comprises a p38aDMAPK inhibitor. In certain instances, the SMA therapy comprises a DcpS inhibitor. In certain instances, the SMA therapy comprises a JNK inhibitor. In certain instances, the control is a pre-established neurofilament cut-off value. In some instances, the control is the neurofilament level (*e.g.*, pNF-H level) in a biological sample or biological samples obtained from one or more human subjects that do not have SMA.

In another aspect, the disclosure features a method for measuring a neurofilament level (*e.g.*, pNF-H level). The method involves providing a biological sample obtained from a human subject having mutations in both copies of the *SMN1* gene that lead to functional SMN protein deficiency; and measuring a neurofilament level (*e.g.*, pNF-H level) in the biological sample.

These embodiments apply to any of the above aspects. In certain instances, the neurofilament is a neurofilament heavy chain (*e.g.*, phosphorylated NF-H). In certain instances, the neurofilament is a neurofilament medium/intermediate chain. In certain instances, the neurofilament is a neurofilament light chain. In certain instances, the neurofilament is internexin. In certain instances, the neurofilament is peripherin. In certain instances, the biological sample is blood, serum, plasma, or cerebrospinal fluid. In some instances, NF-H is detected using a polyclonal anti-NF-H antibody. In some instances, NF-H is detected using a polyclonal anti-NF-H antibody that specifically detects the hyper-phosphorylated form of NF-H (*e.g.*, Encor Biotechnology Cat # RPCA-NF-H; and/or Cat # CPCA-NF-H). In some cases, NF-H is detected using a monoclonal anti-NF-H antibody. In certain embodiments, the human subject is a fetus. In certain embodiments, the human subject is an infant. In certain embodiments, the human subject is less than 6 months of age. In certain embodiments, the human subject is older than 6 months of age. In certain embodiments, the human subject is a child less than 12 years of age. In certain embodiments, the human subject is a child less than 18 years of age. In certain embodiments, the human subject is an adult 18 years or older.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the levels of phosphorylated neurofilament heavy chain (pNF-H) levels in plasma and CSF samples at the same timepoint and in the same subject from EMBRACE and NURTURE clinical studies.

Fig. 2 shows the levels of pNF-H levels in plasma samples in subjects from NURTURE, ENDEAR, EMBRACE and CHERISH clinical studies who have two, three, or four copies of SMN2.

Fig. 3 is a depiction of the levels of pNF-H in subjects who have two SMN2 copies with increasing age.

Fig. 4 is a depiction of the levels of pNF-H in subjects who have three SMN2 copies with increasing age.

Fig. 5 is a depiction of the levels of pNF-H in subjects who have two, three, or four SMN2 copies and age of symptom onset.

Fig. 6 is a table providing baseline characteristics and SMA History by baseline pNF-H level.

Fig. 7 is a table providing baseline characteristics and SMA History by baseline pNF-H level.

Fig. 8 is a table providing baseline characteristics and SMA History by baseline pNF-H level.

Fig. 9 shows the correlation of baseline log(pNF-H) levels and baseline CHOP INTEND scores.

Fig. 10 is a table showing the ability to correlate phenotypes at baseline by pNF-H levels in ENDEAR.

Fig. 11 is a graphical depiction of pNF-H levels in ENDEAR.

Fig. 12 is a graphical depiction of the percentage change in NF-H levels in ENDEAR.

Fig. 13 is a graphical depiction of SMA drug therapy on pNF-H levels in ENDEAR.

Fig. 14 is a graphical depiction of SMA drug therapy on the percentage change in NF-H levels in ENDEAR.

Fig. 15 is a depiction of the change in pNF-H levels at various time points.

Fig. 16 is a graph depicting the association of HINE-2 Score on Day 183 with pNF-H levels on Day 64.

Fig. 17 shows the association of HINE-2 Score on Day 183 with pNF-H levels on Day 64 (with Covariates).

Fig. 18 is a graph depicting the association of CHOP INTEND Score on Day 183 with pNF-H levels on Day 64.

Fig. 19 shows the association of CHOP INTEND Score on Day 183 with pNF-H levels on Day 64 (with Covariates).

Fig. 20 is a depiction of HINE-2 (Responder/Non-responder) category versus pNF-H levels on Day 183.

Fig. 21 is a graphical depiction of HINE-2 (Responder/Non-responder) category versus Day 183 percentage change in pNF-H levels.

Fig. 22 is a graphical depiction of CHOP INTEND (Responder/Non-responder) category versus pNF-H levels on Day 183.

Fig. 23 is a graphical depiction of CHOP INTEND (Responder/Non-responder) category versus Day 183 percentage change in pNF-H levels.

Fig. 24 is a graphical depiction of the maintenance of drug effect based on pNF-H (pg/mL) levels in NURTURE.

Fig. 25 is a graphical depiction of the maintenance of drug effect based on pNF-H (pg/mL) levels in EMBRACE.

Fig. 26 is a graphical depiction of the drug effect on percentage change in pNF-H (pg/mL) levels in NURTURE.

Fig. 27 is a graphical depiction of the drug effect on percentage change in pNF-H (pg/mL) levels in EMBRACE.

Fig. 28 are graphs depicting the association of HINE-2 score on Day 183 with pNF-H levels on Day 64. For top graph: Root MSE: 2.87069; R-square: 0.0184; Adjusted R-square: 0.0002. For bottom graph: Root MSE: 1.70576; R-square: 0.224; Adjusted R-square: 0.1831. DF = degrees of freedom; MSE = mean square error.

Fig. 29 are graphs depicting the association of CHOP INTEND score on Day 183 with pNF-H levels on Day 64. For top graph: Root MSE: 10.09399; R-square: 0.0754; Adjusted R-square: 0.0583. For bottom graph: Root MSE: 9.41166; R-square: 0.2176; Adjusted R-square: 0.1764. DF = degrees of freedom; MSE = mean square error.

Fig. 30 is a graphical illustration of pNF-H levels over time in surviving infants with non-missing values at Day 302.

Fig. 31 is a graphic depiction of the percentage change in pNF-H levels over time in surviving infants with non-missing values at Day 302.

Fig. 32 is a graphical depiction of the association of peroneal CMAP Amplitude on Day 183 with pNF-H levels on Day 64.

Fig. 33 are graphs depicting the association of Peroneal CMAP Amplitude on Day 183 with pNF-H levels on Day 64**.** For top graph: Root MSE: 0.56401; R-square: 0.0164; Adjusted R-square: -0.0029. For bottom graph: Root MSE: 0.18933; R-square: 0.2906; Adjusted R-square: 0.2512. DF = degrees of freedom; MSE = mean square error.

Fig. 34 is a graphical depiction of the association of Ulnar CMAP (compound muscle action potential) Amplitude on Day 183 with pNF-H levels on Day 64. Root MSE: 0.24904; R-square: 0.1746; Adjusted R-square: 0.1633. DF = degrees of freedom; MSE = mean square error.

Fig. 35 are graphs depicting the association of Ulnar CMAP Amplitude on Day 183 with pNF-H levels on Day 64. For top graph: Root MSE: 0.2824; R-square: 0.0517; Adjusted R-square: 0.0338. For bottom graph: Root MSE: 0.11118; R-square: 0.2406; Adjusted R-square: 0.1984. DF = degrees of freedom; MSE = mean square error.

Figs. 36A-36C are receiver operating characteristic (ROC) graphs depicting the effectiveness of measuring percent change in pNF-H levels at Day 29 (Fig. 36A), Day 64 (Fig. 36B), and Day 183 (Fig. 36C) at predicting motor function at Day 302.

Figs. 37A-37B are graphs depicting the effectiveness of measuring percent change in pNF-H levels as a predictor of CHOP INTEND responders (Fig. 37A) and motor milestone responders (Fig. 37B) in ENDEAR.

Figs. 38A-38C are graphs depicting the effectiveness of measuring percent change in pNF-H levels as a predictor of HFMSE responders (Fig. 38A), RULM responders (Fig. 38B), and WHO responders (Fig. 38C) in CHERISH.

Figs. 39A-39B are graphs depicting baseline cerebrospinal fluid pNF-H levels versus age at first dose by patient population (Fig. 39A) and SMN2 copy number (Fig. 39B).

Fig. 40 is a graph depicting decline in cerebrospinal fluid pNF-H levels following treatment with nusinersen.

Figs. 41A-41D are graphs depicting percent change in pNF-H and NF-L levels following treatment with nusinersen in CSF of presymptomatic subjects (Fig. 41A), CSF of infantile-onset subjects (Fig. 41B), plasma of infantile-onset subjects (Fig. 41C), and plasma of later-onset subjects (Fig. 41D).

Figs. 41E-41F are graphs depicting the absolute value change in pNF-H and NF-L levels following treatment with nusinersen in plasma of infantile-onset subjects (Fig. 41E) and plasma of later-onset subjects (Fig. 41F).

DETAILED DESCRIPTION

This disclosure is based, in part, on the surprising finding that neurofilament (NF) levels can serve as an effective biomarker for SMA.

1. Spinal Muscular Atrophy

After cystic fibrosis, spinal muscular atrophy (SMA) is the second most common lethal autosomal recessive disorder among Caucasians. This disease is characterized by the progressive degeneration of the alpha motoneurons in the anterior horn of the spinal cord, which leads to muscle atrophy, paralysis, and sometimes even death. The most common form of SMA is caused by mutations in the 5q13 survival of the motor neuron (*SMN1*) gene. This disorder affects 1 in 6,000 to 10,000 infants, with a carrier frequency of 1 in 40. Several clinical types have been described for SMA, which group the disorder according to the age onset and the progression of the symptoms.

According to the above classification, there are five types of SMA: type 0 (embryonic form), type I (Werdning–Hoffman), type II (intermediate), type III (Kugeleberg–Welander),

and type IV (adult form). Type 0, the most severe, is characterized by reduced movement of the fetus between 30–36 weeks of the pregnancy and a very short life expectancy. Type I is the next most severe form, with an onset before the age of 6 months and a life expectation of around 2 years. Types II and III are known as chronic forms and are less severe, with an onset between 6–18 months (type II) and respectively after 18 months (for type III). In many cases, Type IV mimics the symptoms of type III, but the onset is after 18 years of age (typically around 30 years old). A normal life expectancy is characteristic for this adult form.

The SMA determining gene, *SMN1,* was mapped to the 5q11.2-13.3 region. The homozygous deletion of *SMN1* exon 7 is the most common mutation found in SMA patients; however, there are several cases of compound heterozygous patients in whom deletions and different point mutations have been detected. In humans, two forms of the *SMN* gene exist on each allele: a telomeric form (*SMN1*) and a centromeric form (*SMN2*). Transcription of the *SMN1* gene produces full-length messenger RNA (mRNA) transcripts that encode the SMN protein. The *SMN2* gene is identical to the *SMN1* gene with the exception of a C to T substitution at position 840 that results in the exclusion of exon 7 during transcription. The resultant truncated protein is not functional and is rapidly degraded. Importantly, the exclusion of exon 7 from *SMN2* mRNAs is not complete, and so a small fraction of the total mRNA transcripts (approximately 10% to15%) arising from the *SMN2* gene contain exon 7, which encodes the normal SMN protein. But the full length SMN protein is synthesized in such a small quantity that it is unable to sustain motor neuron survival.

All patients with SMA lack a functioning *SMN1* gene and are thus dependent on their *SMN2* gene to produce the SMN protein necessary for survival. Thus, SMA is caused by a deficiency in the SMN protein that results in selective motor neuron loss. Several genotype/phenotype analyses have shown a positive correlation between *SMN2* copy number and a milder SMA phenotype. Although *SMN2* copy number is a primary determinant of SMA severity, it is clearly not the only phenotypic modifier. The art has described at least three adult patients with mild 3b phenotypes and only 2 copies of *SMN2.* This seemingly incongruous finding was explained by the fact that these individuals had a c.859G>C exon 7 mutation that created an exon splice–enhancing element that resulted in increased full-length SMN protein production and a milder phenotype. Other modifiers have been described and more are expected as the understanding of the molecular pathogenesis of SMA is refined. These findings show that the SMA phenotype cannot always be deduced solely from the *SMN2* copy number determination.

2. Therapies for SMA

One therapy for the treatment of SMA is SPINRAZA®, a compound containing nusinersen (also known as ASO-10-27 / ISIS 396443 / ISIS SMNRx / ISIS-396443 / ISIS-SMNRx). Nusinersen is a modified antisense oligonucleotide compound (modified in that the 2'- hydroxy groups of the ribofuranosyl rings are replaced with 2'-O-2-methoxyethyl groups and the phosphate linkages are replaced with phosphorothioate linkages) that binds to a specific sequence of the SMN2 transcript in the intron downstream of exon 7. The sodium salt of nusinersen (nusinersen sodium) is marketed under the trade name SPINRAZA®. Nusinersen is designed to treat SMA caused by mutations in chromosome 5q that lead to SMN protein deficiency. Using *in vitro* assays and studies in transgenic animal models of SMA, nusinersen was shown to increase exon 7 inclusion in SMN2 messenger ribonucleic acid (mRNA) transcripts and production of full-length SMN protein. Nusinersen acts to counteract the SMN protein deficit that causes SMA, by increasing the splicing efficiency of the SMN2 pre-mRNA.

Nusinersen is an 18-mer 2'-MOE phosphorothioate antisense oligonucleotide. Nusinersen was designed to pair with a specific target sequence on the SMN2 pre-mRNA to displace heterogeneous ribonucleoproteins (hnRNPs) at the intronic splice silencing site-1 (ISS-1) between exons 7 and 8 to allow for more complete translation of SMN protein from the paralogous gene SMN2. Intrathecal injection of nusinersen into the cerebrospinal fluid (CSF) allows it to be distributed from the CSF to the target central nervous system (CNS) tissues. The full sequence of nusinersen is [2'-O-(2-methoxyethyl)](3'-5')(P-thio) (T-5mC-A-5mC-T-T-T-5mC-A-T-A-A-T-G-5mC-T-G-G) (SEQ ID NO:4). The antisense oligonucleotide contains 2'-O-(2-methoxyethyl) (2'-MOE)-oligoribonucleotides to reduce nuclease degradation and to enhance binding affinity towards the complementary RNA.

There are several other therapies for the treatment of SMA. These include, compounds that increase SMN levels such as histone deacetylase inhibitors, aminoglycosides, and quinazoline derivatives. Histone deacetylase inhibitors such as valproic acid, sodium butyrate, phenylbutyrate, and trichostatin A activate the *SMN2* promoter, resulting in increased full-length SMN protein. Other therapies include CK-2127107 (a fast skeletal muscle troponin activator), LMI070 (branaplan, formerly known as NVS-SM1), olesoxime (a cholesterol oximes family member), RG7916 (a splicing modifier), SMN gene therapy (AAV9-SMN1; AVXS-101 (AAV-human SMN transgene)), and SRK-015 (selective and local inhibitor antibody of the latent form of myostatin). In some instances, one or more of

these agents are used in combination for the treatment of SMA. The present methods are intended to cover any treatment of SMA.

3. Biomarkers for SMA

In order to identify subjects who would benefit from treatment with an SMA therapy (such as the one or more of the SMA therapies described above) or to determine if a therapy is working it is helpful to have a biomarker. A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biologic processes, pathologic processes, or pharmacological responses to a therapeutic intervention. Biomarkers can be biological (*e.g.*, small molecules, metabolites, peptides, proteins, RNA, DNA), physiological (*e.g.*, blood pressure, electromyography, respiratory function), or structural measures (*e.g.*, ultrasound, magnetic resonance imaging, or histological assessment). The biomarkers may be prognostic biomarkers that predict a future clinical outcome; disease progression biomarkers that are indicative of the severity of disease impact; predictive biomarkers that predict a future clinical response to therapy and helps stratify therapies; pharmacodynamic biomarkers that monitor or quantify a therapeutic effect; and surrogate end point biomarkers that predict a future clinical response to therapy wherein a change in the end point is associated with a future clinical response.

There are several known biomarkers for SMA. These include prognostic biomarkers such as SMN2 copy number as an indicator of disease severity; disease progression biomarkers such as Compound Muscle Action Potential (CMAP) amplitude that serves as an indicator of motor neuron loss; predictive biomarkers such as reduced CMAP amplitude that is indicative of less response to SMN restoring therapies; pharmacodynamic biomarkers such as increased full-length SMN transcripts and/or increased SMN protein as indicators of effective induction of the *SMN2* gene; and surrogate end point biomarkers such as increased Motor unit number estimation (MUNE) as an indicator of improved physical function.

This disclosure illustrates the use of neurofilament levels as a novel biomarker for SMA. Neurofilaments (NFs) are the predominant cystokeletal element in nerve cells and play a role not only in conferring mechanical stability but also in determining axonal caliber. Human NFs are composed of three protein subunits, NF-L, NF-M, and NF-H. These proteins share the same basic architecture as other intermediate filament subunit proteins. Neurofilaments in the mammalian nervous system also contain the protein internexin and neurofilaments in the peripheral nervous system can also contain the protein peripherin. Thus, as used herein, by "a neurofilament protein" is meant neurofilament heavy chain (NF-

H), neurofilament medium/intermediate chain (NF-M), neurofilament light chain (NF-L), internexin, or peripherin. The SMA biomarker can be one or more of NF-H, NF-M, NF-L, internexin, and peripherin. In certain instances, the SMA biomarker is a phosphorylated NF-H (pNF-H). In certain instances, the SMA biomarker is a phosphorylated NF-L. The levels of the neurofilament biomarker can be assessed using RNA (*e.g.*, mRNA) or protein.

The amino acid sequences of human NF-H are provided in SEQ ID NO:1 and SEQ ID NO:5 and in Lees et al., *EMBO J*, 7(7);1947-1955 (1988), UniProtKB - P12036, NCBI Reference Sequence: NG_008404.1, NCBI Reference Sequence: NP_066554.2.

SEQ ID NO:1

MMSFGGADALLGAPFAPLHGGGSLHYALARKGGAGGTRSAAGSSSGFHSWTRTSVSSVSASPSRFRGAGAASSTD
SLDTLSNGPEGCMVAVATSRSEKEQLQALNDRFAGYIDKVRQLEAHNRSLEGEAAALRQQQAGRSAMGELYEREV
REMRGAVLRLGAARGQLRLEQEHLLEDIAHVRQRLDDEARQREEAEAAARALARFAQEAEAARVDLQKKAQALQE
ECGYLRRHHQEEVGELLGQIQGSGAAQAQMQAETRDALKCDVTSALREIRAQLEGHAVQSTLQSEEWFRVRLDRL
SEAAKVNTDAMRSAQEEITEYRRQLQARTTELEALKSTKDSLERQRSELEDRHQADIASYQEAIQQLDAELRNTK
WEMAAQLREYQDLLNVKMALDIEIAAYRKLLEGEECRIGFGPIPFSLPEGLPKIPSVSTHIKVKSEEKIKVVEKS
EKETVIVEEQTEETQVTEEVTEEEEKEAKEEEGKEEEGGEEEEAEGGEEETKSPPAEEAASPEKEAKSPVKEEAK
SPAEAKSPEKEEAKSPAEVKSPEKAKSPAKEEAKSPPEAKSPEKEEAKSPAEVKSPEKAKSPAKEEAKSPAEAKS
PEKAKSPVKEEAKSPAEAKSPVKEEAKSPAEVKSPEKAKSPTKEEAKSPEKAKSPEKAKSPEKEEAKSPEKAKSP
VKAEAKSPEKAKSPVKAEAKSPEKAKSPVKEEAKSPEKAKSPVKEEAKSPEKAKSPVKEEAKTPEKAKSPVKEEA
KSPEKAKSPEKAKTLDVKSPEAKTPAKEEARSPADKFPEKAKSPVKEEVKSPEKAKSPLKEDAKAPEKEIPKKEE
VKSPVKEEEKPQEVKVKEPPKKAEEEKAPATPKTEEKKDSKKEEAPKKEAPKPKVEEKKEPAVEKPKESKVEAKK
EEAEDKKKVPTPEKEAPAKVEVKEDAKPKEKTEVAKKEPDDAKAKEPSKPAEKKEAAPEKKDTKEEKAKKPEEKP
KTEAKAKEDDKTLSKEPSKPKAEKAEKSSSTDQKDSKPPEKATEDKAAKGK

SEQ ID NO:5

MMSFGGADALLGAPFAPLHGGGSLHYALARKGGAGGTRSAAGSSSGFHSWTRTSVSSVSASPSRFRGAGAASSTD
SLDTLSNGPEGCMVAVATSRSEKEQLQALNDRFAGYIDKVRQLEAHNRSLEGEAAALRQQQAGRSAMGELYEREV
REMRGAVLRLGAARGQLRLEQEHLLEDIAHVRQRLDDEARQREEAEAAARALARFAQEAEAARVDLQKKAQALQE
ECGYLRRHHQEEVGELLGQIQGSGAAQAQMQAETRDALKCDVTSALREIRAQLEGHAVQSTLQSEEWFRVRLDRL
SEAAKVNTDAMRSAQEEITEYRRQLQARTTELEALKSTKDSLERQRSELEDRHQADIASYQEAIQQLDAELRNTK
WEMAAQLREYQDLLNVKMALDIEIAAYRKLLEGEECRIGFGPIPFSLPEGLPKIPSVSTHIKVKSEEKIKVVEKS
EKETVIVEEQTEETQVTEEVTEEEEKEAKEEEGKEEEGGEEEEAEGGEEETKSPPAEEAASPEKEAKSPVKEEAK
SPAEAKSPEKEEAKSPAEVKSPEKAKSPAKEEAKSPPEAKSPEKEEAKSPAEVKSPEKAKSPAKEEAKSPAEAKS
PEKAKSPVKEEAKSPAEAKSPVKEEAKSPAEVKSPEKAKSPTKEEAKSPEKAKSPEKEEAKSPEKAKSPVKAEAK
SPEKAKSPVKAEAKSPEKAKSPVKEEAKSPEKAKSPVKEEAKSPEKAKSPVKEEAKTPEKAKSPVKEEAKSPEKA
KSPEKAKTLDVKSPEAKTPAKEEARSPADKFPEKAKSPVKEEVKSPEKAKSPLKEDAKAPEKEIPKKEEVKSPVK
EEEKPQEVKVKEPPKKAEEEKAPATPKTEEKKDSKKEEAPKKEAPKPKVEEKKEPAVEKPKESKVEAKKEEAEDK

KKVPTPEKEAPAKVEVKEDAKPKEKTEVAKKEPDDAKAKEPSKPAEKKEAAPEKKDTKEEKAKKPEEKPKTEAKA
KEDDKTLSKEPSKPKAEKAEKSSSTDQKDSKPPEKATEDKAAKGK

The amino acid sequence of human NF-L is provided in SEQ ID NO:2 and in Julien et al., *Biochimica et Biohysica Acta*, 909:10-20 (1987), UniProtKB - P07196, NCBI Reference Sequence: NP_006149.2, and NCBI Reference Sequence: NG_008492.1.

SEQ ID NO:2

MSSFSYEPYYSTSYKRRYVETPRVHISSVRSGYSTARSAYSSYSAPVSSSLSVRRSYSSSSGSLMPSLENLDLSQ
VAAISNDLKSIRTQEKAQLQDLNDRFASFIERVHELEQQNKVLEAELLVLRQKHSEPSRFRALYEQEIRDLRLAA
EDATNEKQALQGEREGLEETLRNLQARYEEEVLSREDAEGRLMEARKGADEAALARAELEKRIDSLMDEISFLKK
VHEEEIAELQAQIQYAQISVEMDVTKPDLSAALKDIRAQYEKLAAKNMQNAEEWFKSRFTVLTESAAKNTDAVRA
AKDEVSESRRLLKAKTLEIEACRGMNEALEKQLQELEDKQNADISAMQDTINKLENELRTTKSEMARYLKEYQDL
LNVKMALDIEIAAYRKLLEGEETRLSFTSVGSITSGYSQSSQVFGRSAYGGLQTSSYLMSTRSFPSYYTSHVQEE
QIEVEETIEAAKAEEAKDEPPSEGEAEEEEKDKEEAEEEEAAEEEEAAKEESEEAKEEEEGGEGEEGEETKEAEE
EEKKVEGAGEEQAAKKKD

The amino acid sequences of human NF-M are provided in SEQ ID NO:3 and SEQ ID NO:6 and in Myers et al., *EMBO J.*, 6(6):1617-1626 (1987) and in UniProtKB - P07197.

SEQ ID NO:3

MSYTLDSLGNPSAYRRVTETRSSFSRVSGSPSSGFRSQSWSRGSPSTVSSSYKRSMLAPRLAYSSAMLSSAESSL
DFSQSSSLLNGGSGPGGDYKLSRSNEKEQLQGLNDRFAGYIEKVHYLEQQNKEIEAEIQALRQKQASHAQLGDAY
DQEIRELRATLEMVNHEKAQVQLDSDHLEEDIHRLKERFEEEARLRDDTEAAIRALRKDIEEASLVKVELDKKVQ
SLQDEVAFLRSNHEEEVADLLAQIQASHITVERKDYLKTDISTALKEIRSQLESHSDQNMHQAEEWFKCRYAKLT
EAAEQNKEAIRSAKEEIAEYRRQLQSKSIELESVRGTKESLERQLSDIEERHNHDLSSYQDTIQQLENELRGTKW
EMARHLREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTFAGSITGPLYTHRPPITISSKIQKPKVEAPKLKVQH
KFVEEIIEETKVEDEKSEMEEALTAITEELAVSMKEEKKEAAEEKEEEPEAEEEEVAAKKSPVKATAPEVKEEEG
EKEEEEGQEEEEEEDEGAKSDQAEEGGSEKEGSSEKEEGEQEEGETEAEAEGEEAEAKEEKKVEEKSEEVATKEE
LVADAKVEKPEKAKSPVPKSPVEEKGKSPVPKSPVEEKGKSPVPKSPVEEKGKSPVPKSPVEEKGKSPVSKSPVE
EKAKSPVPKSPVEEAKSKAEVGKGEQKEEEEKEVKEAPKEEKVEKKEEKPKDVPEKKKAESPVKEEAVAEVVTIT
KSVKVHLEKETKEEGKPLQQEKEKEKAGGEGGSEEEGSDKGAKGSRKEDIAVNGEVEGKEEVEQETKEKGSGREE
EKGVVTNGLDLSPADEKKGGDKSEEKVVVTKTVEKITSEGGDGATKYITKSVTVTQKVEEHEETFEEKLVSTKKV
EKVTSHAIVKEVTQSD

SEQ ID NO:6

MARHLREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTFAGSITGPLYTHRPPITISSKIQKPKVEAPKL
KVQHKFVEEIIEETKVEDEKSEMEEALTAITEELAVSMKEEKKEAAEEKEEEPEAEEEEVAAKKSPVKAT

APEVKEEEGEKEEEEGQEEEEEEDEGAKSDQAEEGGSEKEGSSEKEEGEQEEGETEAEAEGEEAEAKEEK
KVEEKSEEVATKEELVADAKVEKPEKAKSPVPKSPVEEKGKSPVPKSPVEEKGKSPVPKSPVEEKGKSPV
PKSPVEEKGKSPVSKSPVEEKAKSPVPKSPVEEAKSKAEVGKGEQKEEEEKEVKEAPKEEKVEKKEEKPK
DVPEKKKAESPVKEEAVAEVVTITKSVKVHLEKETKEEGKPLQQEKEKEKAGGEGGSEEEGSDKGAKGSR
KEDIAVNGEVEGKEEVEQETKEKGSGREEEKGVVTNGLDLSPADEKKGGDKSEEKVVVTKTVEKITSEGG
DGATKYITKSVTVTQKVEEHEETFEEKLVSTKKVEKVTSHAIVKEVTQSD

The amino acid sequence of human internexin is provided in SEQ ID NO:7.

SEQ ID NO:7

MSFGSEHYLCSSSSYRKVFGDGSRLSARLSGAGGAGGFRSQSLSRSNVASSAACSSASSLGLGLAYRRPPASDGL
DLSQAAARTNEYKIIRTNEKEQLQGLNDRFAVFIEKVHQLETQNRALEAELAALRQRHAEPSRVGELFQRELRDL
RAQLEEASSARSQALLERDGLAEEVQRLRARCEEESRGREGAERALKAQQRDVDGATLARLDLEKKVESLLDELA
FVRQVHDEEVAELLATLQASSQAAAEVDVTVAKPDLTSALREIRAQYESLAAKNLQSAEEWYKSKFANLNEQAAR
STEAIRASREEIHEYRRQLQARTIEIEGLRGANESLERQILELEERHSAEVAGYQDSIGQLENDLRNTKSEMARH
LREYQDLLNVKMALDIEIAAYRKLLEGEETRFSTSGLSISGLNPLPNPSYLLPPRILSATTSKVSSTGLSLKKEE
EEEEASKVASKKTSQIGESFEEILEETVISTKKTEKSNIEETTISSQKI

The amino acid sequence of human peripherin is provided in SEQ ID NO:8.

SEQ ID NO:8

MSHHPSGLRAGFSSTSYRRTFGPPPSLSPGAFSYSSSSRFSSSRLLGSASPSSSVRLGSFRSPRAGAGALLRLPS
ERLDFSMAEALNQEFLATRSNEKQELQELNDRFANFIEKVRFLEQQNAALRGELSQARGQEPARADQLCQQELRE
LRRELELLGRERDRVQVERDGLAEDLAALKQRLEEETRKREDAEHNLVLFRKDVDDATLSRLELERKIESLMDEI
EFLKKLHEEELRDLQVSVESQQVQQVEVEATVKPELTAALRDIRAQYESIAAKNLQEAEEWYKSKYADLSDAANR
NHEALRQAKQEMNESRRQIQSLTCEVDGLRGTNEALLRQLRELEEQFALEAGGYQAGAARLEEELRQLKEEMARH
LREYQELLNVKMALDIEIATYRKLLEGEESRISVPVHSFASLNIKTTVPEVEPPQDSHSRKTVLIKTIETRNGEV
VTESQKEQRSELDKSSAHSY

In certain instances, the level of NF (*e.g.*, pNF-H) is used in combination with one or more other SMA biomarkers.

4. Diagnosing SMA

The disclosure features methods of diagnosing whether a subject (*e.g.*, a presymptomatic subject) has biologically active disease (*i.e.*, whether the SMA is active and its severity). The method involves measuring a neurofilament level in a biological sample obtained from the subject. SMA is diagnosed if the neurofilament level in the subject is

higher than a control level. The neurofilament level also predicts the severity of the disease: the higher the NF level relative to a control the more severe the SMA.

In some instances, the method involves measuring a NF-H level in the biological sample obtained from the subject. In some instances, the method involves measuring a pNF-H level in the biological sample obtained from the subject. In some instances, the method involves measuring a NF-M level in the biological sample obtained from the subject. In some instances, the method involves measuring a NF-L level in the biological sample obtained from the subject.

The biological sample can be *e.g.,* blood, serum, plasma, or cerebrospinal fluid. In some instances, the biological sample is plasma.

In certain instances, the subject is a human fetus. In certain instances, the subject is a human who is a new born. In certain instances, the subject is a human who is less than or equal to 6 months of age (*e.g.,* 2 days old, 3 days old, 4 days old, 5 days old, 6 days old, 1 week old, 2 weeks old, 3 weeks old, 1 month old, 2 months old, 3 months old, 4 months old, 5 months old, or 6 months old). In certain instances, the subject is a human who is greater than 6 months of age and less than equal to 18 months of age (*e.g.,* 7 months old, 8 months old, 9 months old, 10 months old, 11 months old, 12 months old, 13 months old, 14 months old, 15 months old, 16 months old, 17 months old, or 18 months old). In some instances, the subject is a human who is greater than 18 months of age. In some instances, the subject is a human who is greater than 18 years of age.

In some instances, the NF level is measured by assessing the level of NF RNA (*e.g.,* mRNA) in the biological sample.

In some instances, the NF level is measured by assessing the level of an NF protein (NF-H, NF-M, or NF-L protein) in the biological sample. In certain instances, the NF protein is pNF-H. The concentration of the protein or proteins of interest can be measured using any method known in the art such as an immunological assay. Non-limiting examples of such methods include enzyme immunoassay, radioimmunoassay, chemiluminescent immunoassay, electrochemiluminescence immunoassay, latex turbidimetric immunoassay, latex photometric immunoassay, immuno-chromatographic assay, and western blotting. In certain embodiments, the concentration of the protein or proteins of interest is measured by mass spectrometry.

In some embodiments, the neurofilament level (*e.g.,* pNF-H) in the biological sample is above a control level. In some embodiments, the neurofilament level in the biological sample is above 300 pg/mL. In some embodiments the neurofilament level in the biological sample

is above 400 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 500 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 600 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 700 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 800 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 900 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,000 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,100 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,200 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,300 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,400 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 1,500 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 2,000 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 3,000 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 4,000 pg/mL. In some embodiments, the neurofilament level in the biological sample is above 5,000 pg/mL.

A human subject who is diagnosed as having SMA can be administered any SMA therapy. In certain cases, a human subject who is previously determined to have SMA (*e.g.*, by measuring NF levels in a biological sample from the subject) is administered a SMA therapy. In some instances, the therapy is SPINRAZA®. In some cases, SPINRAZA® is administered intrathecally at a dose of 12 mg per administration. In some instances, the SMA therapy is a combination therapy.

Exemplary levels of pNF-H in subjects having SMA are provided in the table below:

Clinical Trial	**Age (days)**	**pNF-H Level (pg/mL) (SMN2 =2 copies)**	**pNF-H Level (pg/mL) (SMN2 =3 copies)**
NURTURE	3-42	1,498 – 52,943	959-7,950
ENDEAR	30-262	2,390 – 50,100	13,600

5. Responsiveness to Treatment

The levels of NF can also be used to determine if a subject receiving a SMA therapy is responding to the treatment. This can be assessed by obtaining a first biological sample from

the subject before and a second biological sample after administering a SMA therapy to the subject and measuring the level of NF (*e.g.*, NF-H, NF-M, or NF-L) in such samples. In one instance, the level of NF is a level of pNF-H. In some instances, the therapy is SPINRAZA®. In some cases, SPINRAZA® is administered intrathecally at a dose of 12 mg per administration. In some instances, the SMA therapy is a combination therapy.

In certain instances, the first biological sample or samples can be collected from the subject any time before treatment, *e.g.*, a week before, several days before, a day before, several hours before, an hour before, or less than an hour before, administering the SMA therapy. Similarly, the second biological sample or samples can be collected from the subject any time after administration of the SMA treatment, *e.g.*, less than an hour after, an hour after, several hours after, a day after, several days after, a week after, several weeks after, a month after, two months after, three months after, four months after, five months after, 6 months after, 7 months after, or 8 months after, administering the SMA therapy. A reduction in NF level after commencing SMA therapy is indicative of the effectiveness of the SMA therapy. In such instances, continuation of the SMA therapy is indicated. Failure to reduce NF level after commencing an SMA therapy is indicative of the need for altering the dose (*e.g.*, increasing the dose) of the SMA therapy, or the lack of effectiveness of that particular SMA therapy. In the latter instance, discontinuation of that particular SMA therapy may be suggested and the use of a different SMA therapy or therapies is to be considered.

The level of NF can be assessed by measuring RNA or protein levels. In some instances, the level of pNF-H is determined. The concentration of the NF protein or proteins of interest can be measured using any method known in the art such as an immunological assay. Non-limiting examples of such methods include enzyme immunoassay, radioimmunoassay, chemiluminescent immunoassay, electrochemiluminescence immunoassay, latex turbidimetric immunoassay, latex photometric immunoassay, immuno-chromatographic assay, and western blotting. In certain embodiments, the concentration of the protein or proteins of interest is measured by mass spectrometry.

In certain instances, a neurofilament level (*e.g.*, pNF-H) in the first biological sample is above 300 pg/mL, above 400 pg/mL, above 500 pg/mL, above 600 pg/mL, above 700 pg/mL, above 800 pg/mL, above 900 pg/mL, above 1,000 pg/mL, above 1,500 pg/mL, above 2,000 pg/mL, above 3,000 pg/mL, above 4,000 pg/mL, or above 5,000 pg/mL. In certain embodiments, the neurofilament level measured in the second biological sample is lower than the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample shows a greater than 30% (e.g.,

greater than 31%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 80%, 85%, 90%, or 95%) decline relative to the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 10% to 80% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 20% to 80% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 20% to 85% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 20% to 90% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 20% to 95% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 30% to 80% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 30% to 85% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 30% to 90% of the neurofilament level measured in the first biological sample. In some embodiments, the neurofilament level measured in the second biological sample is between 30% to 95% of the neurofilament level measured in the first biological sample.

In certain instances, the subject is a presymptomatic human. In certain instances, the subject is a human who is a new born. In certain instances, the subject is a human who is less than or equal to 6 months of age (*e.g.*, 2 days old, 3 days old, 4 days old, 5 days old, 6 days old, 1 week old, 2 weeks old, 3 weeks old, 1 month old, 2 months old, 3 months old, 4 months old, 5 months old, or 6 months old). In certain instances, the subject is a human who is greater than 6 months of age and less than equal to 18 months of age (*e.g.*, 7 months old, 8 months old, 9 months old, 10 months old, 11 months old, 12 months old, 13 months old, 14 months old, 15 months old, 16 months old, 17 months old, or 18 months old). In some instances, the subject is a human who is greater than 18 months of age. In some instances, the subject is a human who is greater than 18 years of age.

6. Predicting Disease Progression

The levels of NF in a biological sample from a subject can also serve to predict future phenotypes. For example, the level of NF can predict future motor function. As an

illustration, the level of pNF-H one to two months after commencement of therapy can predict motor function ten months after commencement of therapy. The lower the pNF-H level one to two months after commencement of therapy relative to a control, the more likely the subject will have improved motor function relative to a subject with higher pNF-H levels one to two months after commencement of therapy. This predictive possibility allows the healthcare practitioner to modify or adjust dosing and treatment of the subject.

7. Controls

As described above, the methods of the present disclosure can involve, measuring the expression level (e.g., mRNA or protein concentration) of one or more NF genes or proteins in a biological sample from a subject (*e.g.,* a presymptomatic human subject), wherein the expression level of one or more of the NF genes or proteins, compared to a control, predicts whether a subject has SMA; the severity of the SMA; future phenotypes; and whether or not a subject is a responder to treatment comprising a SMA therapy (*e.g.,* SPINRAZA®).

In certain embodiments, when diagnosing whether a subject has SMA, where the concentration of a NF protein (*e.g.,* pNF-H) in a biological sample from a subject is higher than the control, the subject is identified as likely to have SMA. In this context, the term "control" includes a sample (from the same source – *e.g.,* blood, plasma, serum, CSF) obtained from a subject of the same or similar age who is known to not have SMA. For example, if a subject who is a newborn is being tested, then the control is also from a newborn who does not have SMA; if a subject who is 6-18 months of age is being tested, then the control is also from a subject who is 6-18 months in age who do not have SMA. The term "control" also includes a sample (from the same tissue) obtained in the past from a subject who is known to not have SMA and used as a reference for future comparisons to test samples taken from subjects for whom SMA is to be predicted. The "control" expression level/concentration for a particular NF protein may also be pre-established by an analysis of protein expression in one or more (*e.g.,* two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) human subjects of similar age that do not have SMA. This pre-established reference value (which may be an average or median expression level/concentration taken from multiple subjects that do not have SMA) may then be used for the "control" concentration/expression level of the protein or nucleic acid in the comparison with the test sample. In such a comparison, the subject is predicted to have SMA if the expression level of the NF being analyzed is higher than the pre-established reference.

The methods of the present disclosure can involve, measuring the expression level (*e.g.*, mRNA or protein concentration) of one or more genes (*e.g.*, one or more NF genes) in a biological sample from a subject having or suspected of having SMA, wherein the expression level of one or more of the NF genes, compared to a control, predicts the responsiveness of a subject to treatment comprising a SMA therapy (*e.g.*, SPINRAZA®). In certain embodiments, when the concentration of a NF protein (*e.g.*, pNF-H) in a biological sample from a subject having or suspected of having SMA is lower than the control, the subject is identified as likely to respond to a therapy comprising a SMA therapy. In this context, the term "control" includes a sample (from the same source – *e.g.*, plasma, blood, serum, CSF) obtained from a subject of the same or similar age who is known to not respond to that SMA therapy. The term "control" also includes a sample (from the same tissue) obtained in the past from a subject who is known to not respond to that SMA therapy and used as a reference for future comparisons to test samples taken from subjects for which therapeutic responsiveness is to be predicted. The "control" expression level/concentration for a particular NF protein in a particular cell type or tissue may be pre-established by an analysis of protein expression in one or more (*e.g.*, two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) human subjects of the same or similar age that have not responded to treatment with a SMA therapy (*e.g.*, SPINRAZA®). This pre-established reference value (which may be an average or median expression level/concentration taken from multiple subjects that have not responded to the therapy) may then be used for the "control" concentration/expression level of the protein or nucleic acid in the comparison with the test sample. In such a comparison, the subject is predicted to respond to a therapy comprising a SMA therapy (*e.g.*, SPINRAZA®) if the expression level of the NF gene being analyzed is lower than the pre-established reference.

The "control" concentration for a particular protein (*e.g.*, NF-H) in a particular biological fluid, cell type, or tissue may alternatively be pre-established by an analysis of gene expression in one or more subjects that have responded to treatment with a SMA therapy (*e.g.*, SPINRAZA®). This pre-established reference value (which may be an average or median expression level taken from multiple subjects that have responded to the therapy) may then be used as the "control" expression level in the comparison with the test sample. In such a comparison, the subject is predicted to respond to a SMA therapy (*e.g.*, SPINRAZA®) if the concentration of the protein being analyzed is the same as, or comparable to (at least 85% but less than 100% of), the pre-established reference.

In certain embodiments, the "control" is a pre-determined cut-off value.

In some embodiments, the methods described herein include determining if the concentration of a NF protein(s) of interest falls above or below a predetermined cut-off value.

A cut-off value is typically a concentration of a protein above or below which is considered predictive of something – *e.g.*, likely to develop SMA; or responsiveness of a subject to a therapy of interest. Thus, in accordance with the methods described herein, a reference concentration of a NF protein (*e.g.*, pNF-H) is identified as a cut-off value, above or below of which is predictive of a subject having SMA, or of a subject who shows responsiveness to a SMA therapy (*e.g.*, SPINRAZA®). Some cut-off values are not absolute in that clinical correlations can still remain significant over a range of values on either side of the cutoff; however, it is possible to select an optimal cut-off value (e.g. varying H-scores) of concentration of NF proteins for a particular sample type. Cut-off values determined for use in the methods described herein can be compared with, *e.g.*, published ranges of NF concentrations, but can be individualized to the methodology used and patient population. It is understood that improvements in optimal cut-off values could be determined depending on the sophistication of statistical methods used and on the number and source of samples used to determine reference level values for the different proteins, genes, and sample types. Therefore, established cut-off values can be adjusted up or down, on the basis of periodic re-evaluations or changes in methodology or population distribution.

The reference concentration of one or more NF proteins can be determined by a variety of methods. The reference level can be determined by comparison of the concentration of a NF protein of interest in, *e.g.*, populations of subjects (*e.g.*, patients) that are responsive to a SMA therapy (*e.g.*, SPINRAZA®) or not responsive to a SMA therapy. This can be accomplished, for example, by histogram analysis, in which an entire cohort of patients is graphically presented, wherein a first axis represents the concentration of a protein of interest and a second axis represents the number of subjects in the cohort whose sample contain one or more concentrations. Determination of the reference concentration of a protein can then be made based on an amount or concentration which best distinguishes these separate groups. The reference level can be a single number, equally applicable to every subject, or the reference level can vary, according to specific subpopulations of subjects. For example, older subjects can have a different reference level than younger subjects. In

addition, a subject with more severe disease can have a different reference value than one with a milder form of the disease (*e.g.*, Type I vs. Type IV SMA; Type I vs. Type III SMA; Type I vs. Type II SMA).

The pre-established cut-off value can be a NF protein concentration (*e.g.*, pNF-H) that is determined based on receiver operating characteristic (ROC) analysis. ROC curves are used to determine a cut-off value for a clinical test. Consider the situation where there are two groups of patients and by using an established standard technique one group is known to be responsive to a SMA therapy, and the other is known to not respond to the SMA therapy. A measurement using a biological sample from all members of the two groups is used to test for responsiveness to a SMA therapy. The test will find some, but not all, responders to respond to a SMA therapy. The ratio of the responders found by the test to the total number of responders (known by the established standard technique) is the true positive rate (also known as sensitivity). The test will find some, but not all, non-responders to not respond to a SMA therapy. The ratio of the non-responders found by the test to the total number of non-responders (known by the established standard technique) is the true negative rate (also known as specificity). The hope is that the ROC curve analysis of the SMA therapy-responsiveness test will find a cut-off value that will minimize the number of false positives and false negatives. A ROC is a graphical plot which illustrates the performance of a binary class stratifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives versus the fraction of false positives out of the negatives, at various threshold settings.

In one embodiment, the NF protein concentration is determined based on ROC analysis predicting response to a SMA therapy with a positive predictive value, wherein a concentration of a protein of interest (*e.g.*, pNF-H) equal to or below the pre-established cut-off value is a low concentration of the protein of interest and a value higher than the pre-established cut-off value is a high concentration of the protein of interest. The positive predictive value is the proportion of positive test results that are true positives; it reflects the probability that a positive test reflects the underlying condition being tested for. Methods of constructing ROC curves and determining positive predictive values are well known in the art.

In another embodiment, the pre-established cut-off value can be a NF protein concentration that is determined based on simulation models predicting responsiveness to

SMA therapy, and wherein a concentration of the protein of interest (*e.g.*, pNF-H) equal to or below the pre-established cut-off value is a low concentration of the protein of interest and a value higher than the pre-established cut-off value is a high concentration of the protein of interest.

8. Biological Samples

Suitable biological samples for the methods described herein include any biological fluid, cell, tissue, or fraction thereof, which includes analyte biomolecules of interest such as NF protein or nucleic acid (*e.g.*, RNA (mRNA)). A biological sample can be, for example, a specimen obtained from a human subject or can be derived from such a subject. For example, a sample can be a tissue section obtained by biopsy, archived biological fluid, or cells that are placed in or adapted to tissue culture. In some instances, a biological sample is a biological fluid such as blood, plasma, serum, cerebrospinal fluid (CSF), urine, or such a sample absorbed onto a substrate (*e.g.*, glass, polymer, paper). A biological sample can be further fractionated, if desired, to a fraction containing particular cell types. For example, a blood sample can be fractionated into serum or into fractions containing particular types of blood cells such as red blood cells or white blood cells (leukocytes). If desired, a sample can be a combination of samples from a subject such as a combination of a tissue and fluid sample.

The biological samples can be obtained from a subject having, suspected of having, or at risk of developing, SMA. In certain embodiments, the subject is a human fetus. In certain embodiments, the subject is a presymptomatic human infant. In certain embodiments, the subject is a presymptomatic human child. In certain embodiments, the subject is a presymptomatic human adult.

Any suitable methods for obtaining the biological samples can be employed, although exemplary methods include, *e.g.*, phlebotomy, fine needle aspirate biopsy procedure. Samples can also be collected, *e.g.*, by microdissection (e.g., laser capture microdissection (LCM) or laser microdissection (LMD)).

Methods for obtaining and/or storing samples that preserve the activity or integrity of molecules (*e.g.*, nucleic acids or proteins) in the sample are well known to those skilled in the art. For example, a biological sample can be further contacted with one or more additional

agents such as buffers and/or inhibitors, including one or more of nuclease, protease, and phosphatase inhibitors, which preserve or minimize changes in the molecules (*e.g.,* nucleic acids or proteins) in the sample. Such inhibitors include, for example, chelators such as ethylenediamine tetraacetic acid (EDTA), ethylene glycol bis(P-aminoethyl ether) N,N,N1,Nl-tetraacetic acid (EGTA), protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, antipain, and the like, and phosphatase inhibitors such as phosphate, sodium fluoride, vanadate, and the like. Suitable buffers and conditions for isolating molecules are well known to those skilled in the art and can be varied depending, for example, on the type of molecule in the sample to be characterized (*see, e.g.,* Ausubel et al. Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999); Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press (1988); Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Press (1999); Tietz Textbook of Clinical Chemistry, 3rd ed. Burtis and Ashwood, eds. W.B. Saunders, Philadelphia, (1999)). A sample also can be processed to eliminate or minimize the presence of interfering substances. For example, a biological sample can be fractionated or purified to remove one or more materials that are not of interest. Methods of fractionating or purifying a biological sample include, but are not limited to, chromatographic methods such as liquid chromatography, ion-exchange chromatography, size-exclusion chromatography, or affinity chromatography. For use in the methods described herein, a sample can be in a variety of physical states. For example, a sample can be a liquid or solid, can be dissolved or suspended in a liquid, can be in an emulsion or gel, or can be absorbed onto a material.

9. Determining Expression Levels/Concentrations of Biomarkers

Gene expression can be detected as, *e.g.,* protein or RNA expression of a target gene. That is, the presence or expression level (amount) of a gene can be determined by detecting and/or measuring the level of mRNA or protein expression of the gene. In some embodiments, gene expression can be detected as the activity of a protein encoded by a NF gene.

In one embodiment, the expression of a gene can be determined by detecting and/or measuring expression or concentration of a protein encoded by the gene. Methods of determining protein expression/concentration are well known in the art. A generally used

method involves the use of antibodies specific for the target protein of interest. For example, methods of determining protein expression include, but are not limited to, western blot or dot blot analysis, immunohistochemistry (e.g., quantitative immunohistochemistry), immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISPOT; Coligan, J. E., et al., eds. (1995) Current Protocols in Immunology. Wiley, New York), radioimmunoassay, chemiluminescent immunoassay, electrochemiluminescence immunoassay, latex turbidimetric immunoassay, latex photometric immunoassay, immuno-chromatographic assay, and antibody array analysis (*see, e.g.,* U.S. Publication Nos. 2003/0013208 and 2004/171068, the disclosures of each of which are incorporated herein by reference in their entirety). Further description of many of the methods above and additional methods for detecting protein expression can be found in, *e.g.,* Sambrook et al. (supra).

In one example, the presence or amount of NF protein expression of a NF gene (*e.g.,* NF-H) can be determined using a western blotting technique. For example, a lysate can be prepared from a biological sample, or the biological sample itself, can be contacted with Laemmli buffer and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE-resolved proteins, separated by size, can then be transferred to a filter membrane (e.g., nitrocellulose) and subjected to immunoblotting techniques using a detectably-labeled antibody specific to the protein of interest. The presence or amount of bound detectably-labeled antibody indicates the presence or amount of protein in the biological sample.

In one embodiment, the SimplePlex platform is used to measure the levels of NF-H (e.g., phosphorylated NF-H). SimplePlex is commercially available from Protein Simple (San Jose, CA, USA) (See Dysinger M, et al. J. Immunol. Methods. 451:1-10, 2017).

In one embodiment, an assay for measuring NF-L (e.g., phosphorylated NF-L) is employed. Assays for measuring NF-L in serum have been described (see, e.g., Gaiottino et al., PLoS ONE 8: e75091, 2013; Kuhle et al., J. Neurol. Neurosurg. Psychiatry 86(3): 273-279, 2014). In one example, blood serum from a subject is centrifuged at 1000g for 10 minutes at room temperature and stored at −80°C within 2 hours of collection. Serum NF-L concentrations can be measured (e.g., in duplicate) using ready-to-use enzyme linked immunosorbent assay (ELISA) (Mabtech AB, Nacka Strand, Sweden) or an electrochemiluminescence (ECL) immunoassay described in Gaiottino et al., PLoS ONE 8:

e75091, 2013, or a single molecule array (SIMOA) method described in Disanto et al., Ann. Neurol. 81(6): 857-870, 2017. The assay methods have been compared in Kuhl et al., Clinical Chemistry and Laboratory Medicine 54 (10): 1655-1661, 2016. The SIMOA assay (particularly called the Simoa NF-light Advantage kit) is commercially available from Quanterix Corp. (Lexington, MA, USA).

In another example, an immunoassay can be used for detecting and/or measuring the protein expression of a gene (*e.g.*, NF-H gene). As above, for the purposes of detection, an immunoassay can be performed with an antibody that bears a detection moiety (*e.g.*, a fluorescent agent or enzyme). Proteins from a biological sample can be conjugated directly to a solid-phase matrix (*e.g.*, a multi-well assay plate, nitrocellulose, agarose, sepharose, encoded particles, or magnetic beads) or it can be conjugated to a first member of a specific binding pair (*e.g.*, biotin or streptavidin) that attaches to a solid-phase matrix upon binding to a second member of the specific binding pair (*e.g.*, streptavidin or biotin). Such attachment to a solid-phase matrix allows the proteins to be purified away from other interfering or irrelevant components of the biological sample prior to contact with the detection antibody and also allows for subsequent washing of unbound antibody. Here, as above, the presence or amount of bound detectably-labeled antibody indicates the presence or amount of protein in the biological sample.

There is no particular restriction as to the form of the antibody and the present disclosure includes polyclonal antibodies, as well as monoclonal antibodies. The antiserum obtained by immunizing animals, such as rabbits with a protein or fragment thereof of the invention (i.e., a protein or an immunological fragment thereof of a NF protein), as well polyclonal and monoclonal antibodies of all classes, human antibodies, and humanized antibodies produced by genetic recombination, are also included.

An intact protein or its partial peptide may be used as the antigen for immunization. As partial peptides of the proteins, for example, the amino (N)-terminal fragment of the protein and the carboxy (C)-terminal fragment can be given.

A gene encoding a protein of interest or a fragment thereof (*e.g.*, an immunological fragment) is inserted into a known expression vector, and, by transforming the host cells with the vector described herein, the desired protein or a fragment thereof is recovered from outside or inside the host cells using standard methods. This protein can be used as the

sensitizing antigen. Also, cells expressing the protein, cell lysates, or a chemically synthesized protein of the invention may be also used as a sensitizing antigen.

The mammal that is immunized by the sensitizing antigen is not restricted; however, it is preferable to select animals by considering the compatibility with the parent cells used in cell fusion. Generally, animals belonging to the orders rodentia, lagomorpha, or primates are used. Examples of animals belonging to the order of rodentia that may be used include, for example, mice, rats, and hamsters. Examples of animals belonging to the order of lagomorpha that may be used include, for example, rabbits. Examples of animals belonging to the order of primates that may be used include, for example, monkeys. Examples of monkeys to be used include the infraorder catarrhini (old world monkeys), for example, *Macaca fascicularis*, rhesus monkeys, sacred baboons, and chimpanzees.

Well-known methods may be used to immunize animals with the sensitizing antigen. For example, the sensitizing antigen is injected intraperitoneally or subcutaneously into mammals. Specifically, the sensitizing antigen is suitably diluted and suspended in physiological saline, phosphate-buffered saline (PBS), and so on, and mixed with a suitable amount of general adjuvant if desired, for example, with Freund's complete adjuvant. Then, the solution is emulsified and injected into the mammal. Thereafter, the sensitizing antigen suitably mixed with Freund's incomplete adjuvant is preferably given several times every 4 to 21 days. A suitable carrier can also be used when immunizing and animal with the sensitizing antigen. After the immunization, the elevation in the level of serum antibody is detected by usual methods.

Polyclonal antibodies against the proteins of the present disclosure can be prepared as follows. After verifying that the desired serum antibody level has been reached, blood is withdrawn from the mammal sensitized with antigen. Serum is isolated from this blood using conventional methods. The serum containing the polyclonal antibody may be used as the polyclonal antibody, or according to needs, the polyclonal antibody-containing fraction may be further isolated from the serum. For example, a fraction of antibodies that specifically recognize the protein of the invention may be prepared by using an affinity column to which the protein is coupled. Then, the fraction may be further purified by using a Protein A or Protein G column in order to prepare immunoglobulin G or M.

To obtain monoclonal antibodies, after verifying that the desired serum antibody level has been reached in the mammal sensitized with the above-described antigen, immunocytes

are taken from the mammal and used for cell fusion. For this purpose, splenocytes can be mentioned as preferable immunocytes. As parent cells fused with the above immunocytes, mammalian myeloma cells are preferably used. More preferably, myeloma cells that have acquired the feature, which can be used to distinguish fusion cells by agents, are used as the parent cell.

The cell fusion between the above immunocytes and myeloma cells can be conducted according to known methods, for example, the method by Milstein et al. (Galfre et al., *Methods Enzymol.* 73:3-46, 1981).

The hybridoma obtained from cell fusion is selected by culturing the cells in a standard selection medium, for example, HAT culture medium (medium containing hypoxanthine, aminopterin, and thymidine). The culture in this HAT medium is continued for a period sufficient enough for cells (non-fusion cells) other than the objective hybridoma to perish, usually from a few days to a few weeks. Then, the usual limiting dilution method is carried out, and the hybridoma producing the objective antibody is screened and cloned.

Other than the above method for obtaining hybridomas, by immunizing an animal other than humans with the antigen, a hybridoma producing the objective human antibodies having the activity to bind to proteins can be obtained by the method of sensitizing human lymphocytes, for example, human lymphocytes infected with the EB virus, with proteins, protein-expressing cells, or lysates thereof *in vitro* and fusing the sensitized lymphocytes with myeloma cells derived from human, for example, U266, having a permanent cell division ability.

The monoclonal antibodies obtained by transplanting the obtained hybridomas into the abdominal cavity of a mouse and extracting ascites can be purified by, for example, ammonium sulfate precipitation, protein A or protein G column, DEAE ion exchange chromatography, an affinity column to which the protein of the present disclosure is coupled, and so on.

Monoclonal antibodies can be also obtained as recombinant antibodies produced by using the genetic engineering technique (see, for example, Borrebaeck C.A.K. and Larrick, J.W., THERAPEUTIC MONOCLONAL ANTIBODIES, Published in the United Kingdom by MACMILLAN PUBLISHERS LTD (1990)). Recombinant antibodies are produced by cloning the encoding DNA from immunocytes, such as hybridoma or antibody-producing sensitized lymphocytes, incorporating into a suitable vector, and introducing this vector into a

host to produce the antibody. The present disclosure encompasses such recombinant antibodies as well.

Antibodies or antibody fragments specific for a protein encoded by one or more biomarkers can also be generated by *in vitro* methods such as phage display.

Moreover, the antibody of the present disclosure may be an antibody fragment or modified-antibody, so long as it binds to a protein encoded by a biomarker of the invention. For instance, Fab, F (ab') 2, Fv, or single chain Fv (scFv) in which the H chain Fv and the L chain Fv are suitably linked by a linker (Huston et al., *Proc. Natl. Acad. Sci. USA*, 85:5879-5883, (1988)) can be given as antibody fragments. Specifically, antibody fragments are generated by treating antibodies with enzymes, for example, papain or pepsin. Alternatively, they may be generated by constructing a gene encoding an antibody fragment, introducing this into an expression vector, and expressing this vector in suitable host cells (see, for example, Co et al., *J. Immunol.*, 152:2968-2976, 1994; Better et al., *Methods Enzymol.*, 178:476-496, 1989; Pluckthun et al., *Methods Enzymol.*, 178:497-515, 1989; Lamoyi, *Methods Enzymol.*, 121:652-663, 1986; Rousseaux et al., *Methods Enzymol.*, 121:663-669, 1986; Bird et al., *Trends Biotechnol.*, 9:132-137, 1991).

The antibodies may be conjugated to various molecules, such as fluorescent substances, radioactive substances, and luminescent substances. Methods to attach such moieties to an antibody are already established and conventional in the field (*see, e.g.,* US 5,057,313 and 5,156,840).

Examples of methods that assay the antigen-binding activity of the antibodies include, for example, measurement of absorbance, enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), and/or immunofluorescence. For example, when using ELISA, a protein encoded by a biomarker of the invention is added to a plate coated with the antibodies of the present disclosure, and then, the antibody sample, for example, culture supernatants of antibody-producing cells, or purified antibodies are added. Then, secondary antibody recognizing the primary antibody, which is labeled by alkaline phosphatase and such enzymes, is added, the plate is incubated and washed, and the absorbance is measured to evaluate the antigen-binding activity after adding an enzyme substrate such as p-nitrophenyl phosphate. As the protein, a protein fragment, for example, a fragment comprising a C-terminus, or a fragment comprising an N-terminus may be used. To evaluate the activity of the antibody of the invention, BIAcore (Pharmacia) may be used.

By using these methods, the antibody of the invention and a sample presumed to contain a protein of the invention are contacted, and the protein encoded by a biomarker of the invention is detected or assayed by detecting or assaying the immune complex formed between the above-mentioned antibody and the protein.

Mass spectrometry based quantitation assay methods, for example, but not limited to, multiple reaction monitoring (MRM)-based approaches in combination with stable-isotope labeled internal standards, are an alternative to immunoassays for quantitative measurement of proteins. These approaches do not require the use of antibodies and so the analysis can be performed in a cost- and time- efficient manner (see, for example, Addona et al., *Nat. Biotechnol.,* 27:633–641, 2009; Kuzyk et al., *Mol. Cell Proteomics*, 8:1860–1877, 2009; Paulovich et al., *Proteomics Clin. Appl.*, 2:1386-1402, 2008). In addition, MRM offers superior multiplexing capabilities, allowing for the simultaneous quantification of numerous proteins in parallel. The basic theory of these methods has been well-established and widely utilized for drug metabolism and pharmacokinetics analysis of small molecules.

In another embodiment, the expression level of a NF gene of interest is determined by measuring RNA levels. A variety of suitable methods can be employed to detect and/or measure the level of mRNA expression of a gene. For example, mRNA expression can be determined using Northern blot or dot blot analysis, reverse transcriptase-PCR (RT-PCR; *e.g.,* quantitative RT-PCR), *in situ* hybridization (*e.g.,* quantitative *in situ* hybridization) or nucleic acid array (*e.g.,* oligonucleotide arrays or gene chips) analysis. Details of such methods are described below and in, *e.g.,* Sambrook et al., Molecular Cloning: A Laboratory Manual Second Edition vol. 1, 2 and 3. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, USA, Nov. 1989; Gibson et al. (1999) *Genome Res.*, 6(10):995-1001; and Zhang et al. (2005) *Environ. Sci. Technol.*, 39(8):2777-2785; U.S. Publication No. 2004086915; European Patent No. 0543942; and U.S. Patent No. 7,101,663; the disclosures of each of which are incorporated herein by reference in their entirety.

In one example, the presence or amount of one or more discrete mRNA populations in a biological sample can be determined by isolating total mRNA from the biological sample (*see, e.g.,* Sambrook et al. (supra) and U.S. Patent No. 6,812,341) and subjecting the isolated mRNA to agarose gel electrophoresis to separate the mRNA by size. The size-separated mRNAs are then transferred (*e.g.,* by diffusion) to a solid support such as a nitrocellulose membrane. The presence or amount of one or more mRNA populations in the biological

sample can then be determined using one or more detectably-labeled-polynucleotide probes, complementary to the mRNA sequence of interest, which bind to and thus render detectable their corresponding mRNA populations. Detectable-labels include, *e.g.,* fluorescent (*e.g.,* umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, allophycocyanin (APC), or phycoerythrin), luminescent (*e.g.,* europium, terbium, Qdot™ nanoparticles supplied by the Quantum Dot Corporation, Palo Alto, CA), radiological (*e.g.,* ^{125}I, ^{131}I, ^{35}S, ^{32}P, ^{33}P, or ^{3}H), and enzymatic (horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase) labels.

In another example, the presence or amount of discrete populations of mRNA (*e.g.,* mRNA encoded by one or more NF genes) in a biological sample can be determined using nucleic acid (or oligonucleotide) arrays. For example, isolated mRNA from a biological sample can be amplified using RT-PCR with, e.g., random hexamer or oligo(dT)-primer mediated first strand synthesis. The amplicons can be fragmented into shorter segments. The RT-PCR step can be used to detectably-label the amplicons, or, optionally, the amplicons can be detectably-labeled subsequent to the RT-PCR step. For example, the detectable-label can be enzymatically (*e.g.,* by nick-translation or kinase such as T4 polynucleotide kinase) or chemically conjugated to the amplicons using any of a variety of suitable techniques (*see, e.g.,* Sambrook et al., supra). The detectably-labeled-amplicons are then contacted with a plurality of polynucleotide probe sets, each set containing one or more of a polynucleotide (*e.g.,* an oligonucleotide) probe specific for (and capable of binding to) a corresponding amplicon, and where the plurality contains many probe sets each corresponding to a different amplicon. Generally, the probe sets are bound to a solid support and the position of each probe set is predetermined on the solid support. The binding of a detectably-labeled amplicon to a corresponding probe of a probe set indicates the presence or amount of a target mRNA in the biological sample. Additional methods for detecting mRNA expression using nucleic acid arrays are described in, e.g., U.S. Patent Nos. 5,445,934; 6,027,880; 6,057,100; 6,156,501; 6,261,776; and 6,576,424; the disclosures of each of which are incorporated herein by reference in their entirety.

Methods of detecting and/or for quantifying a detectable label depend on the nature of the label. The products of reactions catalyzed by appropriate enzymes (where the detectable label is an enzyme; see above) can be, without limitation, fluorescent, luminescent, or radioactive or they may absorb visible or ultraviolet light. Examples of detectors suitable for detecting such detectable labels include, without limitation, x-ray film, radioactivity counters,

scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers.

Methods for detecting or measuring gene expression (*e.g.*, protein or mRNA expression) can optionally be performed in formats that allow for rapid preparation, processing, and analysis of multiple samples. This can be, for example, in multi-welled assay plates (*e.g.*, 96 wells or 386 wells) or arrays (e.g., nucleic acid chips or protein chips). Stock solutions for various reagents can be provided manually or robotically, and subsequent sample preparation (*e.g.*, RT-PCR, labeling, or cell fixation), pipetting, diluting, mixing, distribution, washing, incubating (*e.g.*, hybridization), sample readout, data collection (optical data) and/or analysis (computer aided image analysis) can be done robotically using commercially available analysis software, robotics, and detection instrumentation capable of detecting the signal generated from the assay. Examples of such detectors include, but are not limited to, spectrophotometers, luminometers, fluorimeters, and devices that measure radioisotope decay. Exemplary high-throughput cell-based assays (*e.g.*, detecting the presence or level of a target protein in a cell) can utilize ArrayScan® VTI HCS Reader or KineticScan® HCS Reader technology (Cellomics Inc., Pittsburg, PA).

In some embodiments, the expression level of one NF gene, two NF genes, or three NF genes can be assessed and/or measured.

To aid in detecting the presence or level of expression of one or more of the NF genes, any part of the nucleic acid sequence of the genes can be used, *e.g.*, as hybridization polynucleotide probes or primers (*e.g.*, for amplification or reverse transcription). The probes and primers can be oligonucleotides of sufficient length to provide specific hybridization to an RNA, DNA, cDNA, or fragments thereof isolated from a biological sample. Depending on the specific application, varying hybridization conditions can be employed to achieve varying degrees of selectivity of a probe or primer towards target sequence. The primers and probes can be detectably-labeled with reagents that facilitate detection (*e.g.*, fluorescent labels, chemical labels (*see, e.g.*, U.S. Patent Nos. 4,582,789 and 4,563,417), or modified bases).

Standard stringency conditions are described by Sambrook, et al. (*supra*) and Haymes, et al. Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C. (1985). In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded

structure under the particular hybridization conditions (*e.g.*, solvent and salt concentrations) employed.

Hybridization can be used to assess homology between two nucleic acid sequences. A nucleic acid sequence described herein, or a fragment thereof, can be used as a hybridization probe according to standard hybridization techniques. The hybridization of a probe of interest (*e.g.*, a probe containing a portion of a nucleotide sequence described herein or its complement) to DNA, RNA, cDNA, or fragments thereof from a test source is an indication of the presence of DNA or RNA corresponding to the probe in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as hybridization in 2X sodium chloride/sodium citrate (SSC) at 30ºC, followed by a wash in 1 X SSC, 0.1% SDS at 50ºC. Highly stringent conditions are defined as hybridization in 6X SSC at 45ºC, followed by a wash in 0.2 X SSC, 0.1% SDS at 65°C.

Primers can be used in in a variety of PCR-type methods. For example, polymerase chain reaction (PCR) techniques can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. The PCR primers are designed to flank the region that one is interested in amplifying. Primers can be located near the 5' end, the 3' end or anywhere within the nucleotide sequence that is to be amplified. The amplicon length is dictated by the experimental goals. For qPCR, the target length is closer to 100 base pairs and for standard PCR, it is near 500 base pairs. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. PCR primers can be chemically synthesized, either as a single nucleic acid molecule (*e.g.*, using automated DNA synthesis in the 3' to 5' direction using phosphoramidite technology) or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair.

In addition, the nucleic acid sequences or fragments thereof (*e.g.*, oligonucleotide probes) can be used in nucleic acid arrays for detection and/or quantitation of gene expression.

10. Methods of Treatment

The methods disclosed herein enable the assessment of whether or not a subject having or suspected of having SMA is likely to respond to a SMA therapy (*e.g.*, SPINRAZA®). A subject having or suspected of having SMA who is likely to respond to the SMA therapy can be administered the SMA therapy (*e.g.*, SPINRAZA®). Conversely, a subject having or suspected of having SMA who is not likely to respond to a SMA therapy can be administered a different SMA therapy that is suitable for treatment of SMA.

The methods of this disclosure also enable the stratification of subjects having or suspected of having SMA into groups of subjects that are more likely to benefit, and groups of subjects that are less likely to benefit, from treatment comprising a SMA therapy (*e.g.*, SPINRAZA®). The ability to select such subjects from a pool of SMA subjects who are being considered for treatment with a SMA therapy is beneficial for administering an effective treatment to the subject.

The subjects who are considered for treatment comprising a SMA therapy include, but are not limited to, subjects having, suspected of having, or likely to develop SMA. In one embodiment, the subject to be treated with an SMA therapy has, is suspected of having, or is likely to develop Type 0 SMA. In one embodiment, the subject to be treated with an SMA therapy has, is suspected of having, or is likely to develop Type I SMA. In one embodiment, the subject to be treated with an SMA therapy has, is suspected of having, or is likely to develop Type II SMA. In one embodiment, the subject to be treated with an SMA therapy has, is suspected of having, or is likely to develop Type III SMA. In one embodiment, the subject to be treated with an SMA therapy has, is suspected of having, or is likely to develop Type IV SMA.

If the subject having SMA is more likely to respond to a SMA therapy (based on concentrations of one or more of the biomarkers described above (*e.g.*, pNF-H protein)), the subject can then be administered an effective amount of the SMA therapy (*e.g.*, SPINRAZA®). An effective amount of the compound can suitably be determined by a

health care practitioner taking into account, for example, the characteristics of the patient (age, sex, weight, race, etc.), the progression of the disease, and prior exposure to the drug. If the subject is less likely to respond to one SMA therapy, the subject can then be optionally administered a different SMA therapy.

Subjects of all ages can be affected by SMA. Therefore, a biological sample used in a method described herein can be obtained from a human subject of any age, including a fetus, an infant, a child, an adolescent, or an adult, such as an adult having, or suspected of having, SMA.

The methods can also be applied to individuals at risk of developing SMA treatable by a SMA therapy (*e.g.*, SPINRAZA®). Such individuals include those who have (i) a family history of (a genetic predisposition for) such disorders or (ii) one or more risk factors for developing such disorders.

After stratifying or selecting a subject based on whether the subject will be more likely or less likely to respond to a SMA therapy (*e.g.*, SPINRAZA®), a medical practitioner (*e.g.*, a doctor) can administer the appropriate therapeutic modality to the subject. Methods of administering SMA therapies are known in the art.

It is understood that any therapy described herein (*e.g.*, a therapy comprising SPINRAZA® or a therapy that does not comprise SPINRAZA®) can include one or more additional therapeutic agents. That is, any therapy described herein can be co-administered (administered in combination) with one or more additional therapeutic agents such as, but not limited to, other SMA therapies described herein. Furthermore, any therapy described herein can include one or more agents for treating, or more side-effects of a therapy comprising the SMA therapy (*e.g.*, SPINRAZA®).

Combination therapies (*e.g.*, co-administration of a SMA therapy comprising (e.g., SPINRAZA®) and one or more additional SMA therapies or additional therapeutic agents) can be, *e.g.*, simultaneous or successive. For example, a SMA therapy and the additional therapeutic agent(s) can be administered at the same time or at different times. In some embodiments, the one or more additional therapeutic agents can be administered first in time and the SMA therapy (*e.g.*, SPINRAZA®) administered second in time.

In cases where the subject having SMA and predicted to respond to a SMA therapy (*e.g.*, SPINRAZA®) has been previously administered the SMA therapy, the therapy can

replace or augment a previously or currently administered therapy. For example, upon treating with SPINRAZA®, administration of a non-SPINRAZA® therapy can cease or diminish, *e.g.*, be administered at lower levels. Administration of the previous therapy can be maintained while the therapy comprising SPINRAZA® is administered. In some embodiments, a previous therapy can be maintained until the level of SPINRAZA® reaches a level sufficient to provide a therapeutic effect.

In some cases, the method of treatment involves treating a fetus in utero. The fetus to be treated is determined to be in need of treatment with a SMA therapy based on high NF levels (*e.g.*, pNF-H or NF-L) relative to a control. If, for example, the fetus has been identified as having SMA based on genetic testing and has been identified as having elevated NF levels, the fetus can be treated with a SMA therapy (*e.g.*, SPINRAZA®). In some instances, the method of treatment relates to Type 0 patients. If a fetus is identified with very high NF levels and one SMN2 copy, the chances are high that the fetus has Type 0 SMA. Treating such a fetus prenatally with an SMA therapy (*e.g.*, SPINRAZA®) can effectively treat that fetus.

11. Kits

This disclosure also provides kits. In certain embodiments, the kit can include an antibody or antibodies that can be used to detect one or more of the biomarkers disclosed herein or their concentration or expression levels. For example, the kit can include an antibody that specifically binds NF-H (*e.g.*, pNF-H). The antibodies in the kit may be monoclonal or polyclonal and can be further conjugated with a detectable label. In some embodiments, the kit includes probes that can be used to identify or detect any of the biomarkers disclosed herein. In some embodiments, the kit includes any of the nucleic acid arrays. In some embodiments, the kit includes probes and antibodies that can be used to identify or detect any of the biomarkers disclosed herein or their expression or expression levels. The kits can, optionally, contain instructions for detecting and/or measuring the concentration of one or more proteins or the levels of mRNA in a biological sample.

The kits can optionally include, *e.g.*, a control (*e.g.*, a concentration standard for the protein being assessed) or control labeled-amplicon set containing known amounts of one or more amplicons recognized by nucleic acid probes of the array. In some instances, the control can be an insert (*e.g.*, a paper insert or electronic medium such as a CD, DVD, or

floppy disk) containing an expression level or expression level ranges of one or more proteins (*e.g.*, pNF-H) or RNAs predictive of SMA, or of responsiveness to a SMA therapy (*e.g.*, SPINRAZA®).

In some embodiments, the kits can include one or more reagents for processing a biological sample (*e.g.*, calibration reagents, buffers, diluents, color reagents, reagents to stop a reaction). For example, a kit can include reagents for isolating a protein from a biological sample and/or reagents for detecting the presence and/or amount of a protein in a biological sample (*e.g.*, an antibody that binds to the protein that is the subject of the detection assay and/or an antibody that binds the antibody that binds to the protein).

In certain embodiments, the kit includes at least one microplate (*e.g.*, a 96 well plate; *i.e.*, 12 strips of 8 wells). The microplate can be provided with its corresponding plate cover. The microplate can be polystyrene or of any other suitable material. The microplate can have the antibody that is used to identify the presence of a particular biomarker coated inside each well. The antibody may be conjugated to a detectable label. The kit may also include at least one adhesive strip.

In some embodiments, the kits can include a software package for analyzing the results of, *e.g.*, expression profile or a microarray analysis.

The kits can also include one or more antibodies for detecting the protein expression of any of the genes described herein (*e.g.*, NF-H). For example, a kit can include (or in some cases consist of) one or a plurality of antibodies capable of specifically binding to one or more proteins encoded by any of the genes described herein and optionally, instructions for detecting and/or measuring the concentration of one or more proteins and/or a detection antibody comprising a detectably-labeled antibody that is capable of binding to at least one antibody of the plurality. In some embodiments, the kits can include antibodies that recognize NF-H, NF-L, and/or NF-M. In some embodiments, the kits can include antibodies that recognize pNF-H.

In certain embodiments, the kit can also optionally include one or more unit doses of a SMA therapy (*e.g.*, SPINRAZA®).

The kits described herein can also, optionally, include instructions for administering a SMA therapy, where the concentration of one or more proteins or expression level of one or

more RNAs predicts that a subject having or suspected of having SMA will respond to a SMA therapy (*e.g.*, SPINRAZA®).

In a specific embodiment, the kit comprises one or more of the following:

(i) a microplate (e.g., a 96 well plate). The microplate can be coated with an anti-NF-H antibody that is conjugated with a detectable label. The anti-NF-H antibody may monoclonal or polyclonal. The antibody can be *e.g.*, from mouse, rabbit, rat, or guinea pig. The detectable label can be *e.g.*, horse radish peroxidase, biotin, a fluorescent moiety, a radioactive moiety, a histidine tag, or a peptide tag. The microplate can be provided with a cover and optionally, one or more adhesive strips.

(ii) a vial containing anti-NF-H conjugated with a detectable label. The detectable label can be *e.g.*, horse radish peroxidase, biotin, a fluorescent moiety, a histidine tag, a peptide tag. The vial can also include a preservative.

(iii) a vial containing an NF-H standard of known concentration. The NF-H can be a recombinant human NF-H.

(iv) a vial containing an assay diluent.

(v) a vial containing a calibrator diluent.

(vi) a vial containing wash buffer. The buffer may be provided as a concentrate.

(vii) one or more vials containing color reagents.

(viii) a vial containing a stop solution to stop the colorimetric reaction.

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art can develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

EXAMPLES

The Examples below refer to several clinical studies designated: NURTURE, ENDEAR, EMBRACE, and CHERISH.

ENDEAR, was a Phase 3, multicenter, randomized, double-blind, sham-procedure-controlled study of ISIS 396443 in subjects with symptomatic infantile-onset SMA. Results of the final analysis for this study provide clear evidence that subjects treated with intrathecal (IT) ISIS 396443 achieved statistically significant and clinically meaningful improvement in the acquisition of motor milestones as well as sustained and clinically meaningful improvements in event-free survival, overall survival, motor function, and motor neuron health in comparison with a control group of subjects who received a sham procedure. Improvement relative to control was seen as early as 2 months after the initiation of treatment (end of the loading dose period), with clear separation from control at 6 months after the initiation of treatment.

At the time of the final analysis, 121 subjects had received at least 1 dose of ISIS 396443 administered as IT injection by lumbar puncture (LP) [n=80] or sham procedure (n=41). Among these subjects, 89 subjects (65 in the ISIS 396443 arm and 24 in the control arm) had completed the study. A total of 42 subjects (32 subjects in the ISIS 396443 arm and 10 subjects in the control arm) remained in the study at the time the study was closed and were recorded as having discontinued treatment due to early study closure. Two subjects in the ISIS 396443 group and 1 subject in the control group voluntarily withdrew from the study prior to the transition to open label treatment, and 29 subjects (13 in the ISIS 396443 group and 16 in the control group) died.

The demographics and SMA history of the 121 subjects in this Study were consistent with a Type I SMA population.

- There were 45% males and 55% females; 86% of subjects were white.

- Most subjects (86%) had symptom onset at less than 12 weeks of age. Median age was 8.0 weeks (range: 1 to 20 weeks) at SMA symptom onset, 12.0 weeks (range: 0 to 30 weeks) at SMA diagnosis, and 166 days (range: 20 to 211 days) at screening. Median disease duration at the time of enrollment was 13.1 weeks (range: 0.0 to 25.86 weeks).

- Most subjects (99%) had 2 SMN2 gene copies by local laboratory testing at study entry.

- Among the 80 subjects in the ISIS 396443 group, 73 (91%) had received at least 4 doses of ISIS 396443 (i.e., completed the loading dose phase); 32 subjects (40%)

received all 6 planned doses. Of the 41 subjects in the control group, 34 subjects (83%) had at least 4 sham procedures, with 14 subjects (34%) undergoing all 6 procedures.

CHERISH, was a Phase 3, double-blind, randomized, sham-procedure controlled study of ISIS 396443 in subjects with later-onset SMA. Approximately 117 subjects were enrolled into the study and allocated to treatment with ISIS 396443 or sham control in a 2:1 ratio. ISIS 396443 was administered IT using a loading regimen (dosing on Days 1, 29, and 85), followed by a maintenance dose 6 months thereafter (dosing on Day 274).

Results of the interim analysis provide clear evidence that subjects treated with IT ISIS 396443 achieved statistically significant gains in motor function as well as sustained and clinically meaningful improvements in motor milestones in comparison with a control group of subjects who received a sham procedure. Although improvement in motor function was seen at all timepoints, separation from the control group clearly occurred at 6 months after the initiation of treatment. Based on an assessment of risk-benefit of ISIS 396443, the Sponsor decided to terminate the study early, and subjects were given the opportunity to enroll into an open-label extension study.

As of the interim analysis, 126 subjects had received at least 1 dose of ISIS 396443 (n=84) or sham procedure (n=42). No subjects discontinued treatment or withdrew from the study. As of the data cutoff date, 49 of 84 subjects (58%) in the ISIS 396443 group and 23 of 42 subjects (55%) in the control group were continuing in the study. The primary analysis of change from baseline in HFMSE score at 15 months was based on the ITT Set composed of 126 subjects, 84 treated with ISIS 396443 and 42 who underwent the sham procedure. The main analysis of WHO motor milestones was performed using the Interim Efficacy Set, which was composed of 54 subjects (35 subjects treated with ISIS 396443 and 19 subjects who underwent the sham procedure) who had the opportunity to be assessed at the Day 456 Visit (*i.e.*, Month 15). All other secondary and tertiary endpoint analyses were performed on the ITT Set.

The demographics and baseline disease characteristics, including SMA and medical history, of the 126 subjects in this Study were consistent with a population highly likely to develop Type II or III SMA.

- There were 47% males and 53% females; 75% were white.

- The median age was 11 months (range: 6 to 20) at SMA symptom onset, 18 months (range: 0 to 48 months) at SMA diagnosis, and 3.0 years (range: 2 to 9 years) at screening. The median disease duration at the time of enrollment was 35.7 months (range: 8 to 94 months).

- Most subjects (88%) had 3 SMN2 gene copies by laboratory testing at study entry and 8% of subjects had 2 copies.

- Among the 84 subjects in the ISIS 396443 group, all had received at least 3 doses of ISIS 396443 (i.e., completed the loading doses), 76 subjects (90%) received all 4 doses. Of the 42 subjects in the control group, all subjects had received at least 3 sham procedures, with 40 subjects (95%) undergoing all 4 procedures.

- Median time on study was similar between the 2 treatment groups (i.e., 412.5 and 419.5 days for the ISIS 396443 and control groups, respectively). The total number of subject-years on study was 134.06 (88.84 subject-years in the ISIS 396443 group and 45.22 subject-years in the control group).

Subjects treated with ISIS 396443 realized clinically meaningful benefits as compared to subjects who received a sham procedure. These benefits included statistically significantly greater gains in motor function as measured by HFMSE, as well as an improvement in upper limb functional ability.

NURTURE, is an ongoing Phase 2, open-label, multicenter, single-arm study to assess the efficacy, safety, tolerability, and PK of ISIS 396443 in presymptomatic SMA. The study is being conducted in subjects who were ≤6 weeks of age at the time of enrollment with genetic documentation of 5q SMA, 2 or 3 copies of the SMN2 gene, CMAP ≥1 mV, and the absence of signs or symptoms of SMA. Up to 25 subjects are planned. Efficacy data available to date indicate that the development and achievement of motor milestones for most subjects has been more consistent with normal development than with the natural history of Type I SMA.

At the time of the data cutoff for NURTURE, 20 subjects had been enrolled and received at least 1 dose of ISIS 396443. All subjects are continuing in the study. Eighteen subjects who have received all 4 loading doses or have had the opportunity to complete the Day 64 visit comprise the efficacy set.

- Most subjects are male (55%) and white (50%).

- Age at the first dose ranged from 3 to 42 days, with a median of 19 days.
- Of the 18 subjects, 13 subjects (72%) have 2 copies of the SMN2 gene and 5 subjects (28%) have 3 copies.

Efficacy data were available for 18 subjects at Day 64, 16 subjects at Day 183, 11 subjects at Day 302, 9 subjects at Day 365, and 5 subjects at Day 421. Results at the later of these visits demonstrate development that is inconsistent with Type I SMA and the experience of subjects' affected siblings and consistent with age-matched expectations for healthy infants.

EMBRACE, is a Phase 2, randomized, sham-procedure controlled, multicenter study of ISIS 396443 in subjects with SMA who are not eligible to participate in ENDEAR or CHERISH. This study evaluated a unique set of subjects who exhibited symptoms of infantile-onset SMA, at an age too young to be eligible for CHERISH, or subjects who exhibited later-onset symptoms of SMA, at an age too old to be eligible for ENDEAR, and were screened at an age too old or had too many copies of SMN2 to be eligible for ENDEAR. Thus, the population in this study provided the opportunity to explore the safety, tolerability, and efficacy of ISIS 396443 in the context of both infantile-onset and later-onset SMA in subjects with up to 3 copies of SMN2.

This study was initially designed as a double-blind study but evolved into a 2-part study, including a double blind phase (Part 1) and an open-label extension phase (Part 2), after the positive and robust efficacy results from a pivotal study in the ISIS 396443 clinical development program were observed. Part 1 was a randomized, double blind, sham-procedure controlled study. Twenty-one subjects were enrolled into the study from 7 sites in the United States and Germany. Randomization of subjects was stratified based on age of SMA (infantile onset [≤6 months] vs. later onset [>6 months]). Subjects were scheduled to receive a total of 6 intrathecal (IT) injections or 6 sham procedures over the dosing period of approximately 10 months. However, Part 1 of the study was terminated early because of the positive and robust efficacy results observed in an interim analysis of one of the pivotal studies for the ISIS 396443 clinical development program. As a result of the decision by the study Sponsor to terminate Part 1 of the study early based on the assessment of the risk-benefit of ISIS 396443, all subjects were invited to complete the End of Part 1 Evaluation assessments early and participate in Part 2 of the study. The total duration of subject participation in Part 1 was planned to be approximately 15 months, but because Part 1 of the

study was terminated early, eligible subjects were given the opportunity to enroll into Part 2 of the study immediately following their End of Part 1 Evaluation assessments.

A total of 21 subjects were enrolled in this study. Subjects were randomized to ISIS 396443 or control (sham procedure) in a 2:1 ratio. Randomization was stratified based on age at onset of clinical signs and symptoms consistent with SMA (≤6 months [infantile onset] vs. >6 months [later onset]). Fourteen subjects received ISIS 396443 in Part 1 of the study and 7 subjects received the sham procedure.

A total of 21 subjects were screened and enrolled in Part 1 of this study. All subjects were randomized in a 2:1 ratio: 14 subjects received ISIS 396443, and 7 subjects received control treatment (sham procedure). In addition, randomization was stratified based on age at onset of clinical signs and symptoms consistent with infantile onset (≤6 months) and later onset (>6 months) SMA. The first subject was treated on 19 August 2015, and the end of study (Part 1) was on 20 December 2016. All subjects received study treatment according to their randomization assignment.

The 21 subjects randomized and dosed who comprised the ITT set were enrolled at 7 study sites in 2 countries. Sixteen subjects (76%) were enrolled at 6 sites in the United States, and 5 subjects (24%) were enrolled at 1 site in Germany. Subjects were randomized across sites. Of the 21 randomized subjects, 13 had SMA onset at ≤6 months and the remaining 8 subjects had SMA onset at >6 months.

Twenty-one subjects received treatment, and of these, 14 subjects completed due to early termination of the study (6 subjects [86%] in the control group and 8 subjects [57%] in the ISIS 396443 group). Nine subjects (43%) completed treatment up to Day 302 (2 subjects [29%] in the control group and 7 subjects [50%] in the ISIS 396443 group). Six subjects (29%), all in the ISIS 396443 group, completed the Part 1 Final Follow-up Evaluation (Day 422). One subject (5%) assigned to the control group died due to brain death on Study Day 289.

Demographics and Baseline Disease Characteristics:

Of the 21 subjects in the study, 11 (52%) were male and 10 (48%) were female. Age at first dose ranged from 7 to 53 months (median: 17 months). Eleven subjects (52%) were between 7 and 18 months of age, and 10 subjects (48%) were greater than 18 months of age.

Nine subjects (43%) were White, 5 subjects (24%) were Asian, and 2 subjects (10%) were Other.

Efficacy and Pharmacokinetics

- Subjects in the ISIS 396443 group required less ventilator use than did subjects in the control group.

- The proportion of HINE motor milestone responders was greater in the ISIS 396443 group than in the control group, and there was at least 1 responder in each HINE motor milestone category from the ISIS 396443 group, with the greatest number of subjects showing improvement in the categories of head control, rolling, and sitting.

Safety

In this study, ISIS 396443 was well tolerated when administered as multiple IT injections (4 loading doses followed by maintenance doses every 4 months). No new safety concerns were identified in the overall safety profile of ISIS 396443.

Results from Part 1 of this Phase 2, randomized, sham-procedure controlled study of ISIS 396443 have shown clear evidence that intrathecal administration of ISIS 396443 is effective in a population of subjects with either infantile onset or later-onset SMA. When compared with a control group of study subjects who received only sham administration, subjects treated with ISIS 396443 achieved and sustained gains in motor milestones and, based on Investigator and caregiver assessments, generally appeared to grow and thrive. A positive correlation was observed between CSF ISIS 396443 concentration and HINE motor milestone total score, which increased over time. The dosing regimen if ISIS 396443 was safe and well tolerated in this study, as no new safety concerns were identified, and the safety results were consistent with results from other studies in the ISIS 396443 clinical development program. Based on the results observed across all primary safety and exploratory efficacy parameters, and in contrast with the results observed in the untreated control group, ISIS 396443 improves motor function in subjects with infantile-onset and later-onset SMA.

Methods

The plasma at selected time points was assayed to detect the pNF-H concentration. All plasma samples were frozen at -70°C until ready for use. Plasma samples at selected schedule time were diluted at the minimum required dilution (MRD) using assay dilution buffer.

NF-H was assayed using polyclonal anti-NF-H antibodies from Encor Biotechnology (Cat # RPCA-NF-H; and/or Cat # CPCA-NF-H). These antibodies specifically detect the hyper-phosphorylated form of NF-H.

A qualification experiment was conducted before any patient samples were assayed. Such experiment aimed to evaluate the reproducibility and variability of the pNF-H concentration generated by Ella technology platform developed by ProteinSimple®. Ella technology utilizes microfluidic cartridges that include pre-loaded reagents so only the samples and wash buffer are loaded onto the cartridge. Once the cartridge is loaded, the platform will automatically calculate the pNF-H concentration by using its build-in factory-calibrated standard curve.

To evaluate the quality of each cartridge as well as any unforeseen potential confound effects in real-time, 4 quality control (QC) samples are included in each cartridge: three buffer spiked QC, high, middle and low, and an endogenous quality control (EQC) sample from a commercially available Multiple Sclerosis (MS) patient (Bioreclamation) is run. These four QCs are used to monitor the performance of the bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

Samples from same subjects were analyzed on the same cartridge. Two aliquots for each sample were placed on the same cartridge in a randomized order to ensure that the samples analysis take into account possible placement effect.

Example 1: Correlation of CSF vs. Plasma pNF-H Levels

Plasma and CSF samples from the clinical studies NURTURE and EMBRACE were analyzed at the same timepoint and in the same subject to determine the correlation in pNF-H levels. A high correlation (R=0.88) between CSF and plasma levels of pNF-H were identified (**Fig. 1**). This result suggests that plasma levels of pNF-H and other subunits of neurofilament are highly predictive of CSF levels of these same proteins.

Example 2: Plasma Levels of pNF-H as Predictor of Disease and Severity

Plasma samples from clinical studies NURTURE, ENDEAR, EMBRACE, and CHERISH were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 (NURTURE, ENDEAR, EMBRACE, and CHERISH) or SHAM procedure (ENDEAR, EMBRACE, and CHERISH)). Plasma samples from healthy volunteers (age 4-18 years) were also analyzed for levels of pNF-H.

Results from health volunteers demonstrated pNF-H <300 pg/mL for all ages. Results from NURTURE, ENDEAR, EMBRACE, CHERISH demonstrate that plasma levels of pNF-H in clinical trial subjects with SMA are almost all >300 pg/mL. Some subjects have levels up to approximately 50,000 pg/mL. In general, subjects with two SMN2 copies have higher levels than subjects with three SMN2 copies. Furthermore, subjects with symptom onset ≤6 months of age (ENDEAR) have higher levels than subjects with symptom onset >6 months of age (CHERISH). See **Fig. 2**.

However, important exceptions are observed. Within NURTURE, one subject has two SMN2 copies and a pNF-H level more consistent with subjects with 3 SMN2 copies. This subject has a sibling also with two SMN2 copies who developed Type II SMA. So, although this subject has an SMN2 copy number that suggests the subject should develop Type I SMA, the subject has a family history that suggests the subject will develop Type II SMA. The pNF-H level may provide additional diagnostic acumen beyond SMN1 deletion and SMN2 copy number. Within ENDEAR, one subject has three SMN2 copies and a pNF-H levels more consistent with subjects with 2 SMN2 copies. This subject had symptom onset ≤6 months of age and therefore was more consistent with Type I SMA (most often 2 SMN2 copies) than Type II SMA (most often 3 SMN2 copies).

Overall, the results demonstrate that among subjects with SMA, plasma levels of pNF-H are markedly elevated compared to healthy volunteers. And, among subjects with SMA, subjects with a more severe phenotype have higher plasma pNF-H levels than subjects with a less severe phenotype.

Example 3: Correlation of pNF-H Versus Age (SMN2 Copy Number =2)

Plasma samples from clinical studies NURTURE, ENDEAR, EMBRACE, and CHERISH were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 (NURTURE, ENDEAR, EMBRACE, and CHERISH) or SHAM procedure (ENDEAR, EMBRACE, and CHERISH)). For this analysis, subjects were limited to those with two SMN2 copies. Baseline log transformed pNF-H levels were plotted versus age at first dose (or SHAM procedure). In general, younger subjects had higher pNF-H levels than older subjects. See **Fig. 3**.

This result suggests that pNF-H levels decline with increasing age among subjects with 2 SMN2 copies.

Example 4: Correlation of pNF-H Versus Age (SMN2 Copy Number = 3)

Plasma samples from clinical studies NURTURE, ENDEAR, EMBRACE, and CHERISH were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 (NURTURE, ENDEAR, EMBRACE, and CHERISH) or SHAM procedure (ENDEAR, EMBRACE, and CHERISH)). For this analysis, subjects were limited to those with three SMN2 copies. Baseline log transformed pNF-H levels were plotted versus age at first dose (or SHAM procedure). In general, younger subjects had higher pNF-H levels than older subjects. See **Fig. 4**.

This result suggests that pNF-H levels decline with increasing age among subjects with three SMN2 copies.

Example 5: Correlation of pNF-H Versus Age

Plasma samples from clinical studies NURTURE, ENDEAR, EMBRACE, and CHERISH were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 (NURTURE, ENDEAR, EMBRACE, and CHERISH) or SHAM procedure (ENDEAR, EMBRACE, and CHERISH)). For this analysis, subjects were stratified by age of symptom onset (presymptomatic (NURTURE), <6 months, ≥6 months) and by SMN2 copy number (2, 3, 4 or NA). In general, younger subjects had higher pNF-H levels than older subjects within each strata of age of symptom onset and SMN2 copy number. See **Fig. 5**.

This result suggests that pNF-H levels decline with increasing age among subjects independent of age of symptom onset or SMN2 copy number.

Example 6: Correlation of pNF-H Versus Age

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 or SHAM procedure). For this analysis, quartiles of pNF-H were identified within the entire study population. The quartiles were defined as 2390 to 10,900 pg/mL; 10,900 to 15,400 pg/mL; 15,400 to 21,600 pg/mL; and 21,600 to 50,100 pg/mL. In general, subjects with higher pNF-H levels at baseline appeared to be younger at first dose, younger at symptom onset, and younger at SMA diagnosis. Furthermore, subjects with higher pNF-H levels at baseline appeared to have lower mean CHOP INTEND (Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders) scores and peroneal CMAP amplitudes at baseline. See **Figs. 6-8**.

These results suggest that higher pNF-H levels at first dose are associated with a more severe phenotype as demonstrated as a younger age at symptom onset; lower motor function (CHOP INTEND); and worse motor neuron health (CMAP amplitude).

Example 7: pNF-H Levels and Motor Function

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 or SHAM procedure). For this analysis, levels of log transformed pNF-H were plotted versus baseline CHOP INTEND. In general, subjects with higher pNF-H levels had lower CHOP INTEND and subjects with lower pNF-H levels had higher CHOP INTEND with a correlation of -0.3. See **Fig. 9.**

This result suggests that pNF-H levels are inversely associated with motor function. This relationship appears to be linear.

Example 8: Prediction of Motor Function Based on pNF-H Levels

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at baseline (prior to treatment with ISIS 396443 or SHAM procedure). For this analysis, a linear regression model was built to examine the relationship between various potential predictors of baseline CHOP INTEND. A final model was identified that only included those variables that remained statistically significant (Baseline NF-H; treatment group, disease duration (weeks); sex; age of first dose (days); age of SMA symptom onset (weeks); age of

SMA diagnosis (weeks); gestational age (weeks); baseline weight (kg); (gestational age +age of fist dose) x (gestational age + age of SMA symptom onset); (gestational age + age of SMA diagnosis). In this final model, baseline log transformed pNF-H levels were statistically significant. See **Fig. 10**.

This result suggests that pNF-H levels are a significant predictor of baseline motor function.

Example 9: pNF-H (pg/mL) Levels in ENDEAR

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (SHAM procedure). For this analysis, absolute values of pNF-H were log transformed and plotted by study day. Over time, mean pNF-H levels decline nearly linearly from approximately 18,000 pg/mL at Study Day 1 to approximately 5,000 pg/mL at Study Day 302. See **Fig. 11**.

Example 10: Percent Change in pNF-H (pg/mL) Levels in ENDEAR

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (SHAM procedure). For this analysis, percent change from baseline for absolute values of pNF-H were plotted by study day. Over time, mean percent change in pNF-H levels decline nearly linearly from approximately 0% at Study Day 1 to approximately -60% at Study Day 302. See **Fig. 12**.

Example 11: Drug Effect on pNF-H (pg/mL) Levels in ENDEAR

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (ISIS 396443). For this analysis, absolute values of pNF-H were log transformed and plotted by study day. Over time, mean pNF-H levels decline from approximately 18,000 pg/mL at Study Day 1 to approximately 5,000 pg/mL at Study Day 64 and then to approximately 1,000 pg/mL at Study Day 302. See **Fig. 13**.

This result suggests subjects treated with ISIS 396443 have a different pattern in the decline of pNF-H levels compared to subjects who received SHAM control.

Example 12: Drug Effect on Percent Change in pNF-H (pg/mL) Levels in ENDEAR

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (ISIS 396443). For this analysis, percent change from baseline for absolute values of pNF-H were plotted by study day. Over time, mean percent

change in pNF-H levels decline nearly linearly from approximately 0% at Study Day 1 to approximately -70% at Study Day 64 and -90% at Study Day 302. See **Fig. 14**.

This result suggests subjects treated with ISIS 396443 have a different pattern in the decline of pNF-H levels compared to subjects who received SHAM control.

Example 13: Change in pNF-H Levels at Various Time Points

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (ISIS 396443 and SHAM control). For this analysis, the percent of subjects at each study day who achieved specific levels of percent change from baseline for absolute values of pNF-H among subjects who received ISIS 396443 was compared to subjects who received SHAM control. Statistical significance ($p<0.05$) was first identified between the two study groups at study day 64. The greatest difference between percentages of each arm achieving a specific change in pNF-H occurred at study day 183 for -80% change. See **Fig. 15**.

This result suggests that a difference in the groups can be identified as early as study day 64 and the best separation between groups occurs using -80% as the threshold of change.

Example 14: Association of HINE-2 Score on Day 183 with pNF-H Levels of Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the total motor milestone score (HINE-2 (Hammersmith Infant Neurological Examination Section 2)) was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the total HINE-2 score on Study Day 183. pNF-H levels on Study Day 64 were significantly associated with total HINE-2 scores on Study Day 183, $p=0.0006$. See **Fig. 16**.

This result suggests that present pNF-H levels can predict future levels of motor milestones among infants with infantile-onset SMA.

Example 15: Association of HINE-2 Score on Day 183 with pNF-H Levels on Day 64 (With Covariates)

Plasma samples from Study CS3B (ENDEAR) were analyzed for levels of pNF-H at Study Day 64 and the total motor milestone score (HINE-2 (Hammersmith Infant Neurological Examination Section 2)) was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine

whether pNF-H levels on Study Day 64 were associated with total HINE-2 score on Study Day 183 after accounting for multiple potential confounders. After controlling for multiple potential confounders, the final model included Day 64 log transformed pNF-H ($p<0.0001$) and disease duration ($p=0.0029$). See **Fig. 17**.

This result suggests that present pNF-H levels can predict future levels of motor milestones after controlling for treatment and other potential confounders among infants with infantile-onset SMA.

Example 16: Association of CHOP INTEND of Day 183 with pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the CHOP INTEND was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the CHOP INTEND score on Study Day 183. pNF-H levels on Study Day 64 were significantly associated with the CHOP INTEND scores on Study Day 183, $p<0.0001$. See **Fig. 18**.

This result suggests that present pNF-H levels can predict future levels of general motor function among infants with infantile-onset SMA.

Example 17: Association of CHOP INTEND Score on Day 183 with pNF-H Levels on Day 64 (With Covariates)

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the CHOP INTEND score was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with CHOP INTEND score on Study Day 183 after accounting for multiple potential confounders. After controlling for multiple potential confounders, the final model included Day 64 log transformed pNF-H ($p=0.0001$), treatment group ($p=0.005$), and disease duration ($p=0.0003$). See **Fig. 19**.

This result suggests that present pNF-H levels can predict future levels of general motor function after controlling for treatment and other potential confounders among infants with infantile-onset SMA.

Example 18: HINE-2 (Responder/Non-Responder) Category Versus pNF-H Levels on Day 183

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 183 and the HINE-2 (Hammersmith Infant Neurological Examination Section 2) responder status was assessed at Study Day 183; between Study Day 183 and Study Day 302; after Study Day 302 (ISIS 396443 and SHAM control). If a subject became a responder based upon the protocol definition of a HINE-2 responder, the time of this achievement was noted. If a subject did not become a responder within Study CS3B, that was noted. No subjects in the SHAM control group became a HINE-2 responder during Study CS3B. Most subjects who received ISIS 396443 who became a responder did so by Study Day 183. Among SHAM control subjects, almost all subjects had pNF-H levels above the overall median (2130 pg/mL). Among subjects who received ISIS 396443 and became responders by Study Day 183, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 183 and 302, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 302 and end of study, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and did not become responders within ENDEAR, approximately half had pNF-H levels below the study median (2130 pg/mL) at Study Day 183. See **Fig. 20**.

These results suggest that achieving a certain threshold of pNF-H by 183 days after treatment initiation is able to differentiate treatment from SHAM control and predict eventual responders on treatment.

Example 19: HINE-2 (Responder/Non-Responder) Category Versus pNF-H Levels on Day 183 Percent Change in pNF-H Levels

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 183 and the HINE-2 responder status was assessed at Study Day 183; between Study Day 183 and Study Day 302; after Study Day 302 (ISIS 396443 and SHAM control). The percent change in pNF-H was calculated between baseline and Study Day 183. If a subject became a responder based upon the protocol definition of a HINE-2 responder, the time of this achievement was noted. If a subject did not become a responder within Study CS3B, that was noted. No subjects in the SHAM control group became a HINE-2 responder during Study CS3B. Most subjects who received ISIS 396443 who became a responder did

so by Study Day 183. Among SHAM control subjects, all subjects had a decline in pNF-H levels < 80%. Among subjects who received ISIS 396443 and became responders by Study Day 183, almost half had a decline in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 183 and 302, most had a change in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 302 and end of study, most had a change in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and did not become responders within ENDEAR, approximately half had a change in pNF-H levels >80% at Study Day 183. See **Fig. 21**.

These results suggest that achieving a certain threshold of decline in pNF-H by 183 days after treatment initiation can differentiate treatment from SHAM control and predict eventual responders on treatment.

Example 20: CHOP INTEND (Responder/Non-Responder) Category Versus pNF-H Levels on Day 183

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 183 and the CHOP INTEND responder status was assessed at Study Day 183; between Study Day 183 and Study Day 302; after Study Day 302 (ISIS 396443 and SHAM control). If a subject became a responder based upon the protocol definition of a CHOP INTEND responder, the time of this achievement was noted. If a subject did not become a responder within Study CS3B, that was noted. One subject in the SHAM control group became a CHOP INTEND responder during Study CS3B. Most subjects who received ISIS 396443 who became a responder did so by Study Day 183. Among SHAM control subjects, almost all subjects had pNF-H levels above the overall median. Among subjects who received ISIS 396443 and became responders by Study Day 183, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 183 and 302, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 302 and end of study, most had pNF-H levels below the study median at Study Day 183. Among subjects who received ISIS 396443 and did not become responders within ENDEAR, approximately half had pNF-H levels below the study median at Study Day 183. See **Fig. 22**.

These results suggest that achieving a certain threshold of pNF-H by 183 days after treatment initiation can differentiate treatment from SHAM control and predict eventual responders on treatment.

Example 21: CHOP INTEND (Responder/Non-Responder) Category Versus pNF-H Levels on Day 183 Percent Change in pNF-H Levels

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 183 and the CHOP INTEND responder status was assessed at Study Day 183; between Study Day 183 and Study Day 302; after Study Day 302 (ISIS 396443 and SHAM control). The percent change in pNF-H was calculated between baseline and Study Day 183. If a subject became a responder based upon the protocol definition of a HINE-2 responder, the time of this achievement was noted. If a subject did not become a responder within Study CS3B, that was noted. One subject in the SHAM control group became a CHOP INTEND responder during Study CS3B. Most subjects who received ISIS 396443 who became a responder did so by Study Day 183. Among SHAM control subjects, all but one subject had a decline in pNF-H levels < 80%. Among subjects who received ISIS 396443 and became responders by Study Day 183, almost half had a decline in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 183 and 302, most had a change in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and became responders between Study Days 302 and end of study, most had a change in pNF-H levels > 80% at Study Day 183. Among subjects who received ISIS 396443 and did not become responders within ENDEAR, approximately half had a change in pNF-H levels >80% at Study Day 183. See **Fig. 23**.

These results suggest that achieving a certain threshold of decline in pNF-H by 183 days after treatment initiation can differentiate treatment from SHAM control and predict eventual responders on treatment.

Example 22: Maintenance of Drug Effect: pNF-H (pg/mL) Levels in NURTURE

Plasma samples from clinical study NURTURE were analyzed for levels of pNF-H at Study Days 1, 64, 183, 302, and 421 (ISIS 396443). In this analysis, absolute levels of pNF-H were plotted by Study Day. See **Fig. 24**.

The results of this analysis suggest that significant decline occurs by Study Day 64 and this new level appears stable to Study Day 421.

Example 23: Maintenance of Drug Effect: pNF-H (pg/mL) Levels in EMBRACE

Plasma samples from clinical study EMBRACE were analyzed for levels of pNF-H at Study Days 1, 64, and 183 (ISIS 396443). In this analysis, absolute levels of pNF-H were plotted by Study Day. See **Fig. 25**.

The results of this analysis suggest that significant decline occurs by Study Day 64 and this new level appears stable to Study Day 183.

Example 24: Maintenance of Drug Effect: Percentage Change in pNF-H (pg/mL) Levels in NURTURE

Plasma samples from Study 232SM201 (NURTURE) were analyzed for levels of pNF-H at Study Days 1, 64, 183, 302, and 421 (ISIS 396443). In this analysis, levels of percent change in pNF-H from baseline were plotted by Study Day. See **Fig. 26**.

The results of this analysis suggest that significant decline (-80%) occurs by Study Day 64 and this new level appears stable to Study Day 421.

Example 25: Maintenance of Drug Effect: pNF-H (pg/mL) Levels in EMBRACE

Plasma samples from clinical study EMBRACE were analyzed for levels of pNF-H at Study Days 1, 64, and 183 (ISIS 396443). In this analysis, absolute levels of pNF-H were plotted by Study Day. See **Fig. 27**.

The results of this analysis suggest that significant decline (-50%) occurs by Study Day 64 and this new level appears stable to Study Day 183.

Example 26: Association of HINE-2 Score on Day 183 With pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the total motor milestone score (HINE-2) was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the total HINE-2 score on Study Day 183. Separate analyses were conducted among subjects who received ISIS 396443 and SHAM Control. Among subjects who received ISIS 396443, log transformed pNF-H levels on Study Day 64 were not statistically significantly associated

with total HINE-2 scores on Study Day 183, p=0.3186, but trended such that higher pNF-H levels were associated with a lower total HINE-2 score. Among subjects who received SHAM control, log transformed pNF-H levels on Study Day 64 were statistically significantly associated with total HINE-2 scores on Study Day 183, p=0.0302, such that higher pNF-H levels were associated with a lower total HINE-2 score. See **Fig. 28**.

This result suggests that present pNF-H levels can predict future levels of motor milestones among infants with infantile-onset SMA.

Example 27: Association of CHOP INTEND Score on Day 183 With pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the CHOP INTEND score was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the CHOP INTEND score on Study Day 183. Separate analyses were conducted among subjects who received ISIS 396443 and SHAM Control. Among subjects who received ISIS 396443, log transformed pNF-H levels on Study Day 64 were statistically significantly associated with CHOP INTEND scores on Study Day 183, p=0.0406, such that higher pNF-H levels were associated with a lower total CHOP INTEND score. Among subjects who received SHAM control, log transformed pNF-H levels on Study Day 64 were statistically significantly associated with CHOP INTEND scores on Study Day 183, p=0.0330, such that higher pNF-H levels were associated with a lower CHOP INTEND score. See **Fig. 29**.

This result suggests that present pNF-H levels can predict future levels of motor function among infants with infantile-onset SMA.

Example 28: pNF-H Levels over Time in Surviving Infants with non-Missing Values at Day 302

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (ISIS 396443 and SHAM procedure). For this analysis, the population was limited to those subjects who survived to Study Day 302 and had a pNF-H level measured at Study Day 302. Absolute values of pNF-H were plotted by study day. Over time among subjects who received the SHAM procedure, mean pNF-H levels decline nearly linearly from approximately 16,000 pg/mL at Study Day 1 to approximately

7,000 pg/mL at Study Day 302. Over time among subjects who received ISIS 396443, mean pNF-H decline from approximately 18,000 pg/mL at Study Day 1 to approximately 4,000 at Study Day 64 and then remain stable at approximately 1,000 pg/ML at Study Day 302. See **Fig. 30**.

The results of this analysis suggest that the decline in both cohorts is true and not a survival bias.

Example 29: Percentage Change in pNF-H Levels Over Time in Surviving Infants with non-Missing Values at Day 302

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Days 1, 2, 29, 64, 183, and 302 (ISIS 396443 and SHAM procedure). For this analysis, the population was limited to those subjects who survived to Study Day 302 and had a pNF-H level measured at Study Day 302. Change from baseline were plotted by study day. Over time among subjects who received the SHAM procedure, change in pNF-H levels decline nearly linearly to approximately -50% at Study Day 302. Over time among subjects who received ISIS 396443, change in pNF-H decline to approximately -70% at Study Day 64 and then remain stable at approximately -90% at Study Day 302. See **Fig. 31**.

The results of this analysis suggest that the decline in both cohorts is true and not a survival bias.

Example 30: Association of Peroneal CMAP Amplitude on Day 183 with pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the Peroneal CMAP Amplitude was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the Peroneal CMAP Amplitude on Study Day 183. pNF-H levels on Study Day 64 were significantly associated with Peroneal CMAP Amplitude on Study Day 183, p=0.0021. See **Fig. 32**.

This result suggests that present pNF-H levels may predict future levels of general motor nerve health among infants with infantile-onset SMA.

Example 31: Association of Peroneal CMAP Amplitude on Day 183 With pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the Peroneal CMAP Amplitude was assessed at Study Day 183 (ISIS

396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the Peroneal CMAP Amplitude on Study Day 183. Separate analyses were conducted among subjects who received ISIS 396443 and SHAM Control. Among subjects who received ISIS 396443, log transformed pNF-H levels on Study Day 64 were not statistically significantly associated with Peroneal CMAP Amplitude on Study Day 183, p=0.3612, such that higher pNF-H levels were associated with a trend toward lower Peroneal CMAP Amplitude. Among subjects who received SHAM control, log transformed pNF-H levels on Study Day 64 were statistically significantly associated with Peroneal CMAP Amplitude on Study Day 183, p=0.0142, such that higher pNF-H levels were associated with a lower Peroneal CMAP Amplitude. See **Fig. 33**.

This result suggests that present pNF-H levels may predict future levels of general motor neuron health among infants with infantile-onset SMA.

Example 32: Association of Ulnar CMAP Amplitude on Day 183 with pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the Peroneal CMAP Amplitude was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the Ulnar CMAP Amplitude on Study Day 183. pNF-H levels on Study Day 64 were significantly associated with Ulnar CMAP Amplitude on Study Day 183, p=0.0002. See **Fig. 34**.

This result suggests that present pNF-H levels may predict future levels of general motor nerve health among infants with infantile-onset SMA.

Example 33: Association of Ulnar CMAP Amplitude on Day 183 With pNF-H Levels on Day 64

Plasma samples from clinical study ENDEAR were analyzed for levels of pNF-H at Study Day 64 and the Ulnar CMAP Amplitude was assessed at Study Day 183 (ISIS 396443 and SHAM control). For this analysis, a linear regression model was constructed to determine whether pNF-H levels on Study Day 64 were associated with the Ulnar CMAP Amplitude on Study Day 183. Separate analyses were conducted among subjects who received ISIS 396443 and SHAM Control. Among subjects who received ISIS 396443, log transformed pNF-H levels on Study Day 64 were not statistically significantly associated with Ulnar CMAP Amplitude on Study Day 183, p=0.0952, such that higher pNF-H levels were associated with a trend toward lower Ulnar CMAP Amplitude. Among subjects who

received SHAM control, log transformed pNF-H levels on Study Day 64 were statistically significantly associated with Ulnar CMAP Amplitude on Study Day 183, p=0.0281, such that higher pNF-H levels were associated with a lower Ulnar CMAP Amplitude. See **Fig. 35**.

This result suggests that present pNF-H levels can predict future levels of general motor neuron health among infants with infantile-onset SMA.

Example 34: ENDEAR Baseline Characteristics Dichotomized by Median pNF-H Levels

Below is a table that provides baseline characteristics dichotomized (<15,400 and ≥15,400 pg/mL) by median pNF-H levels from the ENDEAR trial.

	Baseline plasma pNF-H (pg/mL)		***P* value[b]**
	<15,400[a]	**≥15,400[a]**	
Individuals[c], n	58	59	
Female, n (%)	32 (55)	33 (56)	1.000
Mean (range) age at first dose, wk	**25.8 (7.4, 37.4)**	**22.6 (4.3, 33.6)**	**.0165**
Mean (range) age of symptom onset, wk	**9.5 (3, 20)**	**7.3 (2, 19)**	**.0049**
Mean (range) age at SMA diagnosis, wk	**16.02 (4, 29)**	**12.31 (0, 30)**	**.0046**
Mean (range) disease duration, wks	13.8 (0.6, 25.9)	13.2 (0, 23.1)	.5723
Use of ventilation support, n (%)	14 (24)	13 (22)	.8290
Mean ± SD total HINE-2 score	1.6 ± 1.30	1.2 ± 0.94	.0593
Mean ± SD total CHOP INTEND score	**29.49 ± 7.125**	**25.11 ± 7.850**	**.0020**
Mean ± SD peroneal CMAP amplitude	**0.40 ± 0.328**	**0.28 ± 0.244**	**.0353**
Mean ± SD ulnar CMAP amplitude	0.21 ± 0.162	0.23 ± 0.164	.6259

CHOP INTEND = Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders;
CMAP = compound muscle action potential;
HINE-2 = Hammersmith Infant Neurological Examination Section 2.
[a]The median of baseline pNF-H.
[b]Results for continuous variables are from Student's two-sample *t*-test and results for proportions are from Fisher's exact test.
[c]Number of participants in the table refers to participants who have non-missing baseline pNF-H in either category.

Example 35: Correlations Between Baseline Characteristics and Log(pNF-H) Levels in ENDEAR

The table below provides correlations between baseline characteristics and log(pNF-H) levels in the ENDEAR trial.

	Pearson correlation coefficient	Probability > r under H0: Rho=0
Weight (kg), n=117	−0.11	.2201
Age of symptom onset (weeks), n=117	**−0.20**	**.0344**
Age at first dose (weeks), n=117	**−0.24**	**.0106**
Age at SMA diagnosis (weeks), n=117	**−0.25**	**.0064**
Gestational age (weeks), n=117	0.01	.9438
Disease duration (weeks), n=117	−0.09	.3183
HINE motor milestone score, n=117	−0.13	.1786
CHOP INTEND, n=117	**−0.30**	**.0012**
CMAP: peroneal nerve amplitude, n=108	−0.13	.1842
CMAP: ulnar amplitude, n=111	−0.16	.1002

Example 36: CHERISH Baseline Characteristics Dichotomized by Median pNF-H Levels

Below is a table that provides baseline characteristics dichotomized (<1,200 and ≥1,200 pg/mL) by median pNF-H levels from the CHERISH trial.

	Baseline plasma pNF-H (pg/mL)		*P* value[b]
	<1,200[a]	≥1,200[a]	
Individuals[c], n	62	64	
Mean (range) weight, kg	**16.77 (10.0, 36.4)**	**12.59 (8.5, 24.0)**	**<.0001**
Mean (range) age of symptom onset (weeks)	48.57 (26.1, 78.2)	48.34 (26.1, 86.9)	.9306
Mean (range) age at first dose (weeks)	**269.10 (131.9, 482.1)**	**167.45 (107.3, 379.9)**	**<.0001**
Mean (range) age at SMA diagnosis (weeks)	89.99 (43.5, 208.6)	78.28 (0.0, 165.1)	.0542
Mean (range) disease duration (weeks)	**217.49 (97.4, 408.9)**	**116.10 (34.8, 315.1)**	**<.0001**
Mean ± SD HFMSE total score	21.58 ± 8.231	21.53 ± 7.904	.9726

Mean ± SD Upper Limb Module Test score	**20.81 ± 5.847**	**17.45 ± 5.741**	**.0015**

HFMSE = Hammersmith Functional Motor Scale – Expanded.
[a]The median of baseline pNF-H.
[b]Results for continuous variables are from Student's two-sample *t*-test and results for proportions are from Fisher's exact test.
[c]Number of participants in the table refers to participants who have non-missing baseline pNF-H in either category.

Example 37: Correlations Between Baseline Characteristics and Log(pNF-H) Levels in CHERISH

The table below provides correlations between baseline characteristics and log(pNF-H) levels in the CHERISH trial.

	Pearson correlation coefficient	**Probability > r under H0: Rho=0**
Weight (kg), n=126	−0.44	<.0001
Age of symptom onset (weeks), n=126	0.05	.5469
Age at first dose (weeks), n=126	**−0.63**	**<.0001**
Age at SMA diagnosis (weeks), n=126	−0.03	.7257
Disease duration (weeks), n=126	−0.64	<.0001
HFMSE score, n=126	0.10	.2436
WHO motor milestones, n=126	0.14	.1154
Upper Limb Module Test score, n=126	**−0.20**	**.0280**

HFMSE = Hammersmith Functional Motor Scale – Expanded.
WHO = World Health Organization

Example 38: Predicting Future Motor Function by Measuring Percent Change in pNF-H Levels After Treatment

Receiver operating characteristic (ROC) curves were used in an effort to select the time period for measuring percent change in neurofilament levels that best predicts future motor function in a treated individual. In ENDEAR, percent change in pNF-H levels were measured at each of Day 29, Day 64, and Day 183 and compared with motor function of the treated subject at Day 302. ROC curves revealed that percent change in pNF-H levels at Day 64 is better at predicting motor function at Day 302 than percent change in pNF-H levels at Day 29 or Day 183. See **Figs. 36A-36C**. In ENDEAR, even after controlling for age of first

dose, percent change in pNF-H levels at Day 64 predicted CHOP INTEND (≥ 4 point improvement) and motor milestone (more HINE-2 motor milestones with improvement than worsening) responders at Day 302. See **Figs. 37A-B**. In CHERISH, even after controlling for disease duration, percent change in pNF-H levels at Day 85 predicted HFMSE (Hammersmith Functional Motor Scale – Expanded; ≥ 3 point improvement), RULM (Revised Upper Limb Module; ≥ 2 point improvement), and WHO (World Health Organization; attainment of ≥ 1 motor milestone) responders at Day 456. See **Figs. 38A-38C**.

Example 39: Cerebral Spinal Fluid pNF-H Levels at Baseline and Following Treatment with Nusinersen

Baseline cerebral spinal fluid (CSF) pNF-H levels were measured in presymptomatic, infantile-onset, and later-onset patients as well as patients with 2 or 3 copies of SMN2. Baseline pNF-H levels in CSF were highest in presymptomatic infants and the youngest infants with 2 copies of SMN2. See **Figs. 39A-39B**. In the NURTURE study, treatment with nusinersen was associated with a rapid decline in pNF-H levels in CSF, followed by stabilization. See **Fig. 40**.

Example 40: Comparison of Phosphorylated Neurofilament Heavy Chain (pNF-H) and Neurofilament Light Chain (NF-L) in Different Matrices Among Multiple Spinal Muscular Atrophy Populations

Concentrations of pNF-H and NF-L were compared in plasma and cerebrospinal fluid (CSF) in individuals from nusinersen clinical trials with presymptomatic (most likely to develop Type I/II), infantile-onset (has or most likely to develop Type I/II) or later-onset SMA (has or most likely to develop Type II/III). pNF-H concentrations in plasma and CSF were evaluated using the ProteinSimple™ SimplePlex ELLA immunoassay. NF-L concentrations were evaluated using the SIMOA assay (Quanterix™). Results reported below are in units of pg/mL.

For each SMA population, pNF-H and NF-L concentrations were similar in CSF; however, in plasma, NF-L concentrations were lower than those of pNF-H. The difference between NF-L and pNF-H concentrations in plasma was more pronounced in presymptomatic infants versus those with infantile-onset SMA. In CSF, pNF-H and NF-L concentrations declined over time on nusinersen treatment at similar rates in the presymptomatic (percentage reduction at Day 183: **Fig. 41A**) and the infantile-onset (percentage reduction at Day 302: **Fig. 41B**) SMA cohorts. Comparable results were demonstrated in plasma pNF-H and NF-L concentrations in the infantile-onset (percentage reduction at Day 64: **Fig. 41C**) and the later-onset (percentage reduction at Day 169: **Fig. 41D**) SMA cohorts. The absolute values of the

data shown in **Fig. 41C** are presented in **Fig. 41E**, and the absolute values of the data shown in **Fig. 41D** are presented in **Fig. 41F**. Additionally, the rates of change in pNF-H concentrations in plasma and CSF were similar in both the presymptomatic and infantile-onset SMA cohorts. For NF-L, plasma and CSF concentrations declined over a similar trajectory in the infantile-onset cohort.

Overall, the baseline pNF-H and NF-L geometric mean (95% CI) concentrations were 20139 (10075-40257) and 7272 (3287-16090) pg/mL, respectively, among presymptomatic infants with 2 SMN2 copies and 952 (367-2470) and 519 (231-1164) pg/mL, respectively, among those with 3 SMN2 copies. In infantile-onset SMA participants, baseline pNF-H and NF-L geometric mean (95% CI) concentrations were 3791 (2980-4823) and 3718 (2832-4882) pg/mL, respectively. In later-onset SMA participants, baseline pNF-H and NF-L geometric mean (95% CI) concentrations were 381 (331-438) and 185 (154-222) pg/mL, respectively.

Thus, like plasma pNF-H levels, CSF pNF-H and NF-L levels appear highest in presymptomatic SMA participants with 2 SMN2 copies and lowest in those with later-onset SMA.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of treating spinal muscular atrophy (SMA) in a human subject in need thereof, comprising administering to the human subject a therapeutically effective amount of an SMA therapy, wherein the human subject has been previously determined to have, in a biological sample obtained from the human subject, a neurofilament level prior to initiation of the SMA therapy that is higher than a control.

2. A method of treating spinal muscular atrophy (SMA) in a human subject in need thereof, comprising:

measuring a neurofilament level in a biological sample obtained from the human subject before initiation of an SMA therapy; and

administering a therapeutically effective amount of the SMA therapy to the human subject.

3. The method of claim 1 or 2, wherein the neurofilament level in the biological sample is above 400 pg/mL.

4. A method of treating spinal muscular atrophy (SMA) in a human subject in need thereof, comprising:

measuring a neurofilament level in a first biological sample obtained from the human subject before initiation of an SMA therapy;

administering an SMA therapy to the human subject; and

measuring a neurofilament level in a second biological sample obtained from the human subject after initiation of the SMA therapy.

5. The method of claim 4, wherein the neurofilament level measured in the second biological sample is lower than the neurofilament level measured in the first biological sample.

6. The method of claim 5, wherein the neurofilament level measured in the second biological sample is between 10% to 95% of the neurofilament level measured in the first biological sample.

7. The method of any one of claims 4 to 6, wherein the second biological sample is obtained from the human subject 40-90 days after initiation of the SMA therapy.

8. The method of any one of claims 4 to 6, wherein the second biological sample is obtained from the human subject 50-80 days after initiation of the SMA therapy.

9. The method of any one of claims 4 to 6, wherein the second biological sample is obtained from the human subject 60-70 days after initiation of the SMA therapy.

10. The method of any one of claims 4 to 6, wherein the second biological sample is obtained from the human subject about 64 days after initiation of the SMA therapy.

11. The method of any one of claims 7 to 10, wherein the neurofilament level measured in the second biological sample is reduced by at least 50% compared to the neurofilament level measured in the first biological sample.

12. The method of any one of claims 7 to 10, wherein the neurofilament level measured in the second biological sample is reduced by at least 60% compared to the neurofilament level measured in the first biological sample.

13. The method of any one of claims 7 to 10, wherein the neurofilament level measured in the second biological sample is reduced by at least 70% compared to the neurofilament level measured in the first biological sample.

14. The method of any one of claims 7 to 10, wherein the neurofilament level measured in the second biological sample is reduced by less than 50% compared to the neurofilament level measured in the first biological sample.

15. The method of any one of claims 7 to 10, wherein the neurofilament level measured in the second biological sample is reduced by less than 40% compared to the neurofilament level measured in the first biological sample.

16. The method of any one of claims 7 to 15, wherein the dose of the SMA therapy is changed for a subsequent administration to the human subject based upon the percent reduction in neurofilament level measured in the second biological sample as compared to the neurofilament level measured in the first biological sample.

17. The method of claim 4, wherein the neurofilament level measured in the second biological sample is higher than the neurofilament level measured in the first biological sample.

18. The method of any one of claims 4 to 17, wherein administration of the SMA therapy is continued.

19. The method of claim 17, wherein administration of the SMA therapy is discontinued.

20. A method of treating spinal muscular atrophy (SMA) in a human subject in need thereof, comprising:

measuring a neurofilament level in a first biological sample obtained from the human subject before administration of a candidate amount of an SMA therapy;

measuring a neurofilament level in a second biological sample obtained from the human subject after administration of the candidate amount of the SMA therapy, wherein the neurofilament level in the second biological sample is lower than the neurofilament level in the first biological sample, thereby indicating that the candidate amount of the SMA therapy is a therapeutically effective amount;

administering the therapeutically effective amount of the SMA therapy to the human subject after having measured the lowered neurofilament level in the second biological sample.

21. A method of predicting the prognosis of spinal muscular atrophy (SMA), comprising:

measuring a neurofilament level in a biological sample obtained from a human subject having mutations in both copies of the SMN1 gene that lead to functional SMN protein deficiency; and

comparing the neurofilament level measured in the biological sample to a control,

wherein the neurofilament level measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the subject will develop.

22. The method of claim 21, wherein the biological sample is obtained from the human subject before initiation of an SMA therapy, and wherein the neurofilament level measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the human subject will develop in the absence of treatment.

23. The method of claim 21, wherein the biological sample is obtained from the human subject after initiation of an SMA therapy, and wherein the neurofilament level measured in the biological sample, as compared to the control, is predictive of the severity or type of SMA that the subject will develop while receiving the SMA therapy.

24. The method of claim 23, wherein the biological sample is obtained from the human subject at least two weeks after initiation of the SMA therapy.

25. The method of claim 23, wherein the biological sample is obtained from the human subject at least two months after initiation of the SMA therapy.

26. A method of predicting the prognosis of spinal muscular atrophy (SMA), comprising:

measuring, before initiation of an SMA therapy, a neurofilament level in a first biological sample obtained from a human subject having mutations in both copies of the SMN1 gene that lead to functional SMN protein deficiency;

measuring a neurofilament level in a second biological sample obtained from the human subject after initiation of the SMA therapy; and

comparing the neurofilament level measured in the second biological sample to the neurofilament level measured in the first biological sample,

wherein the neurofilament level measured in the second biological sample, as compared to the neurofilament level measured in the first biological sample, is predictive of the severity or type of SMA that the subject will develop.

27. The method of claim 26, wherein the second biological sample is obtained from the human subject 40-90 days after initiation of the SMA therapy.

28. The method of claim 26, wherein the second biological sample is obtained from the human subject 50-80 days after initiation of the SMA therapy.

29. The method of claim 26, wherein the second biological sample is obtained from the human subject 60-70 days after initiation of the SMA therapy.

30. The method of claim 26, wherein the second biological sample is obtained from the human subject about 64 days after initiation of the SMA therapy.

31. The method of any one of claims 26 to 30, wherein the neurofilament level measured in the second biological sample is reduced by at least 50% compared to the neurofilament level measured in the first biological sample.

32. The method of any one of claims 26 to 30, wherein the neurofilament level measured in the second biological sample is reduced by at least 60% compared to the neurofilament level measured in the first biological sample.

33. The method of any one of claims 26 to 30, wherein the neurofilament level measured in the second biological sample is reduced by at least 70% compared to the neurofilament level measured in the first biological sample.

34. The method of any one of claims 26 to 30, wherein the neurofilament level measured in the second biological sample is reduced by less than 50% compared to the neurofilament level measured in the first biological sample.

35. The method of any one of claims 26 to 30, wherein the neurofilament level measured in the second biological sample is reduced by less than 40% compared to the neurofilament level measured in the first biological sample.

36. The method of any one of the preceding claims, wherein the SMA therapy is nusinersen or a nusinersen salt.

37. The method of any one of claims 1 to 35, wherein the SMA therapy is nusinersen sodium.

38. The method of any one of claims 1 to 35, wherein the SMA therapy is olesoxime, AVX-101, CK-2127107, RG7916, RG7800, RO7034067, LMI070, or SRK-015.

39. The method of any one of the preceding claims, wherein the control is a pre-established neurofilament cut-off value.

40. The method of any one of claims 1 to 38, wherein the control is the neurofilament level in a biological sample or biological samples obtained from one or more human subjects that do not have SMA.

41. A method for measuring a neurofilament level, comprising:

providing a biological sample obtained from a human subject having mutations in both copies of the SMN1 gene that lead to functional SMN protein deficiency; and

measuring a neurofilament level in the biological sample.

42. The method of any one of the preceding claims, wherein the neurofilament is a neurofilament heavy chain.

43. The method of any one of the preceding claims, wherein the neurofilament is a phosphorylated neurofilament heavy chain.

44. The method of any one of claims 1 to 41, wherein the neurofilament is a neurofilament medium/intermediate chain.

45. The method of any one of claims 1 to 41, wherein the neurofilament is a neurofilament light chain.

46. The method of any one of the preceding claims, wherein the biological sample is blood, serum, plasma, or cerebrospinal fluid.

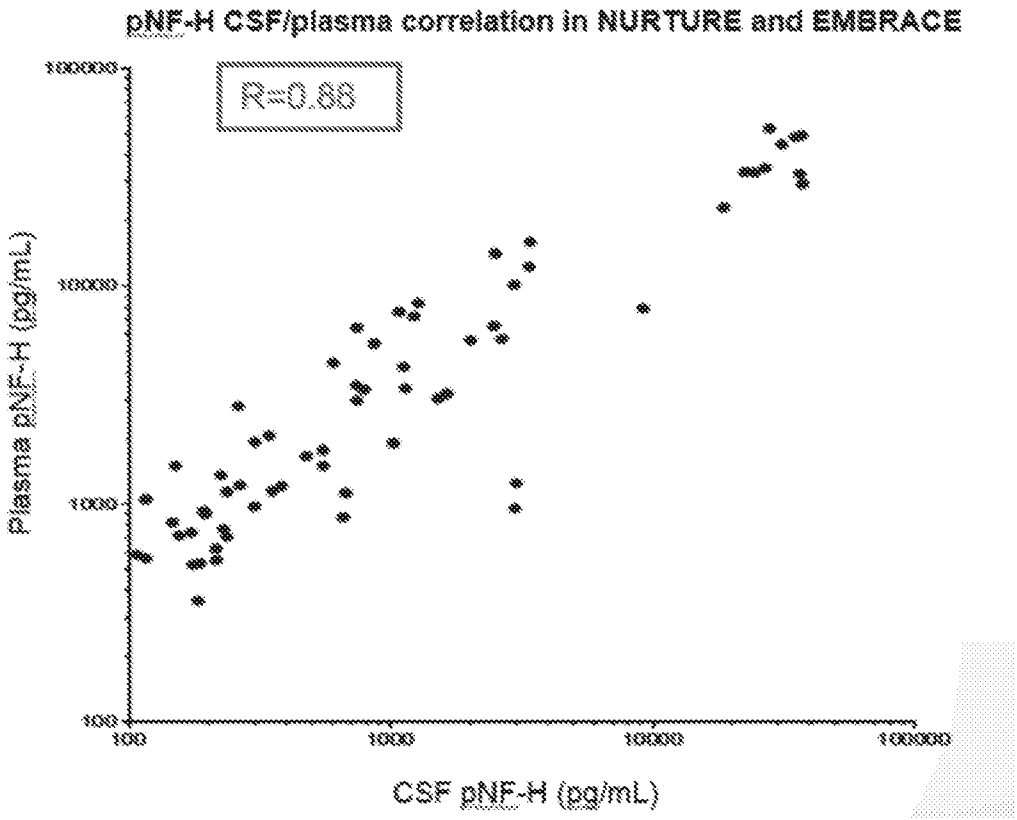

pNF-H CSF/plasma correlation in NURTURE and EMBRACE
R=0.86
Plasma pNF-H (pg/mL)
CSF pNF-H (pg/mL)
100
1000
10000
100000

FIG. 1

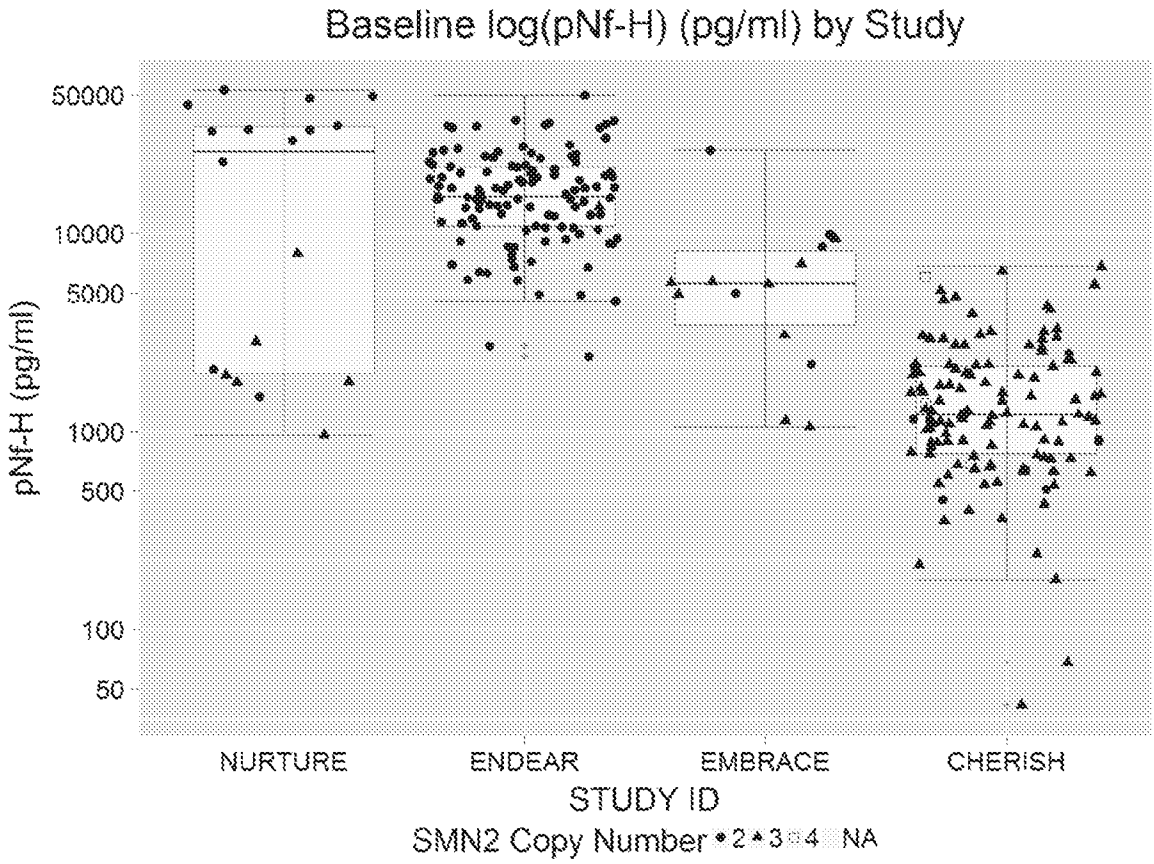

Baseline log(pNf-H) (pg/ml) by Study
pNf-H (pg/ml)
50000
10000
5000
1000
500
100
50
NURTURE
ENDEAR
EMBRACE
CHERISH
STUDY ID
SMN2 Copy Number 2 3 4 NA

FIG. 2

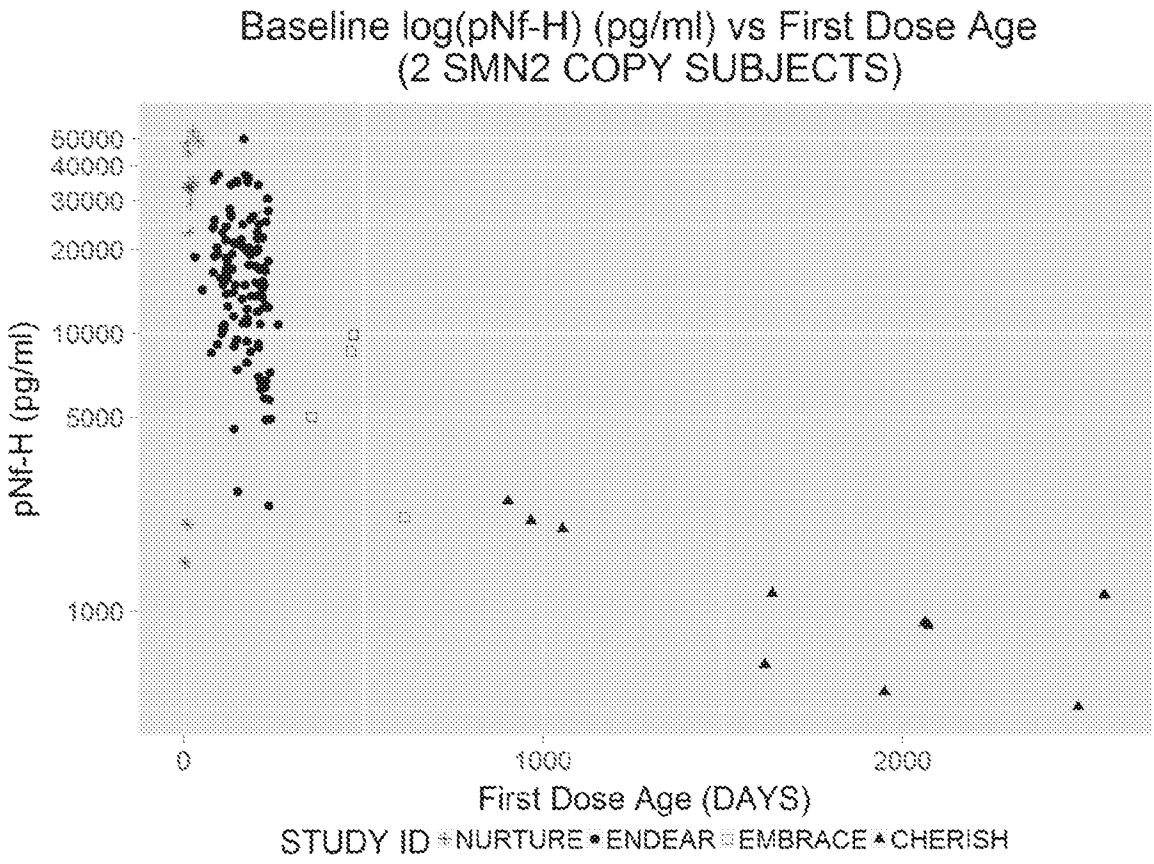

Baseline log(pNf-H) (pg/ml) vs First Dose Age
(2 SMN2 COPY SUBJECTS)
pNf-H (pg/ml)
50000
40000
30000
20000
10000
5000
1000
0
1000
2000
First Dose Age (DAYS)
STUDY ID NURTURE ENDEAR EMBRACE CHERISH

FIG. 3

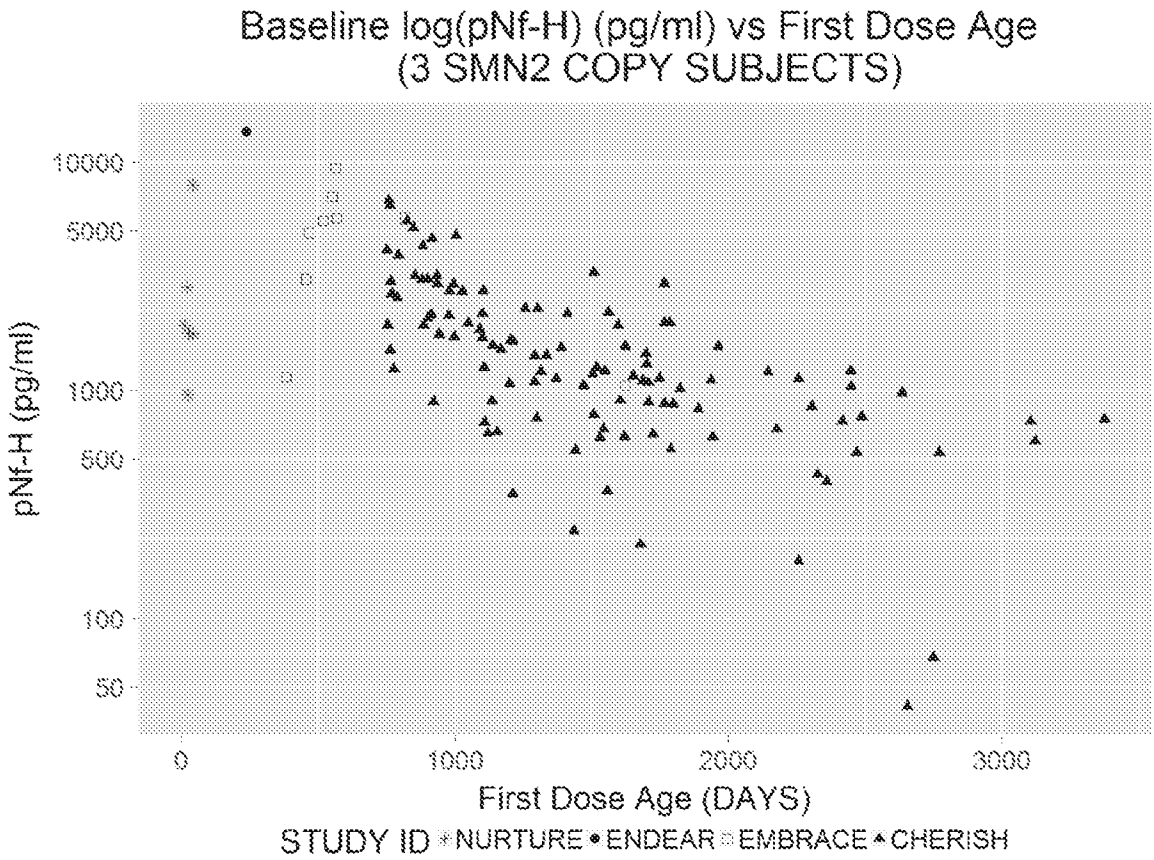

Baseline log(pNf-H) (pg/ml) vs First Dose Age
(3 SMN2 COPY SUBJECTS)
pNf-H (pg/ml)
10000
5000
1000
500
100
50
0
1000
2000
3000
First Dose Age (DAYS)
STUDY ID NURTURE ENDEAR EMBRACE CHERISH

FIG. 4

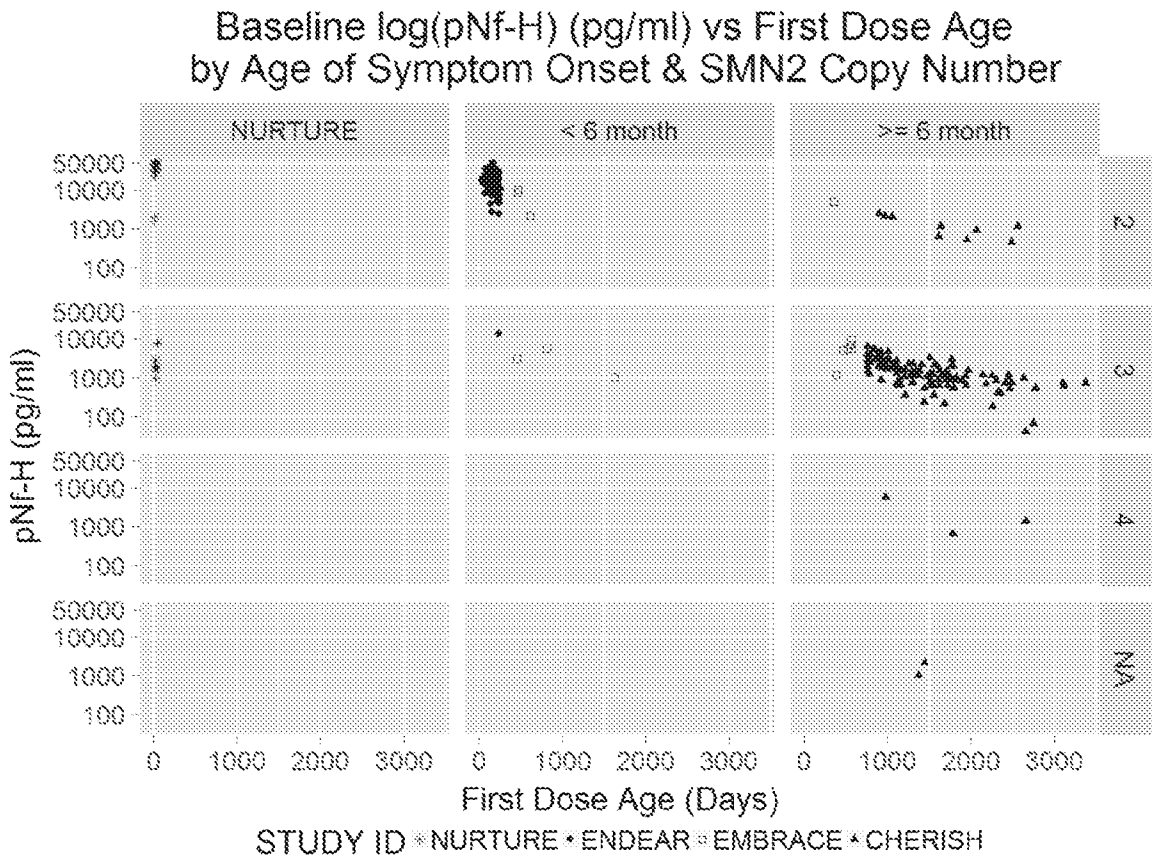

Baseline log(pNf-H) (pg/ml) vs First Dose Age
by Age of Symptom Onset & SMN2 Copy Number
NURTURE
< 6 month
>= 6 month
pNf-H (pg/ml)
50000
10000
1000
100
0
1000
2000
3000
First Dose Age (Days)
STUDY ID NURTURE ENDEAR EMBRACE CHERISH

FIG. 5

Summary of Baseline Characteristics and SMA History by Baseline pNF-H Concentrations (pg/ml) Quartiles
Page 1 of 3

	Quartiles of Baseline pNF-H			
	1st Quartile (2838 - 10800)	2nd Quartile (10855 - 15450)	3rd Quartile (15459 - 21600)	4th Quartile (21650 - 50100)
Number of subjects	29	29	29	30
Female sex n (%)	18 (66%)	13 (45%)	16 (55%)	17 (57%)
Age at first dose (Days) mean (min,max)	181.4 (77, 292)	179.4 (62, 237)	154.2 (30, 235)	161.8 (61, 235)
Age of symptom onset at Baseline (weeks) mean (min,max)	8.8 (6, 20)	8.3 (5, 18)	6.8 (1, 12)	7.8 (3, 16)
Age at SMA diagnosis (weeks) mean (min,max)	16.93 (6, 29)	15.19 (6, 28)	11.34 (0, 26)	13.33 (2, 30)
Disease duration at screening (weeks) mean (min,max)	13.4 (5, 21.1)	14.1 (8.6, 25.9)	13.1 (0, 22)	13.3 (0, 23.1)

Note: (a) Number of subjects in the table refers to subjects who have non-missing baseline value in each quartile
(b) SD=Standard Deviation

SOURCE: I:\Stat\449\Biomarker\C335-A08CC\T-SUM-BASCHAR-SMA-BNFRQUART.SAS DATE: 06MAY2017

FIG. 6

Summary of baseline Characteristics and SMA History by baseline pNF-H concentrations (pg/ml) quartiles
Page 2 of 3

	Quartiles of Baseline pNf-H			
	1st Quartile (2390 - 10900)	2nd Quartile (10900 - 15400)	3rd Quartile (15400 - 21600)	4th Quartile (21600 - 50100)
Symptoms of SMA				
Hyptonia	29 (100%)	29 (100%)	29 (100%)	30 (100%)
Dev Delay of Motor Funtion	28 (97%)	28 (97%)	24 (83%)	26 (87%)
Paradoxical Breathing	23 (79%)	23 (79%)	24 (83%)	25 (83%)
Pneumonia/Resp. Symptoms	8 (28%)	10 (34%)	12 (41%)	6 (20%)
Limb weakness	29 (100%)	29 (100%)	29 (100%)	29 (97%)
Swallowing Abnormalities	15 (52%)	11 (38%)	14 (48%)	12 (40%)
Other	8 (28%)	4 (14%)	9 (31%)	13 (43%)
Use of ventilator support n (%)	7 (24%)	7 (24%)	8 (28%)	5 (17%)

Note: (a) Number of subjects in the table refers to subjects who have non-missing baseline value in each quartile (b) SD=Standard Deviation

SOURCE: ISIS396443/BIOMARKER/CS3B-ADHOC/T-SUM-BASCHAR-SMA-PNFHQUART.SAS

DATE: 09NOV2017

FIG. 7

Summary of baseline Characteristics and SMA History by baseline pNF-H concentrations (pg/ml) quartiles
Page 3 of 3

	Quartiles of Baseline pNf-H			
	1st Quartile (2390 - 10900)	2nd Quartile (10900 - 15400)	3rd Quartile (15400 - 21600)	4th Quartile (21600 - 50100)
Total HINE-2 score mean +/- SD (b)	1.7 +/- 1.41	1.4 +/- 1.18	1.0 +/- 0.85	1.4 +/- 1.00
CHOP INTEND score mean +/- SD	30.90 +/- 6.607	28.09 +/- 7.456	24.36 +/- 7.295	25.83 +/- 8.412
Peroneal mean +/- SD	0.39 +/- 0.311	0.41 +/- 0.348	0.27 +/- 0.199	0.29 +/- 0.283
Ulnar mean +/- SD	0.24 +/- 0.150	0.19 +/- 0.171	0.29 +/- 0.202	0.17 +/- 0.089

Note: (a) Number of subjects in the table refers to subjects who have non-missing baseline value in each quartile (b) SD=Standard Deviation

SOURCE: ISIS396443/BIOMARKER/CS3B-ADHOC/T-SUM-BASCHAR-SMA-PNFHQUART.SAS

DATE: 09NOV2017

FIG. 8

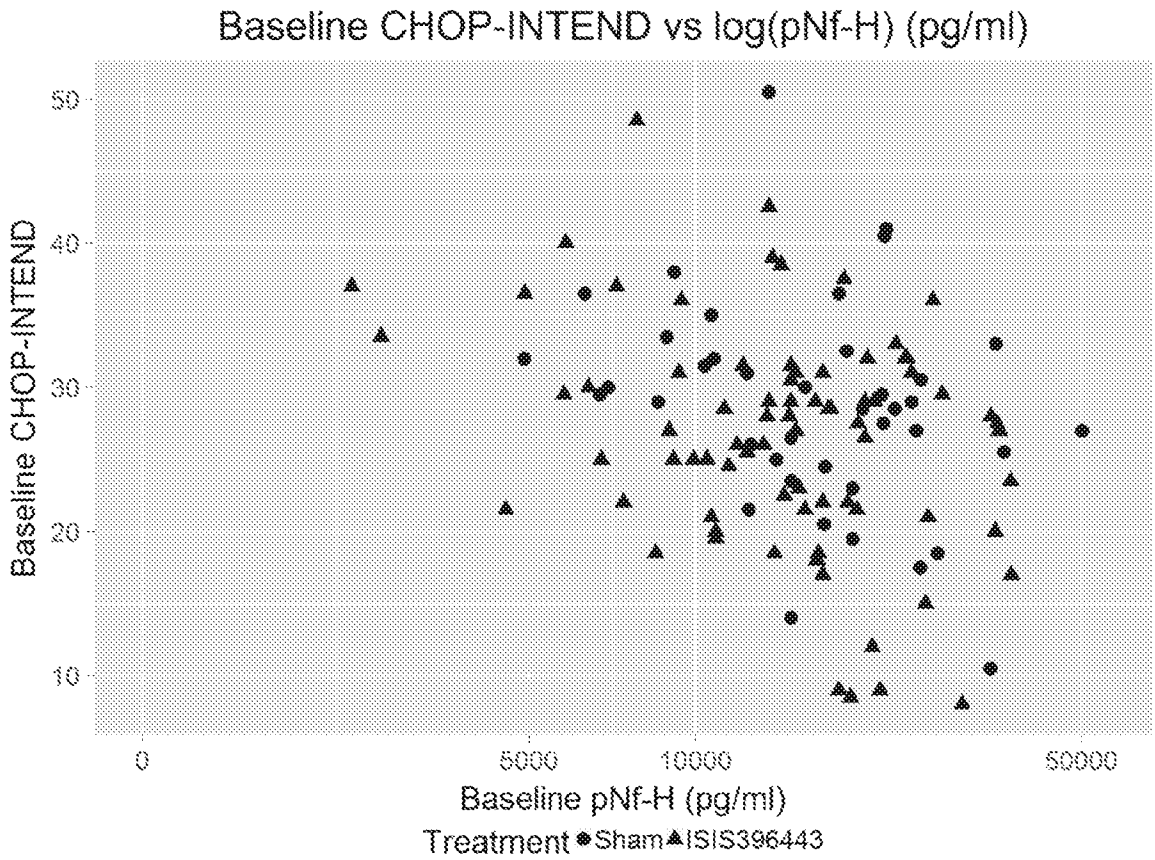

Baseline CHOP-INTEND vs log(pNf-H) (pg/ml)
Baseline CHOP-INTEND
50
40
30
20
10
0
5000
10000
50000
Baseline pNf-H (pg/ml)
Treatment Sham ISIS396443

FIG. 9

Variables in the initial model:

- Baseline log(pNF-H)
- Treatment group
- Disease duration (weeks)
- Sex
- Age of first dose (days)
- Age of SMA symptom onset (weeks)
- Age of SMA diagnosis (weeks)
- Gestational age (weeks)
- Baseline weight (kg)
- (Gestational age + age of first dose [weeks]) x (Gestational age + age of SMA symptom onset [weeks])
- Gestational age + age of SMA diagnosis (weeks)

Variables in the Final Model	*P*-value
Intercept	<0.0001
Baseline log(pNF-H)	0.0062
Age of SMA diagnosis (weeks)	0.0016
Baseline weight (kg)	0.0075

FIG. 10

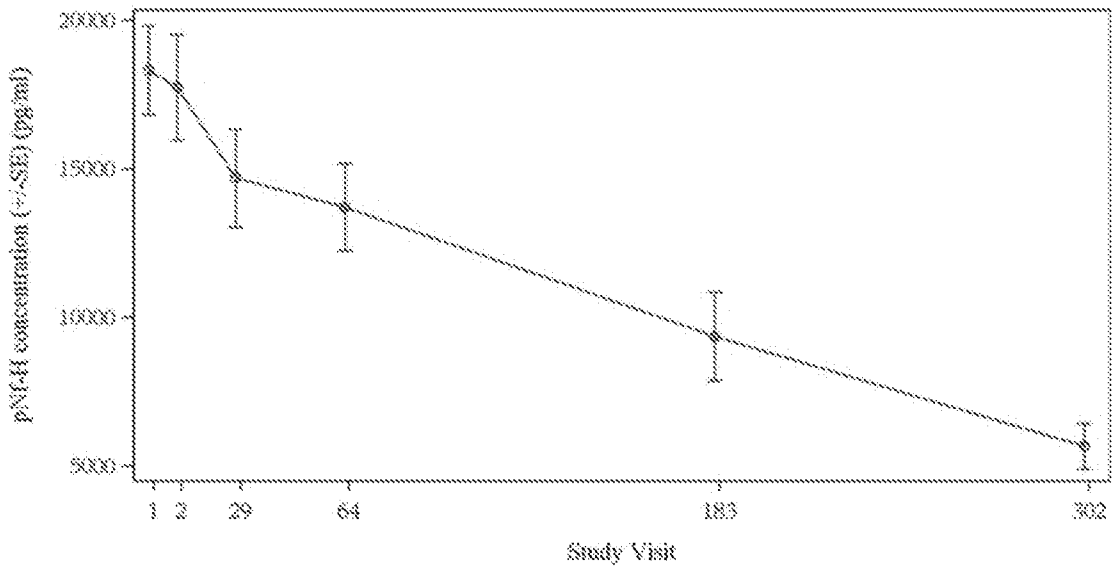

20000
15000
10000
5000
PCSK9 concentration (ng/mL)
1
2
29
64
183
302
Study Visit

FIG. 11

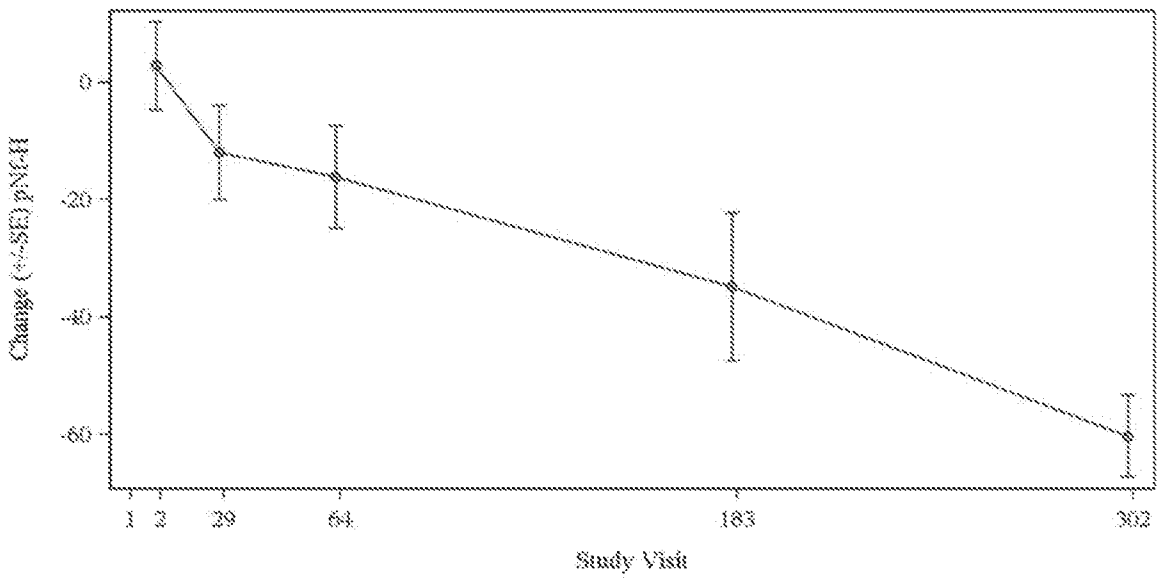


FIG. 12

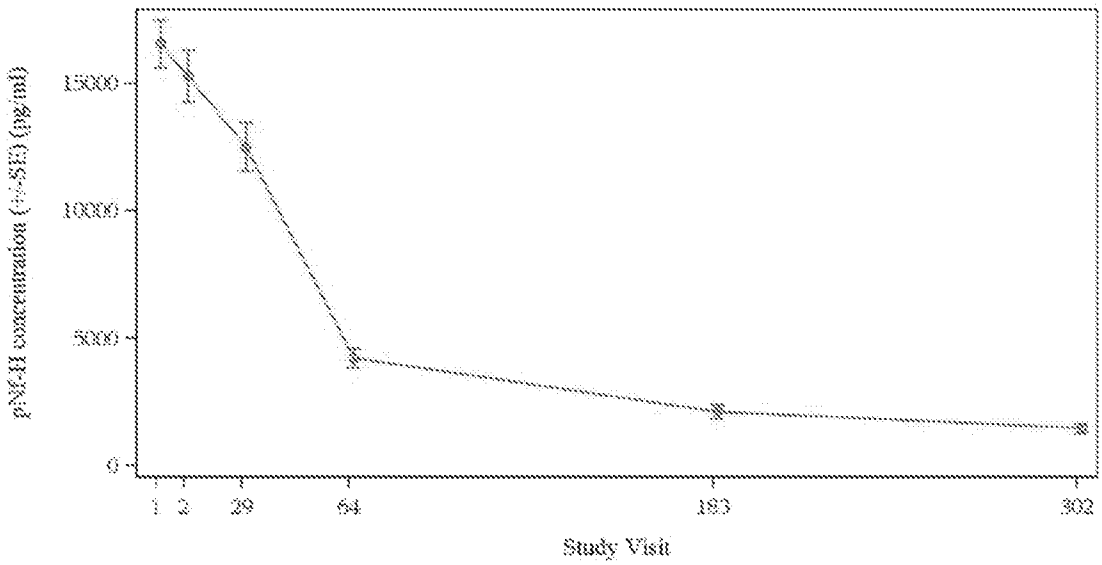

pNF-H concentration (+/-SE) (pg/ml)
15000
10000
5000
0
1
2
29
64
183
302
Study Visit

FIG. 13

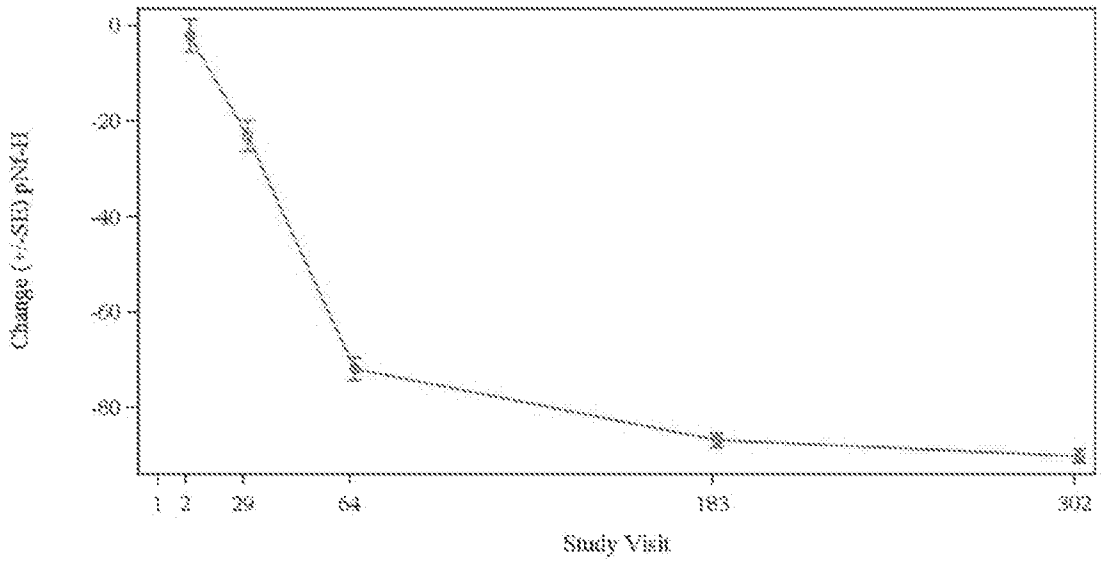


FIG. 14

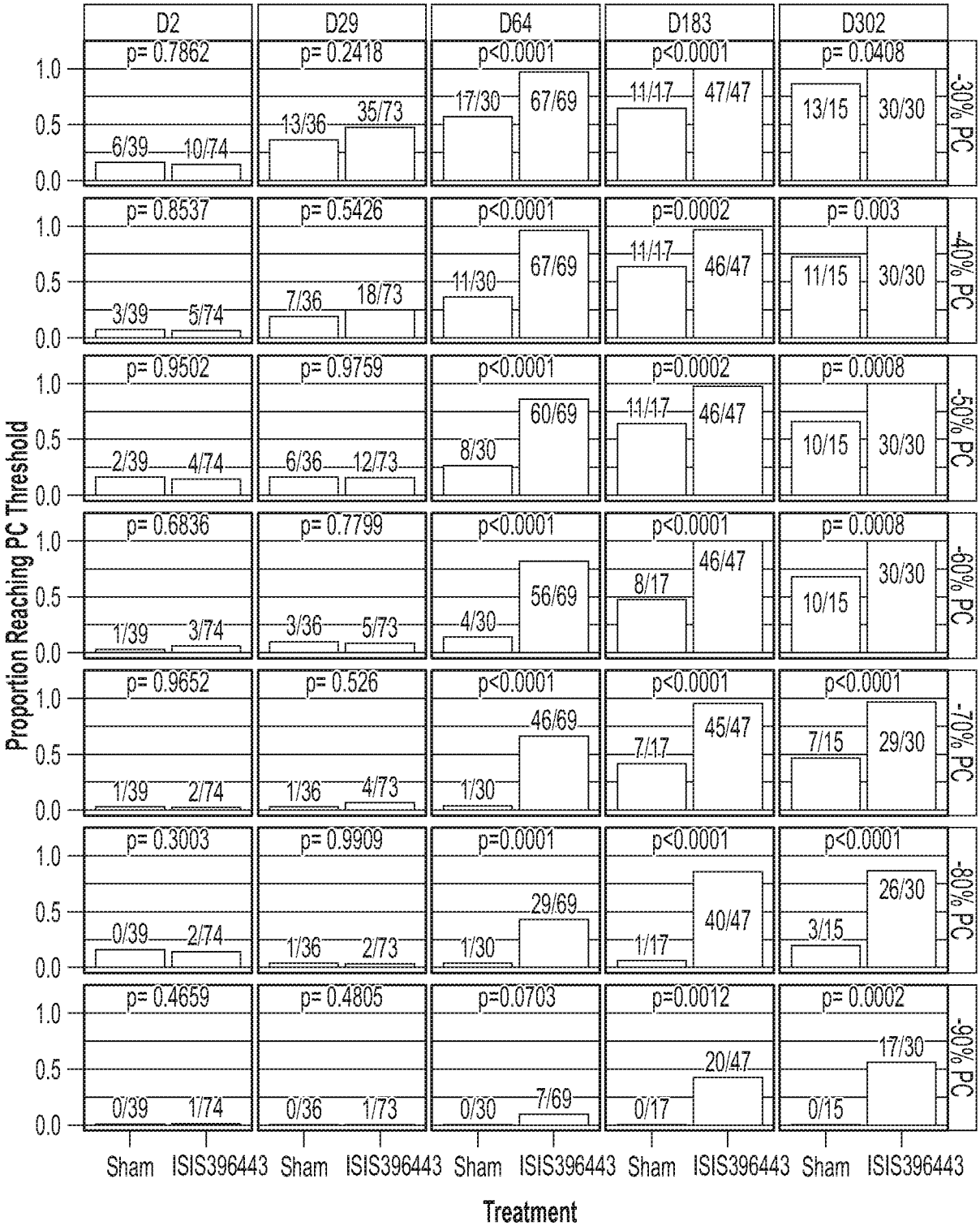
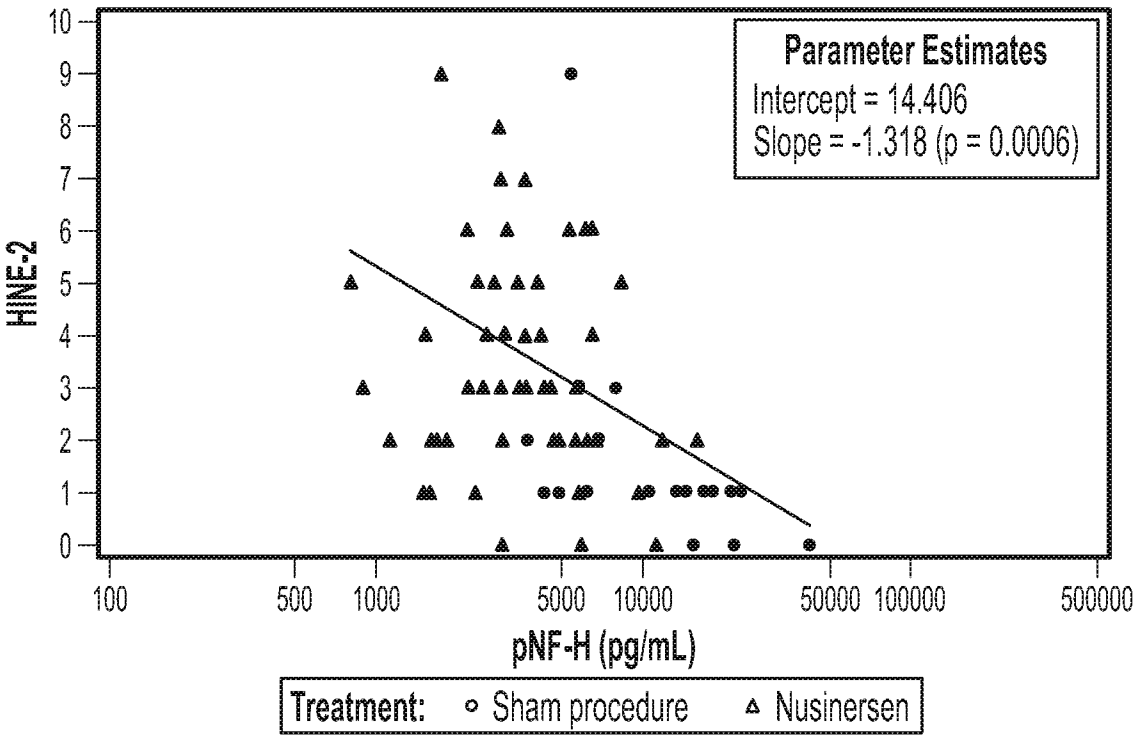

D2
D29
D64
D183
D302
-30% PC
-40% PC
-50% PC
-60% PC
-70% PC
-80% PC
-90% PC
p= 0.7862
p= 0.2418
p<0.0001
p<0.0001
p= 0.0408
6/39
10/74
13/36
35/73
17/30
67/69
11/17
47/47
13/15
30/30
p= 0.8537
p= 0.5426
p<0.0001
p=0.0002
p= 0.003
3/39
5/74
7/36
18/73
11/30
67/69
11/17
46/47
11/15
30/30
p= 0.9502
p= 0.9759
p<0.0001
p=0.0002
p= 0.0008
2/39
4/74
6/36
12/73
8/30
60/69
11/17
46/47
10/15
30/30
p= 0.6836
p= 0.7799
p<0.0001
p<0.0001
p= 0.0008
1/39
3/74
3/36
5/73
4/30
56/69
8/17
46/47
10/15
30/30
p= 0.9652
p= 0.526
p<0.0001
p<0.0001
p<0.0001
1/39
2/74
1/36
4/73
1/30
46/69
7/17
45/47
7/15
29/30
p= 0.3003
p= 0.9909
p=0.0001
p<0.0001
p<0.0001
0/39
2/74
1/36
2/73
1/30
29/69
1/17
40/47
3/15
26/30
p= 0.4659
p= 0.4805
p=0.0703
p=0.0012
p= 0.0002
0/39
1/74
0/36
1/73
0/30
7/69
0/17
20/47
0/15
17/30
1.0
0.5
0.0
Proportion Reaching PC Threshold
Sham
ISIS396443
Treatment

FIG. 15



Variable	DF	Parameter Estimate	Standard Error	t Value	pr > ltl
Intercept	1	14.406	3.1279	4.6058	<.0001*
Day 64 log(pNF-H)	1	-1.318	0.3677	-3.584	0.0006*

Root MSE: 2.63579 R-square: 0.1462 Adjusted R-square: 0.1349

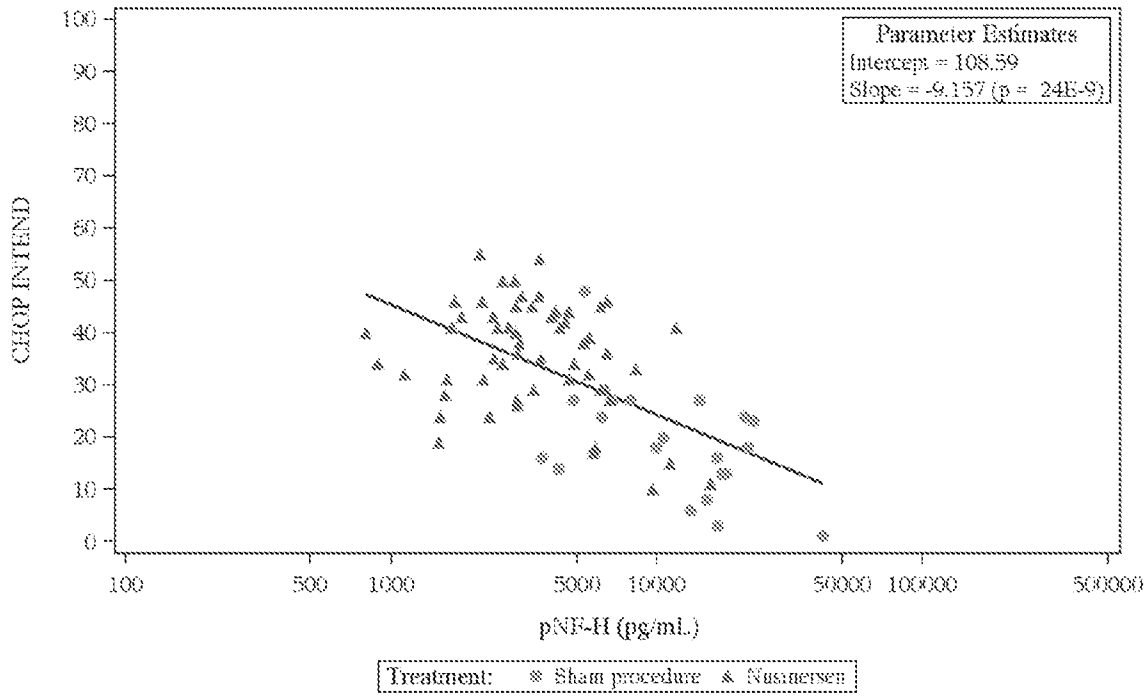
FIG. 16

Variables in the initial model:

- Day 64 log(pNF-H)
- Treatment group
- Disease duration (weeks)
- Sex
- Age of first dose (days)
- Age of SMA symptom onset (weeks)
- Age of SMA diagnosis (weeks)
- Gestational age (weeks)
- Baseline weight (kg)
- (Gestational age + age of first dose [weeks]) x (Gestational age + age of SMA symptom onset [weeks])
- Gestational age + age of SMA diagnosis (weeks)

Variables in the Final Model	P-value
Intercept	<0.0001
Day 64 log(pNF-H)	<0.0001
Disease duration	0.0029

FIG. 17



Variable	DF	Parameter Estimate	Standard Error	t Value	pr > |t|
Intercept	1	108.59	13.484	8.6981	<.0001*
Day 64 log(pNF-H)	1	-9.157	1.4678	-6.239	<.0001*

Root MSE: 10.5204 R-square: 0.3417 Adjusted R-square: 0.3329

FIG. 18

Variables in the initial model:

- Day 64 log(pNF-H)
- Treatment group
- Disease duration (weeks)
- Sex
- Age of first dose (days)
- Age of SMA symptom onset (weeks)
- Age of SMA diagnosis (weeks)
- Gestational age (weeks)
- Baseline weight (kg)
- (Gestational age + age of first dose [weeks]) x (Gestational age + age of SMA symptom onset [weeks])
- Gestational age + age of SMA diagnosis (weeks)

Variables in the Final Model	P-value
Intercept	<0.0001
Day 64 log(pNF-H)	0.0001
Treatment group: nusinersen	0.0050
Disease duration	0.0003

FIG. 19

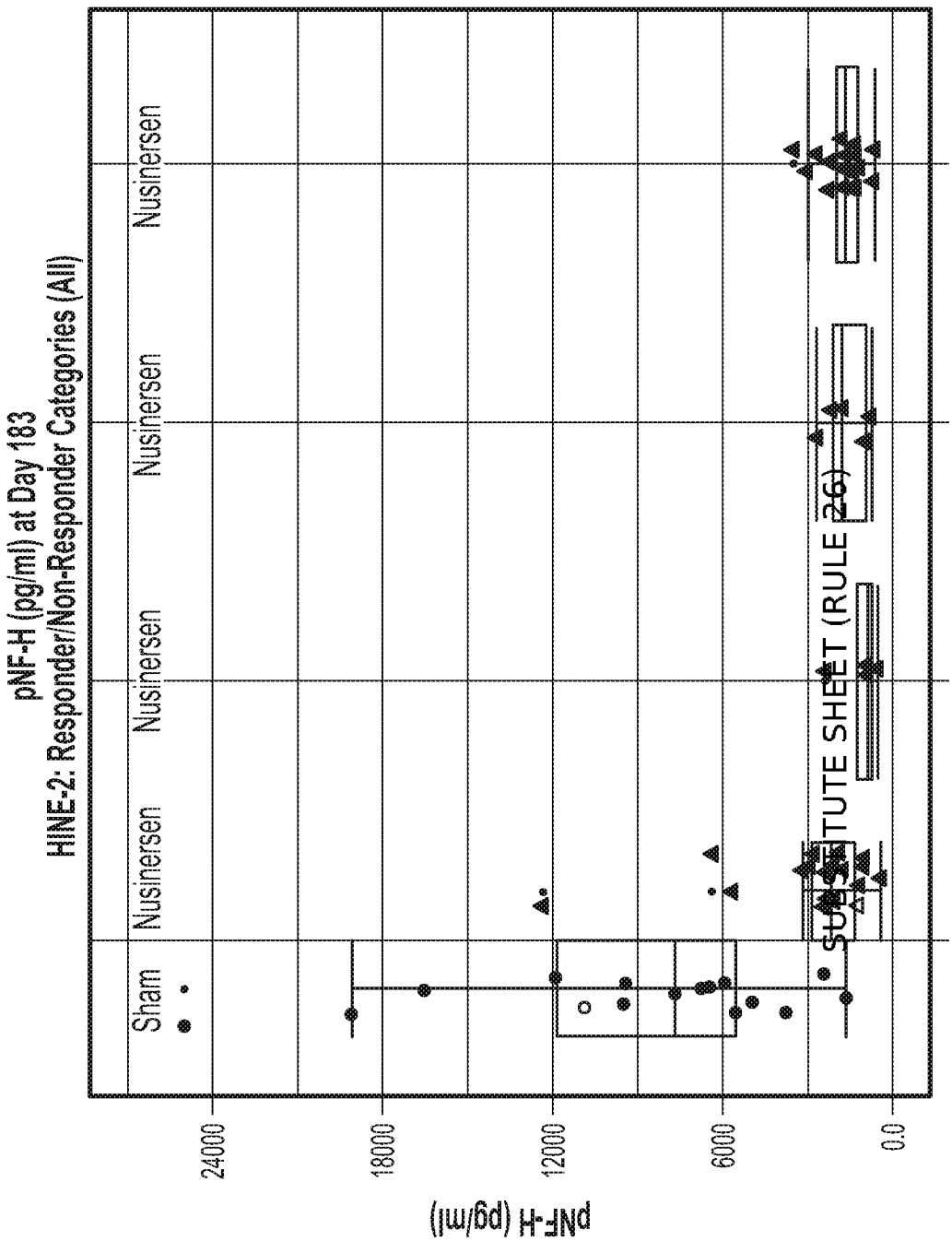

pNF-H (pg/ml) at Day 183
HINE-2: Responder/Non-Responder Categories (All)
Sham
Nusinersen
Nusinersen
Nusinersen
Nusinersen
pNF-H (pg/ml)
24000
18000
12000
6000
0.0
Non-responder at all time points
Became responder after Day 302
Became responder after Day 183 and before Day 302
Became responder before Day 183
ARM: Status ○ Sham: Death ● Sham: Alive △ Nusinersen: Death ▲ Nusinersen: Alive
SUBSTITUTE SHEET (RULE 26)

FIG. 20

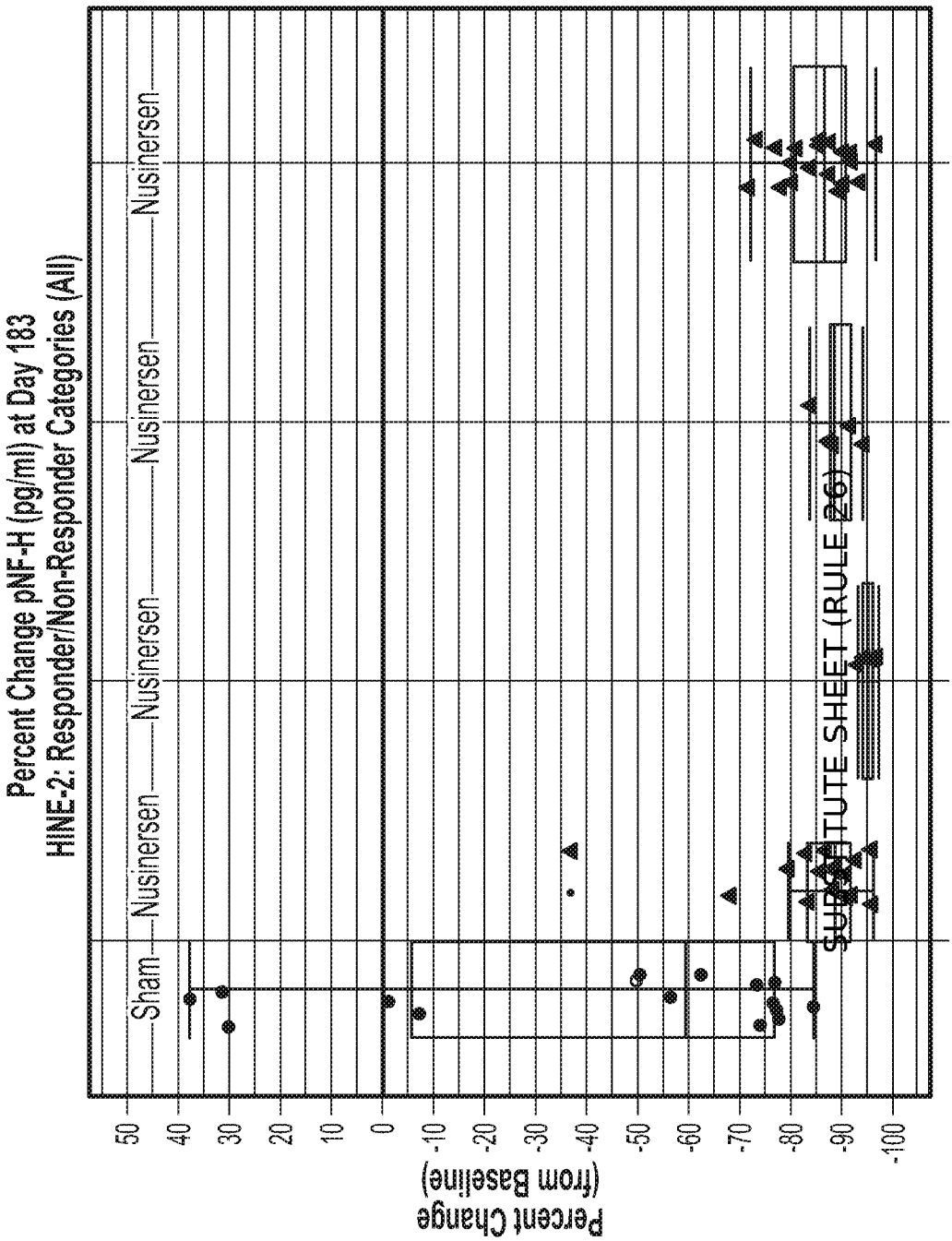

Percent Change pNF-H (pg/ml) at Day 183
HINE-2: Responder/Non-Responder Categories (All)
Sham
Nusinersen
Nusinersen
Nusinersen
Nusinersen
Percent Change (from Baseline)
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Non-responder at all time points
Became responder after Day 302
Became responder Day 183 and before Day 302
Became responder before Day 183
ARM: Status ○ Sham: Death ● Sham: Alive △ Nusinersen: Death ▲ Nusinersen: Alive
SUBSTITUTE SHEET (RULE 26)

FIG. 21

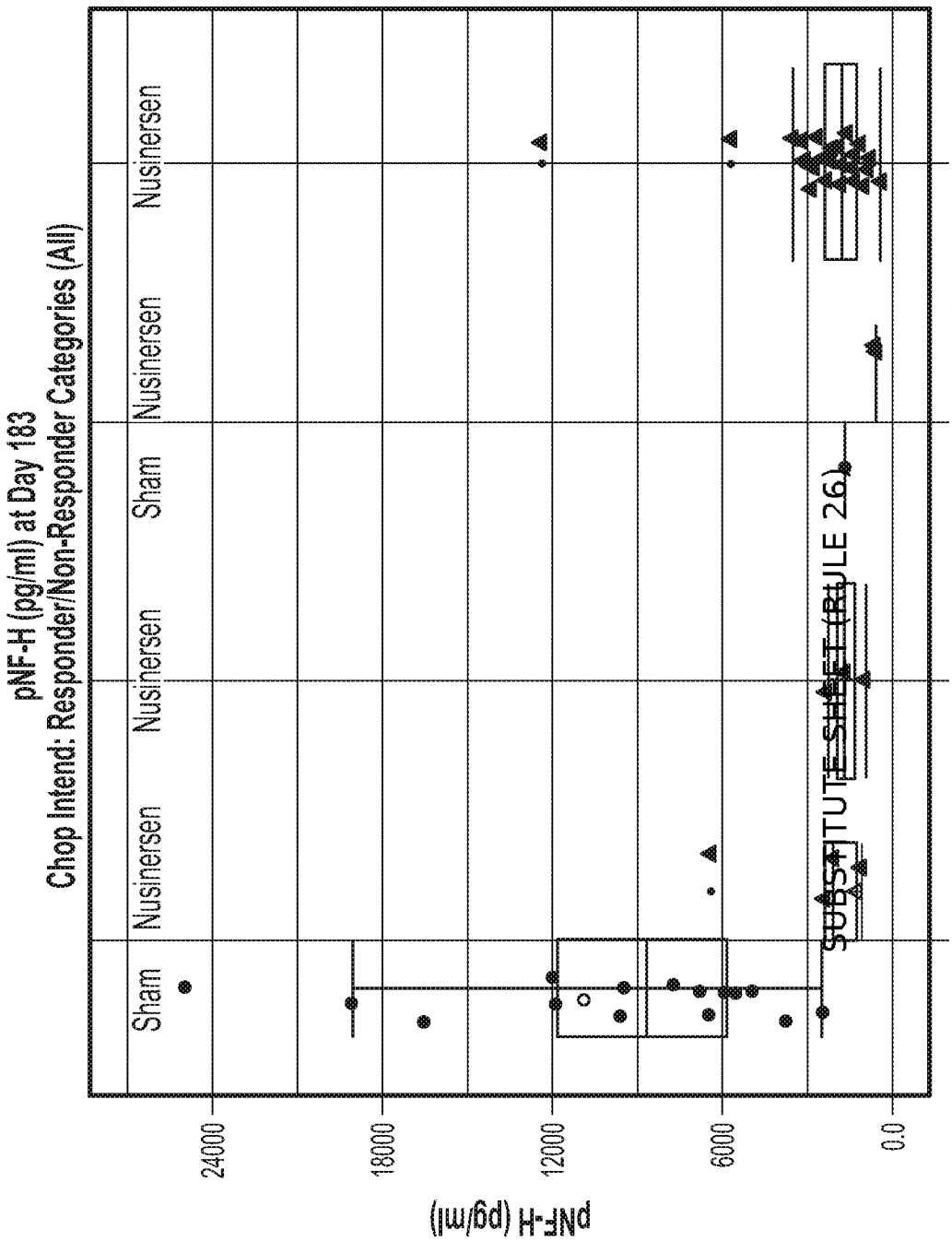

pNF-H (pg/ml) at Day 183
Chop Intend: Responder/Non-Responder Categories (All)
Sham
Nusinersen
Nusinersen
Sham
Nusinersen
Nusinersen
24000
18000
12000
6000
0.0
pNF-H (pg/ml)
Non-responder at all time points
Became responder after Day 302
Became responder after Day 183 and before Day 302
Became responder before Day 183
ARM: Status ○ Sham: Death ● Sham: Alive △ Nusinersen: Death ▲ Nusinersen: Alive
SUBSTITUTE SHEET (RULE 26)

FIG. 22

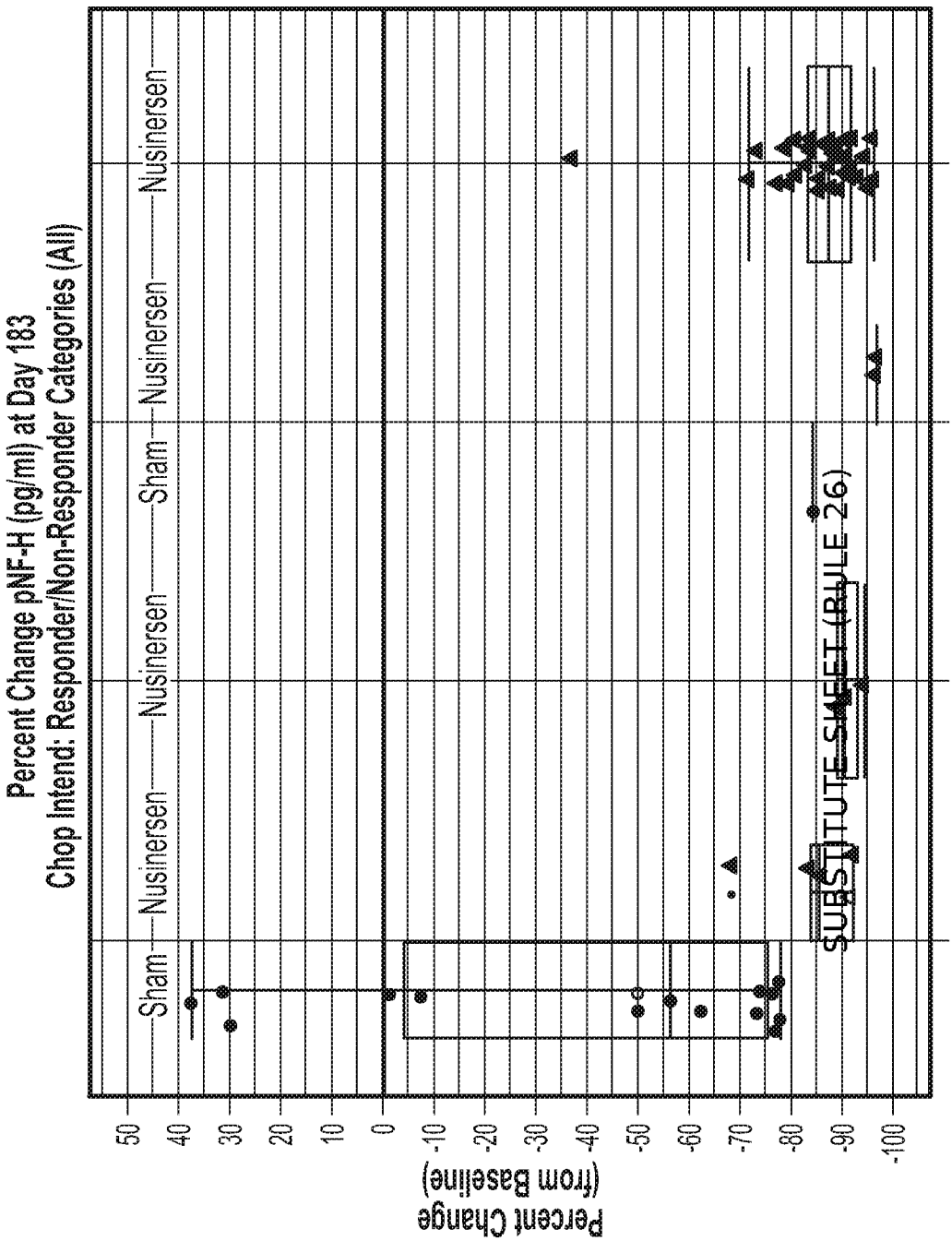

Percent Change pNF-H (pg/ml) at Day 183
Chop Intend: Responder/Non-Responder Categories (All)
Sham
Nusinersen
Nusinersen
Sham
Nusinersen
Nusinersen
Percent Change (from Baseline)
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
Non-responder at all time points
Became responder after Day 302
Became responder Day 183 and before Day 302
Became responder before Day 183
ARM: Status ○ Sham: Death ● Sham: Alive △ Nusinersen: Death ▲ Nusinersen: Alive
SUBSTITUTE SHEET (RULE 26)

FIG. 23

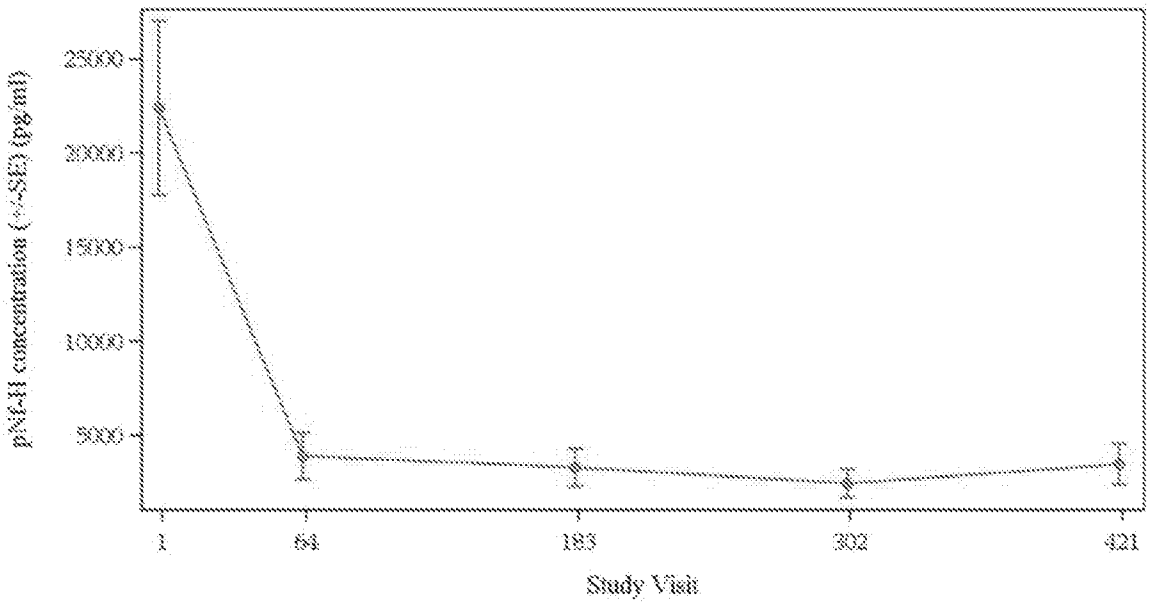


FIG. 24

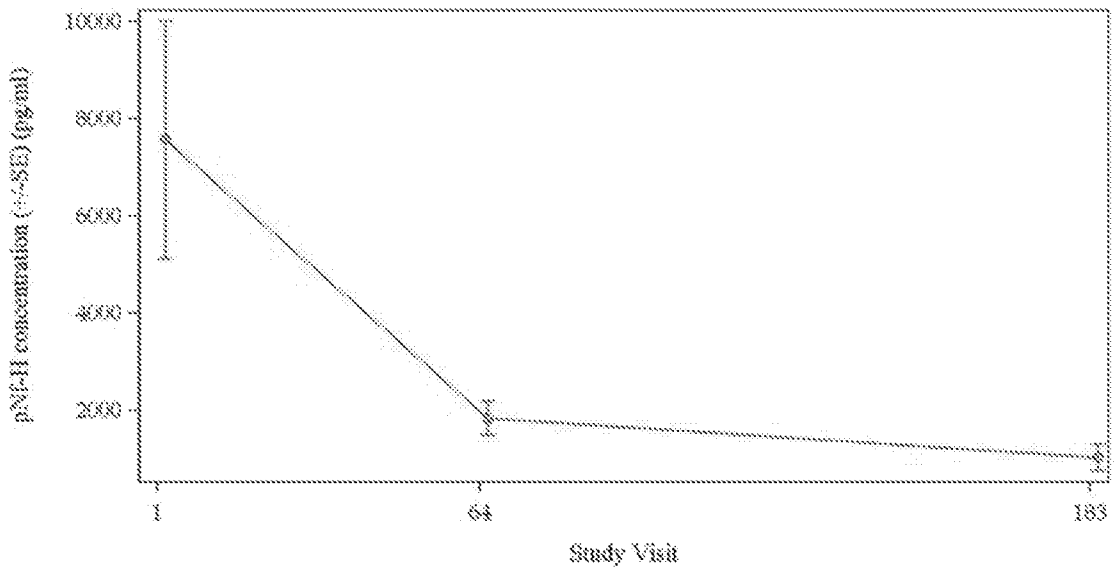

10000
8000
6000
4000
2000
pNGAL concentration (+/-SE) (pg/ml)
1
64
183
Study Visit

FIG. 25

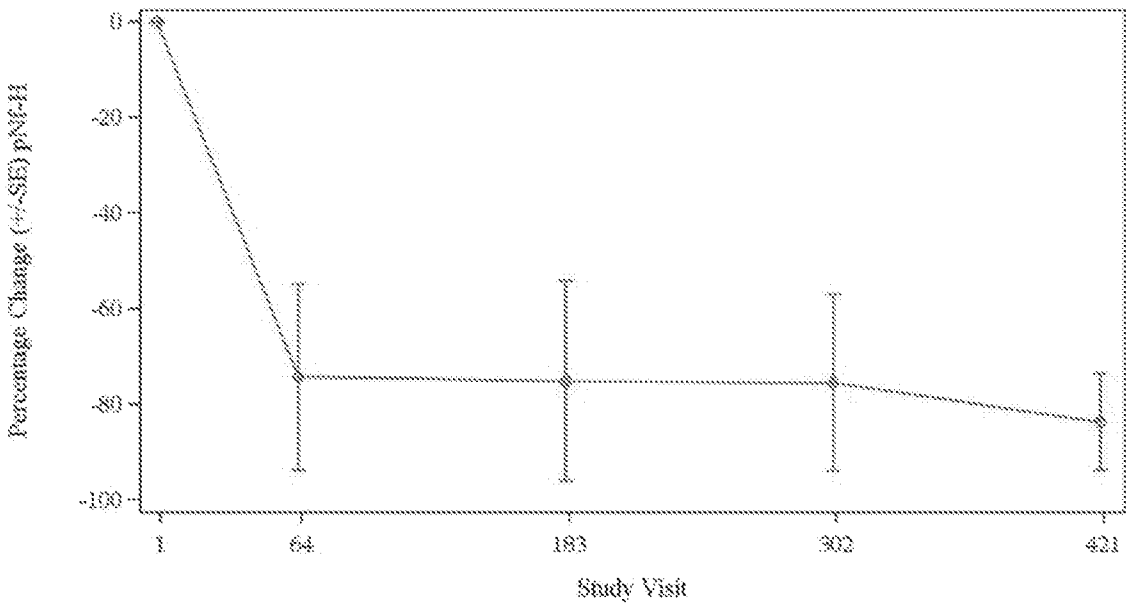

Percentage Change (+/-SD) pNF-H
0
-20
-40
-60
-80
-100
1
64
183
302
421
Study Visit

FIG. 26

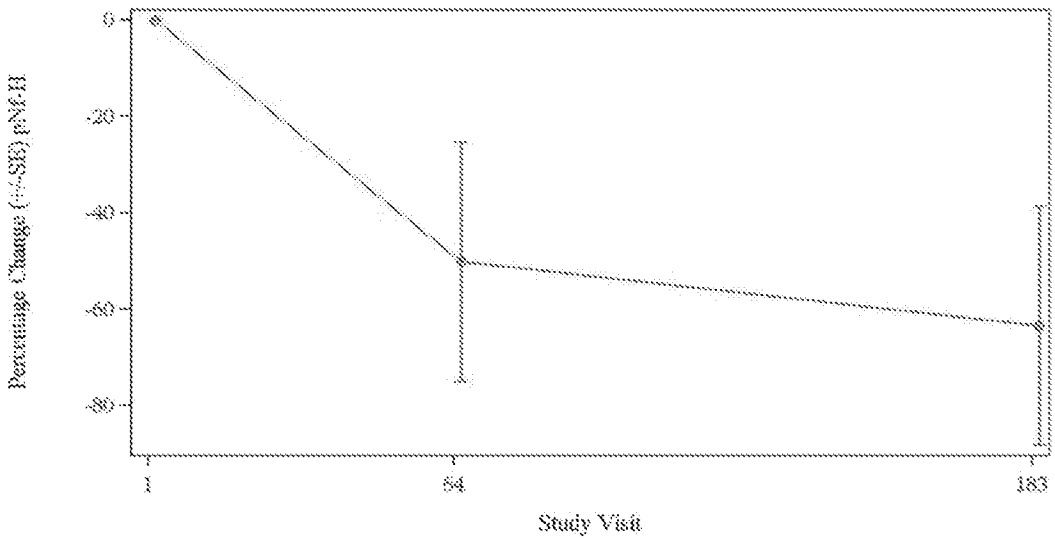
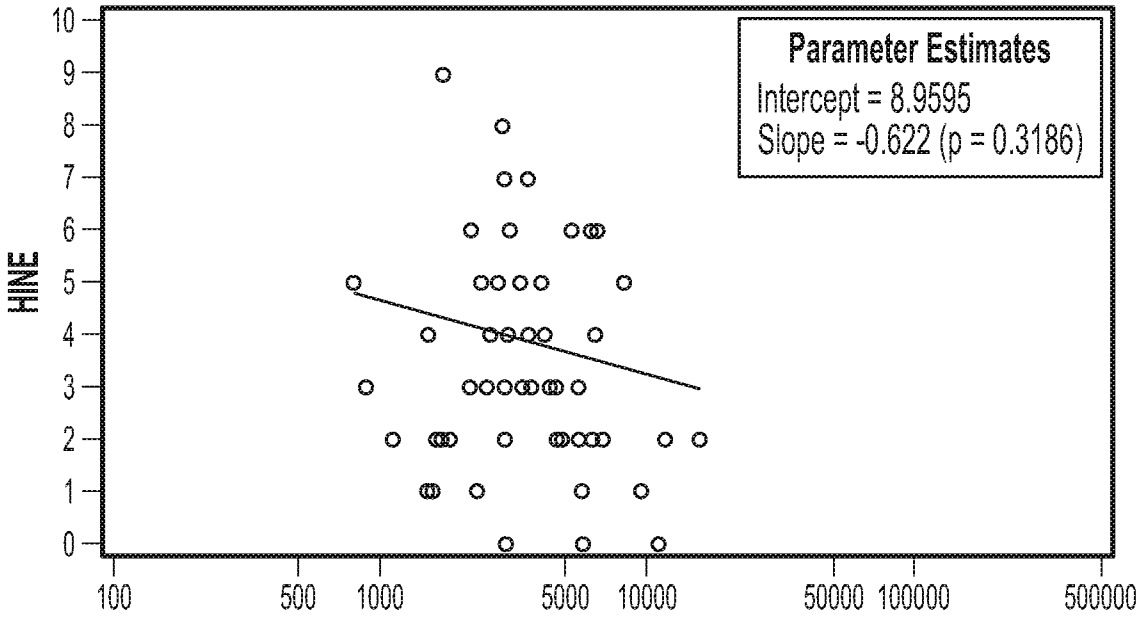
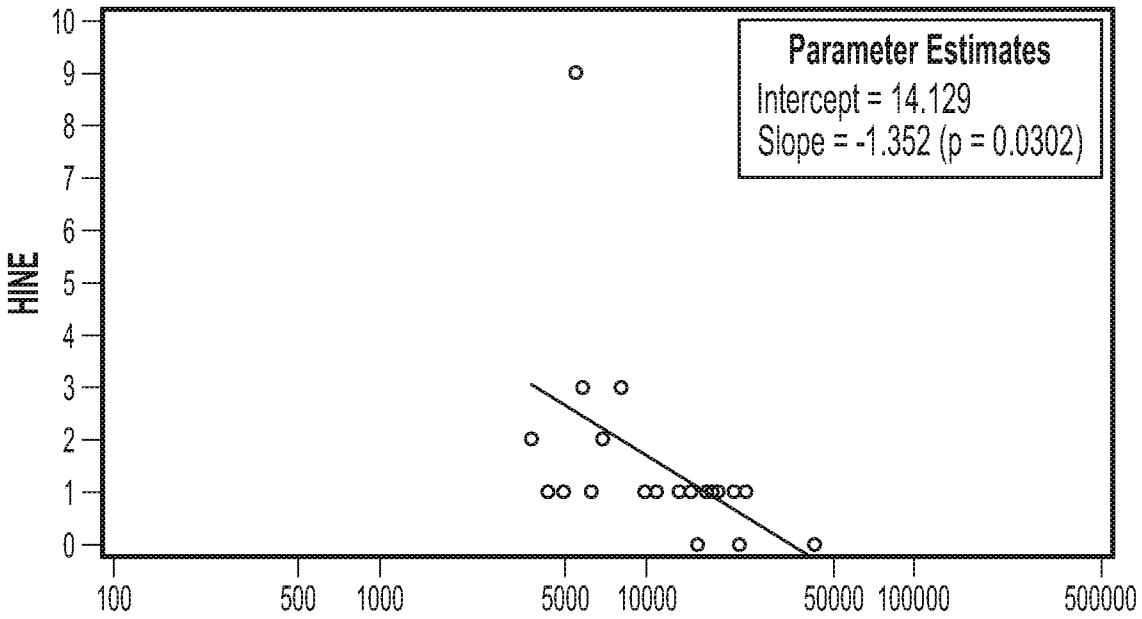


FIG. 27

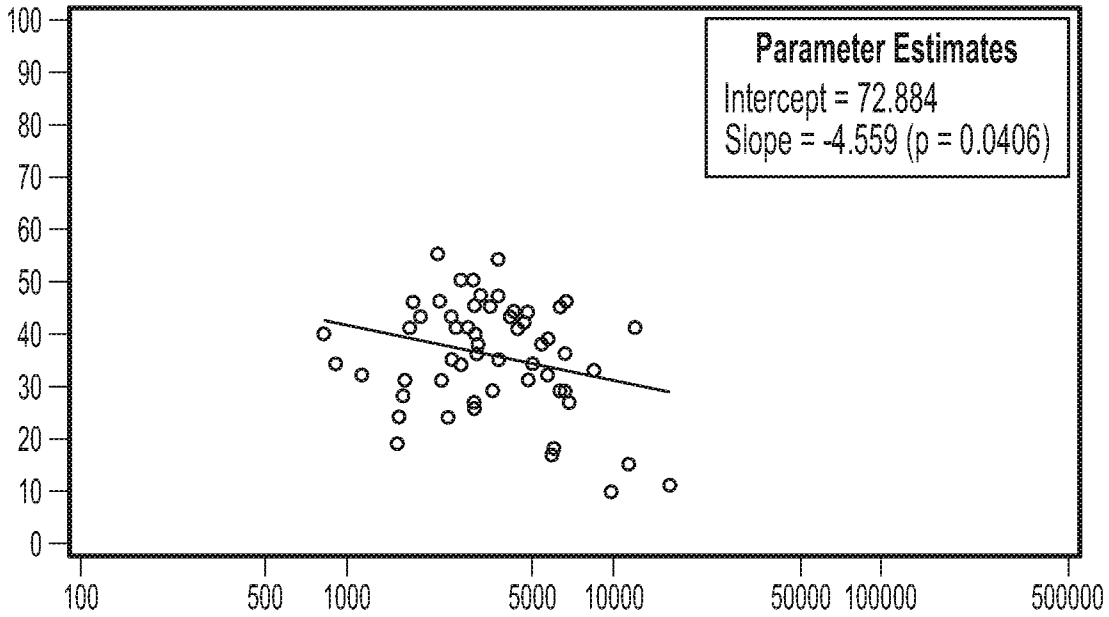


Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	8.9595	5.0481	1.7748	0.0816
Day 64 log(pNF-H)	1	-0.622	0.6179	-1.007	0.3186

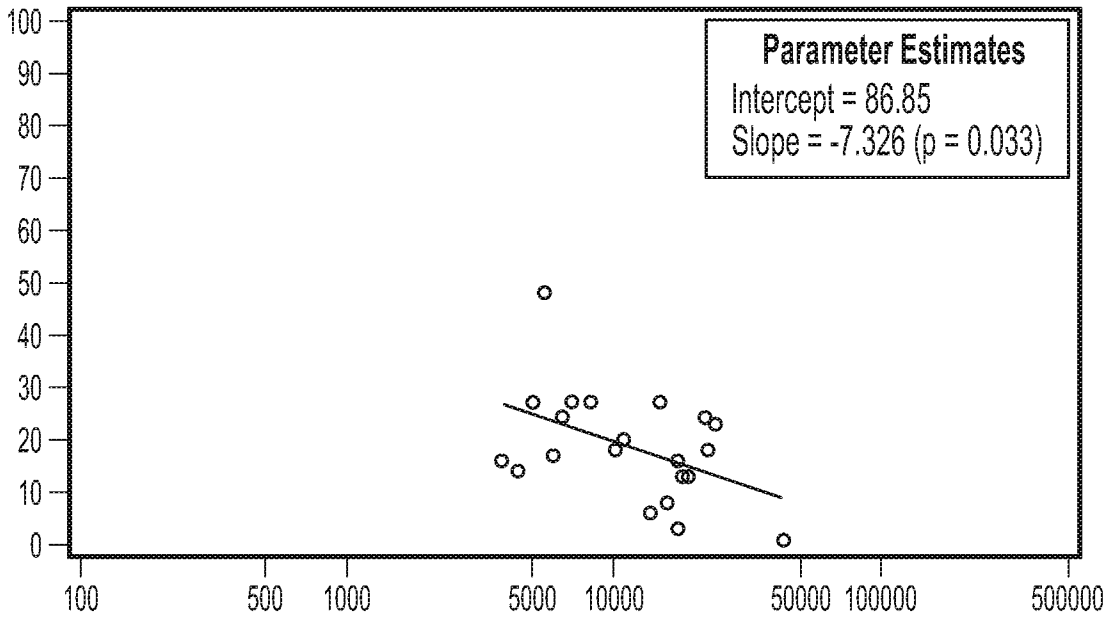


Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	14.129	5.3958	2.6186	0.0169
Day 64 log(pNF-H)	1	-1.352	0.5775	-2.342	0.0302

FIG. 28



Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	72.884	17.75	4.1061	0.0001
Day 64 log(pNF-H)	1	-4.559	2.1727	-2.098	0.0406



Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	86.85	29.772	2.9172	0.0088
Day 64 log(pNF-H)	1	-7.326	3.1866	-2.299	0.0330

FIG. 29

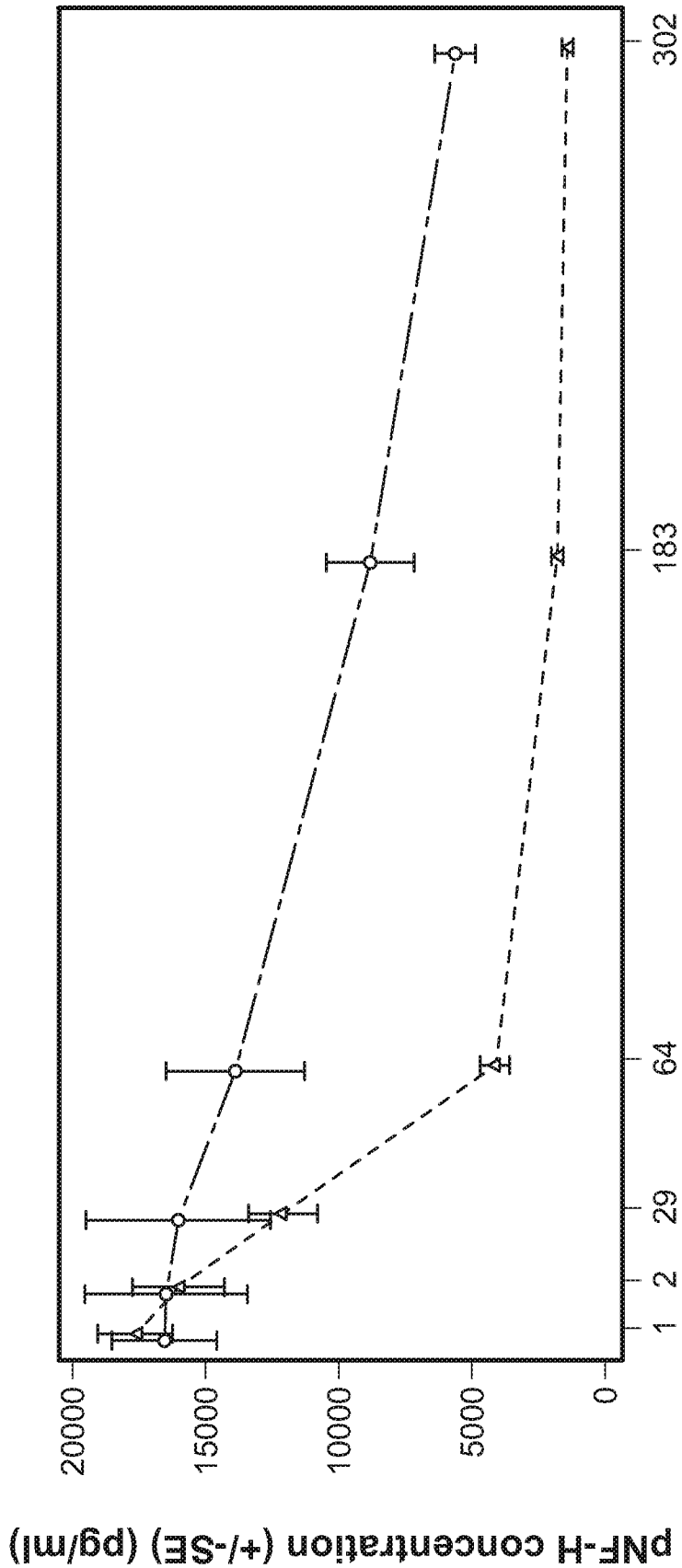

pNF-H concentration (+/-SE) (pg/ml)
20000
15000
10000
5000
0
1
2
29
64
183
302
Study Visit
Treatment: Sham procedure Nusinersen
Number of Subjects
Sham procedure 15 15 15 14 14 15
Nusinersen 30 30 29 30 30 30

FIG. 30

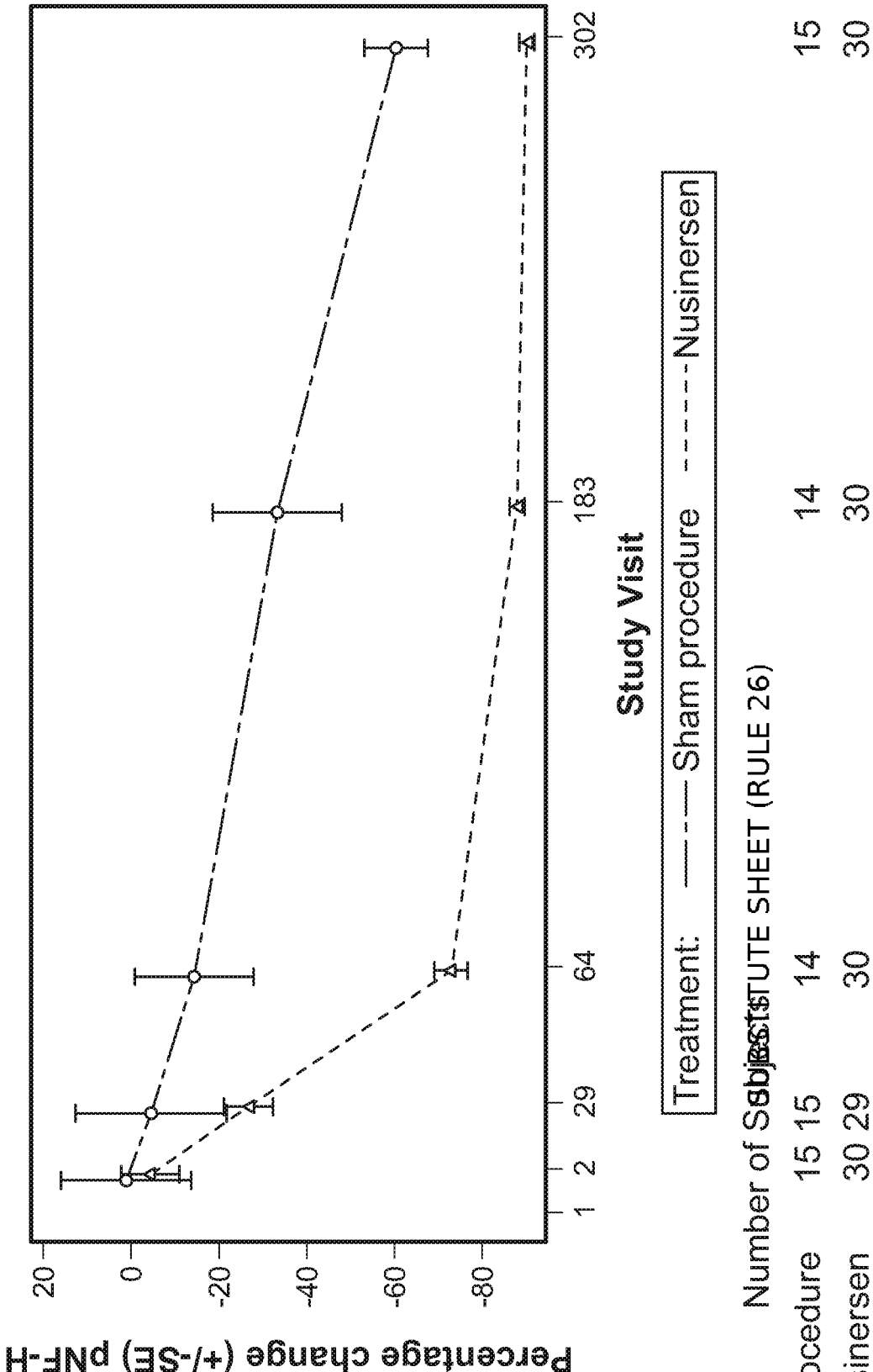
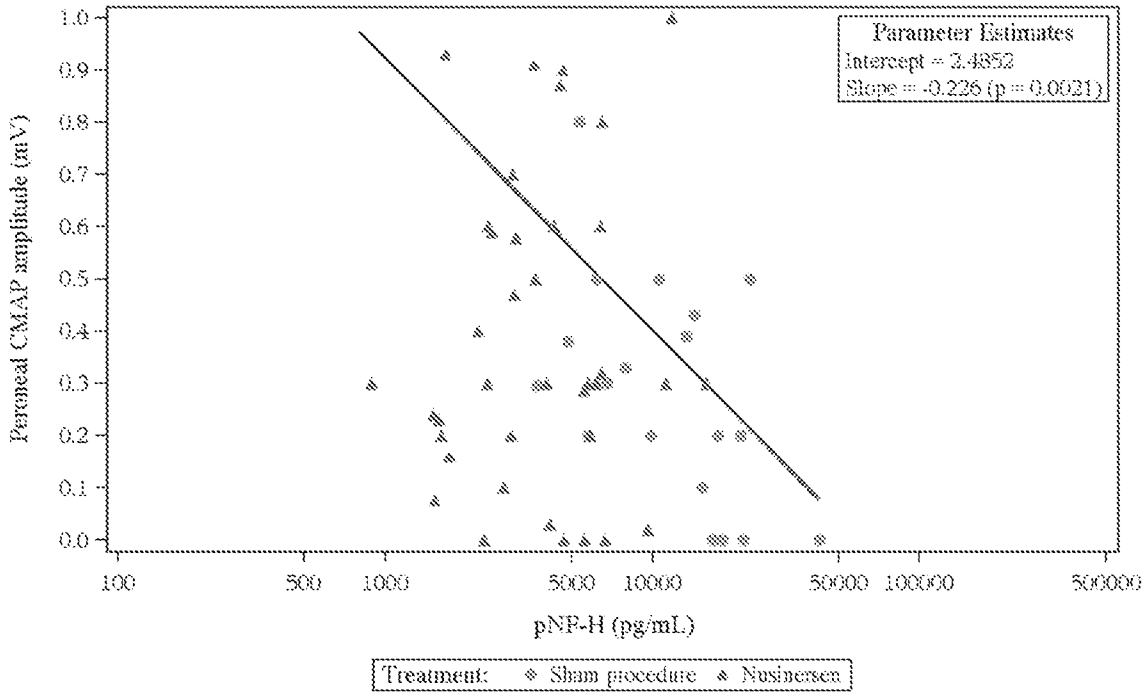

Percentage change (+/-SE) pNF-H
20
0
-20
-40
-60
-80
1
2
29
64
183
302
Study Visit
Treatment: Sham procedure Nusinersen
Number of Subjects
Sham procedure 15 15 14 14 15
Nusinersen 30 29 30 30 30

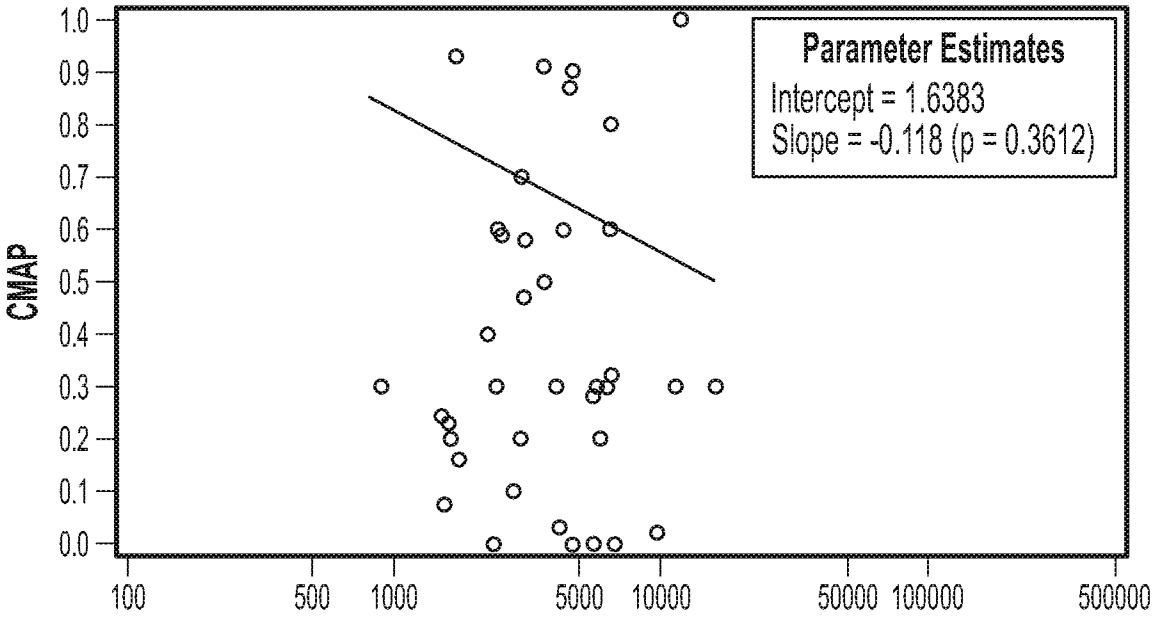
FIG. 31



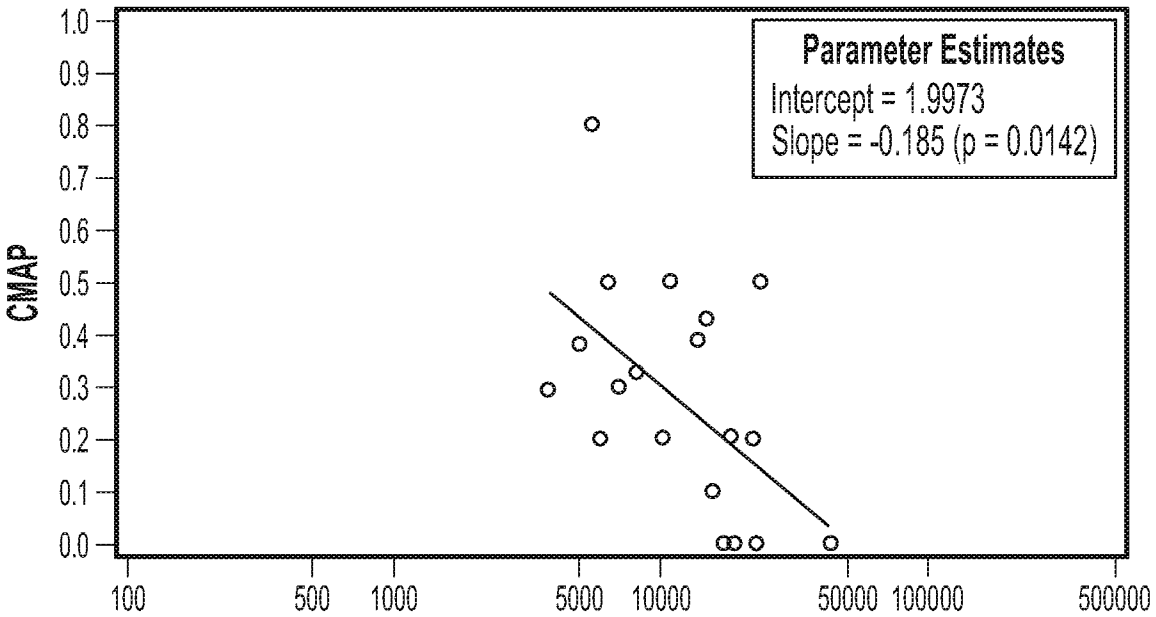
Variable	DF	Parameter Estimate	Standard Error	t Value	pr > |t|
Intercept	1	2.4852	0.6046	4.1104	0.0001*
Day 64 log(pNP-H)	1	-0.226	0.0709	-3.189	0.0021*

Root MSE: 0.49489 R-square: 0.1253 Adjusted R-square: 0.113

FIG. 32

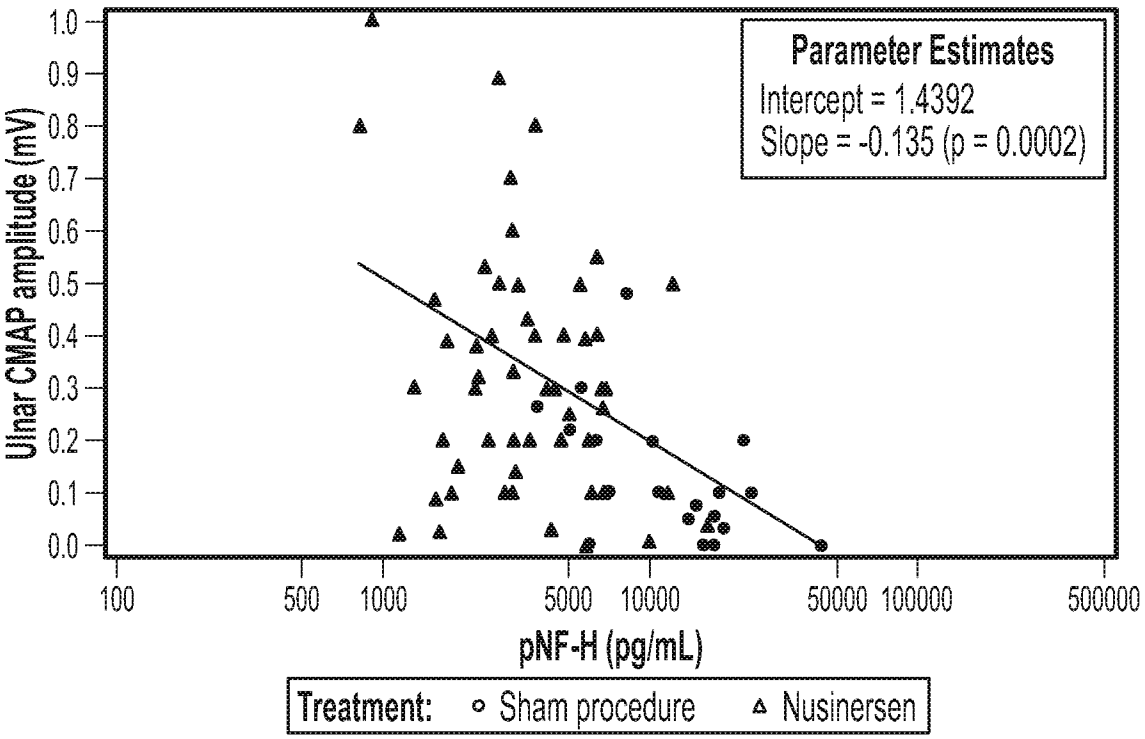


Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	1.6383	1.0438	1.5696	0.1227
Day 64 log(pNF-H)	1	-0.118	0.1277	-0.921	0.3612



Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	1.9973	0.6388	3.1265	0.0058
Day 64 log(pNF-H)	1	-0.185	0.068	-2.716	0.0142

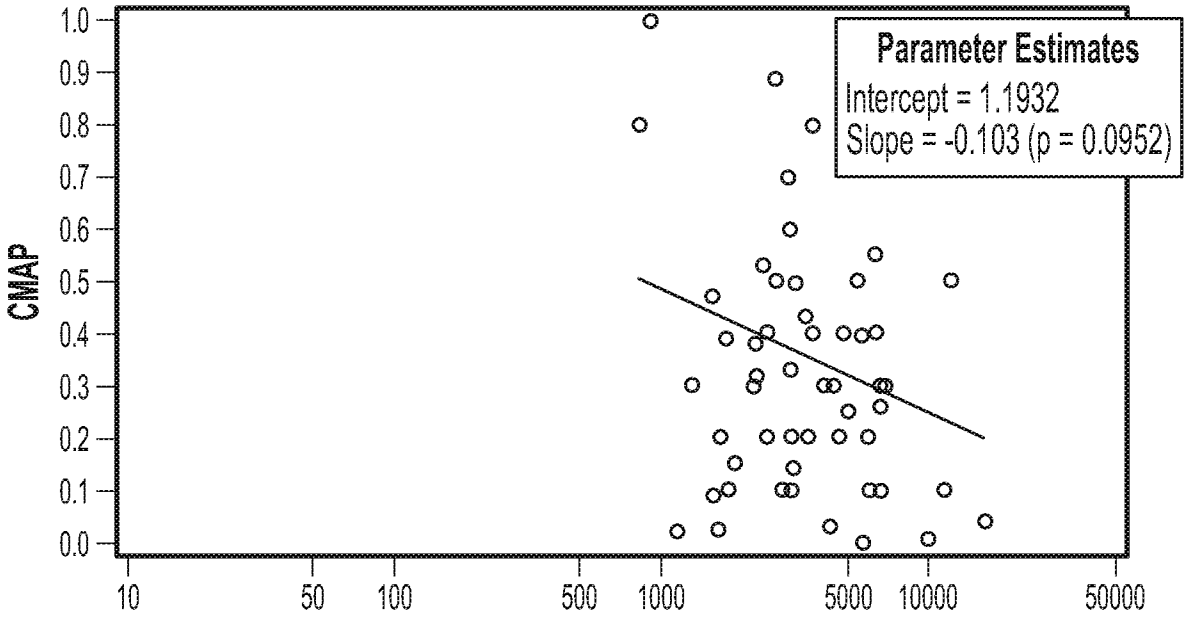
FIG. 33



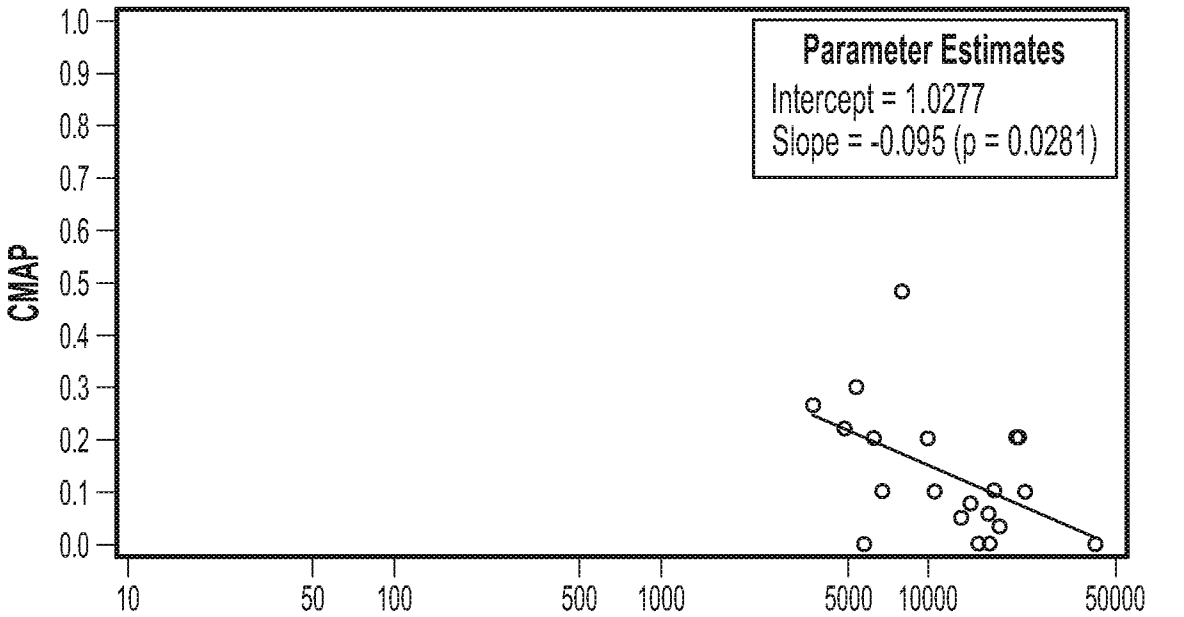
Variable	DF	Parameter Estimate	Standard Error	t Value	pr > ltl
Intercept	1	1.4392	0.2918	4.9325	<.0001*
Day 64 log(pNF-H)	1	-0.135	0.0344	-3.93	0.0002*

Root MSE: 0.24904 R-square: 0.1746 Adjusted R-square: 0.1633

FIG. 34



Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	1.1932	0.4932	2.4193	0.0190
Day 64 log(pNF-H)	1	-0.103	0.0606	-1.699	0.0952



Variable	DF	Parameter Estimate	Standard Error	t value	pr > |t|
Intercept	1	1.0277	0.3751	2.7396	0.0135
Day 64 log(pNF-H)	1	-0.095	0.04	-2.388	0.0281

FIG. 35

Day 29
AUC = 60.1%

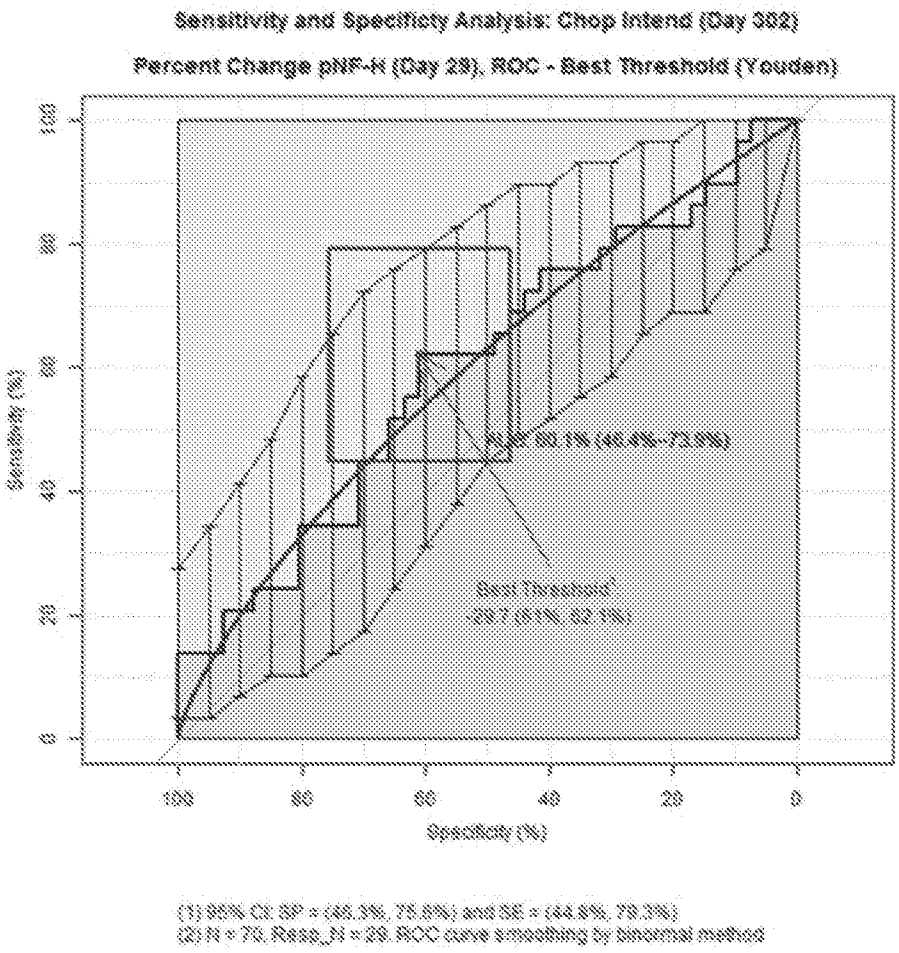


FIG. 36A

Day 64
AUC = 87.5%

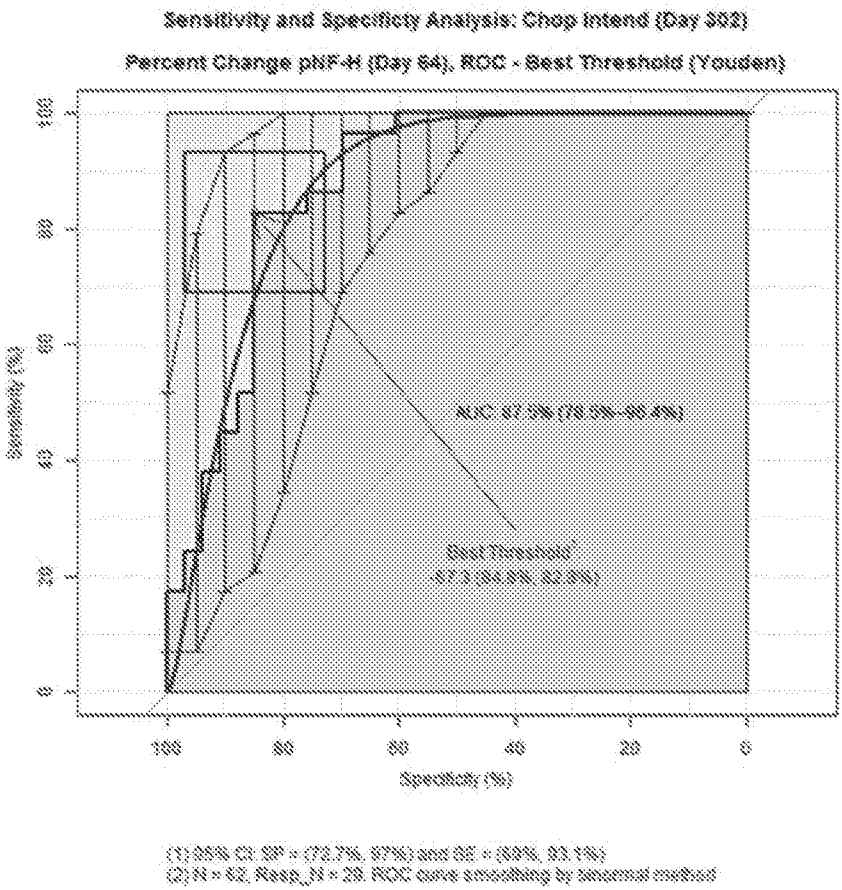


FIG. 36B

Day 183
AUC = 81.5%

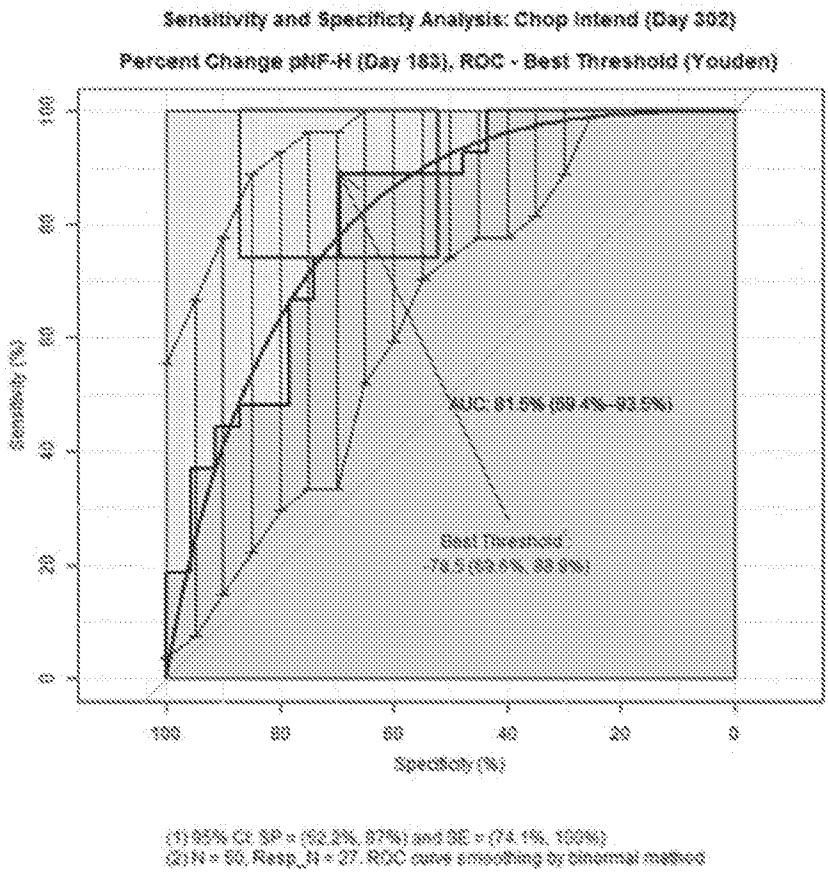
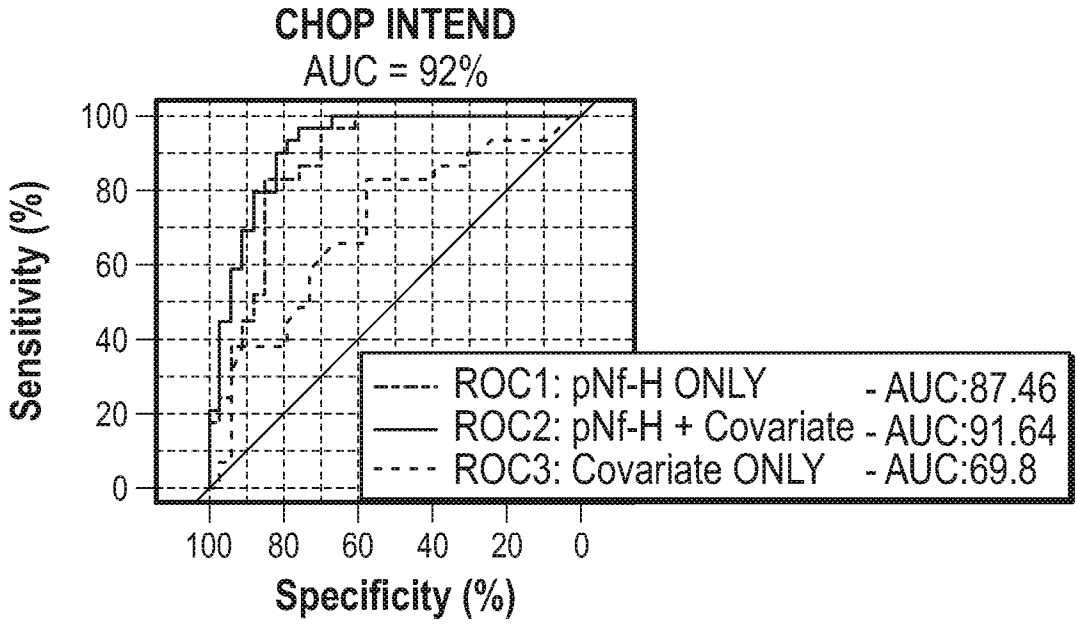
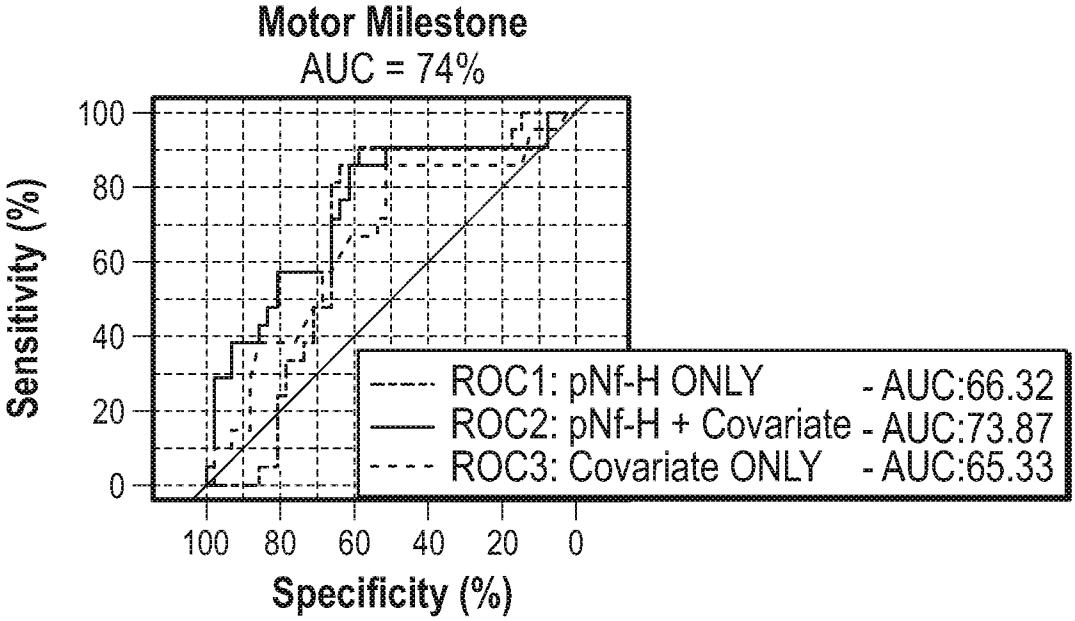


FIG. 36C



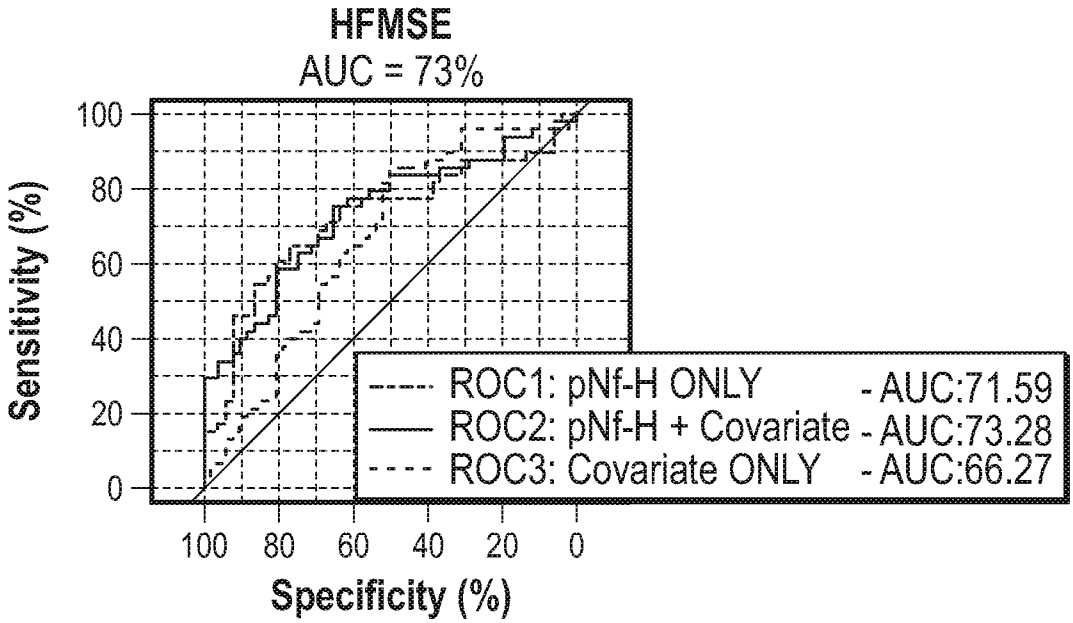
Notes: (1) Significance, ROC1 vs ROC2: p-value = 0.15124
(2) Significance, ROC3 vs ROC2: p-value = 0.00096
(3) N = 62; Resp_N = 29

FIG. 37A



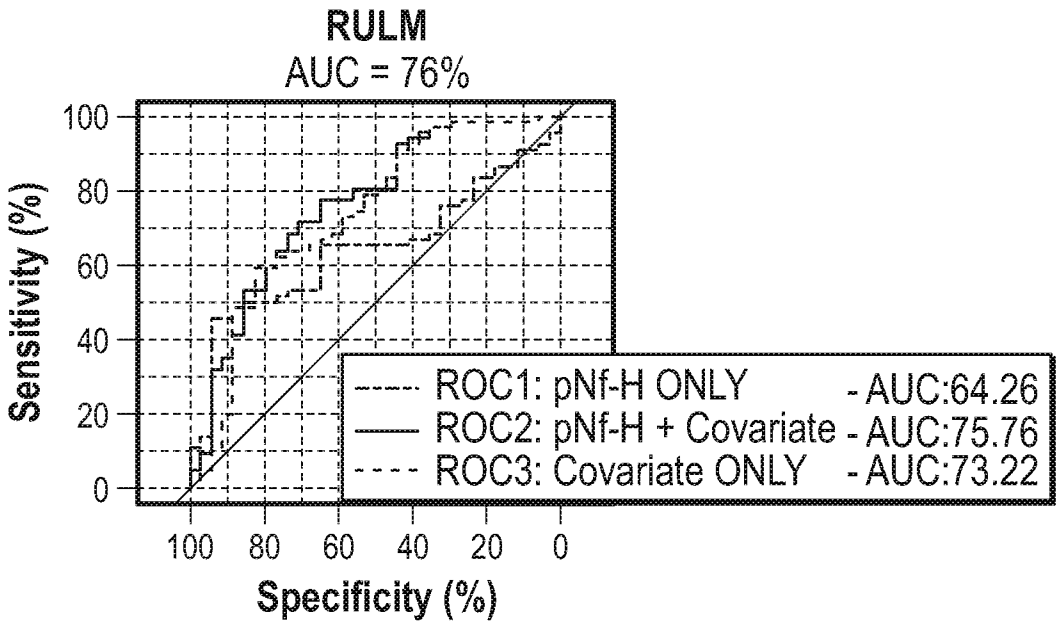
Notes: (1) Significance, ROC1 vs ROC2: p-value = 0.20867
(2) Significance, ROC3 vs ROC2: p-value = 0.05903
(3) N = 62; Resp_N = 21

FIG. 37B



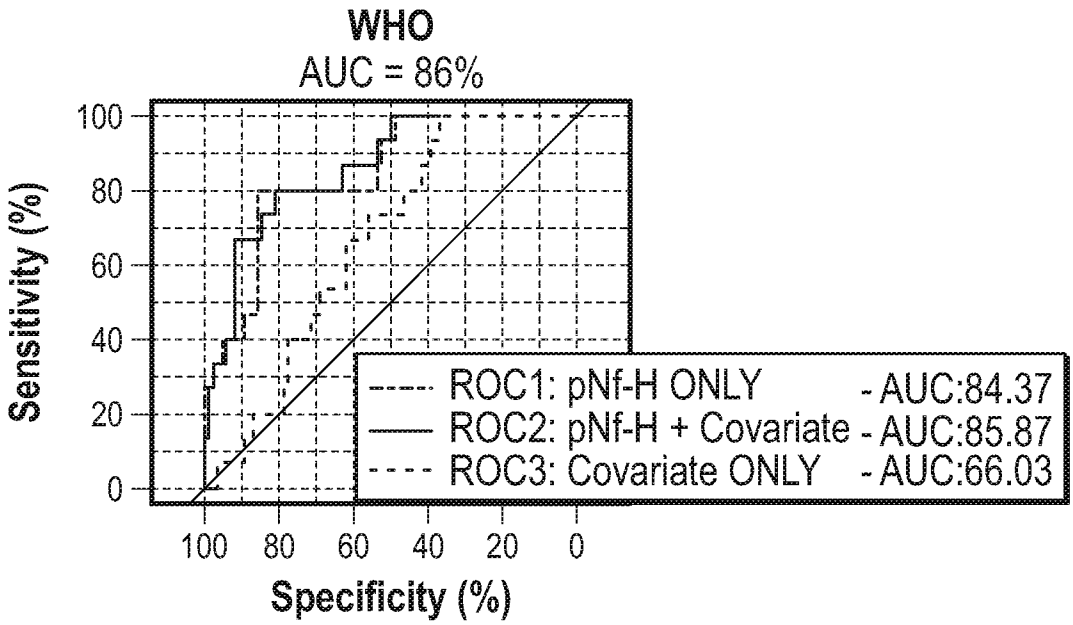
Notes: (1) Significance, ROC1 vs ROC2: p-value = 0.68253
(2) Significance, ROC3 vs ROC2: p-value = 0.09021
(3) N = 100; Resp_N = 48

FIG. 38A



Notes: (1) Significance, ROC1 vs ROC2: p-value = 0.09997
(2) Significance, ROC3 vs ROC2: p-value = 0.08413
(3) N = 100; Resp_N = 66

FIG. 38B



Notes: (1) Significance, ROC1 vs ROC2: p-value = 0.30269
(2) Significance, ROC3 vs ROC2: p-value = 0.00032
(3) N = 99; Resp_N = 15

FIG. 38C

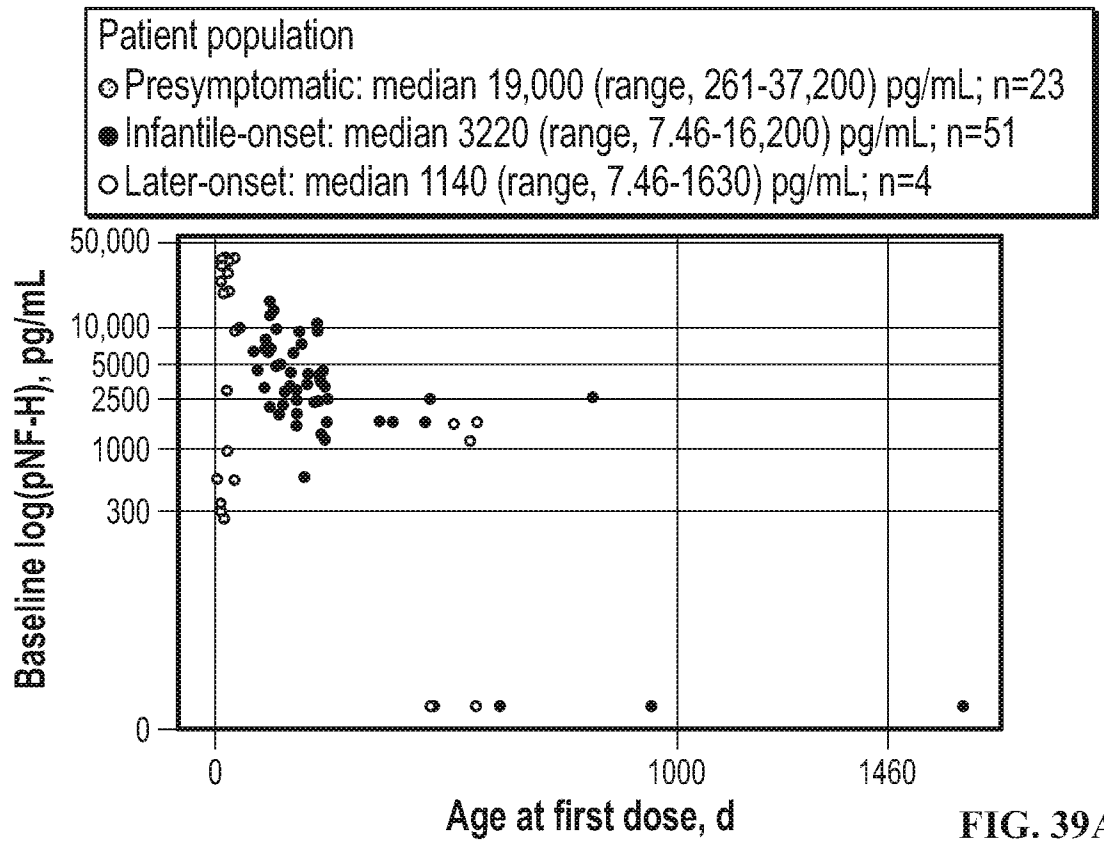


FIG. 39A

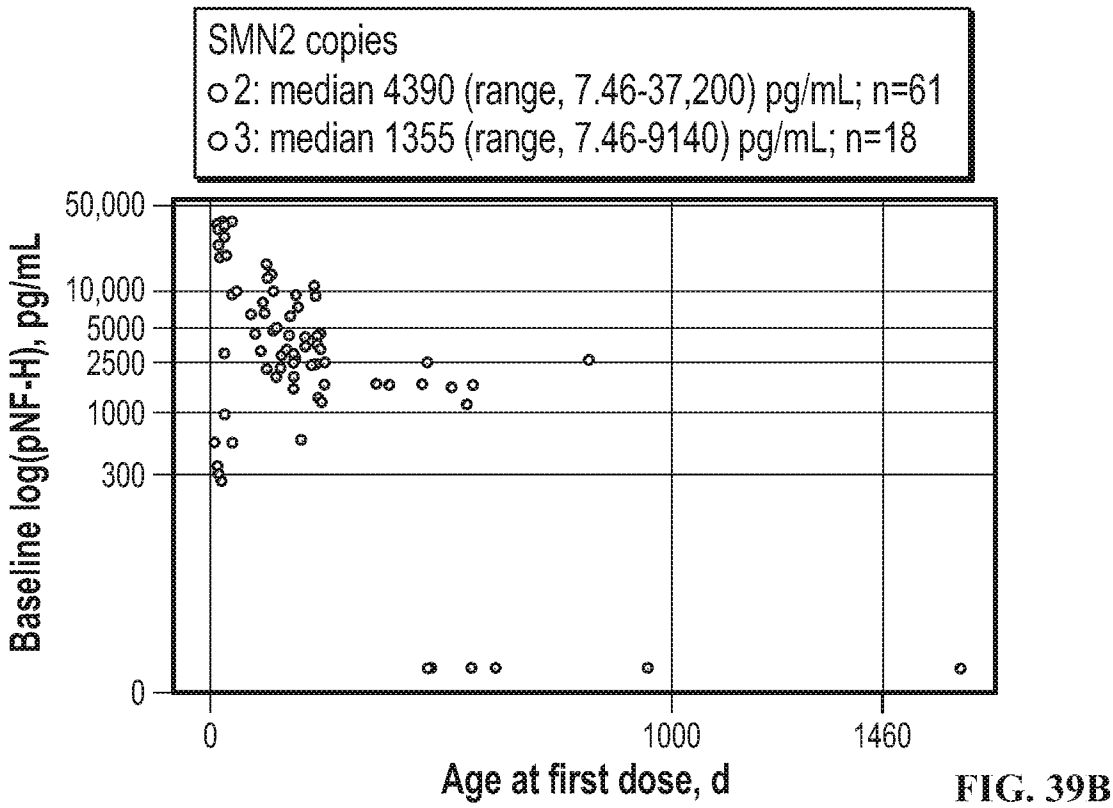


FIG. 39B

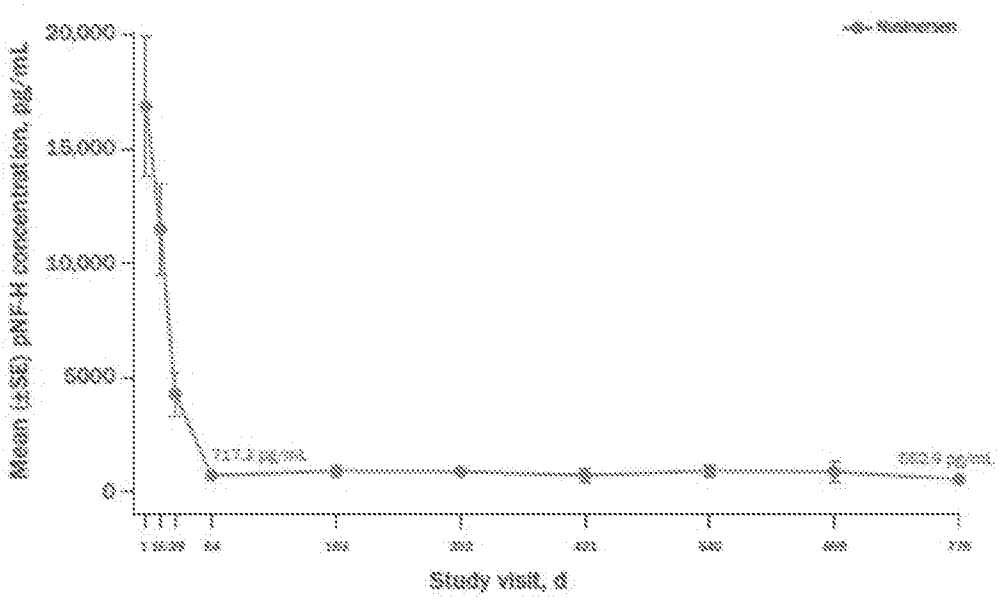


FIG. 40

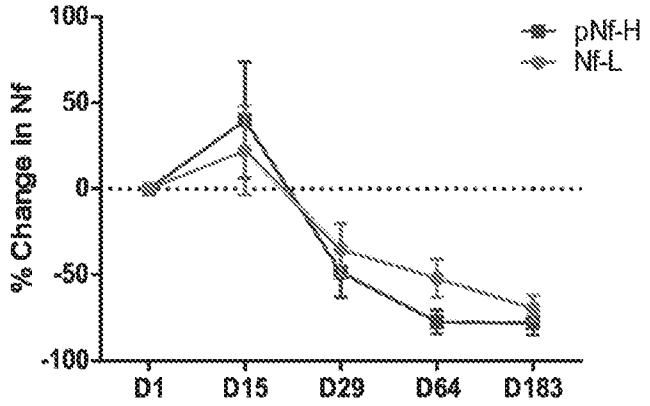


FIG. 41A

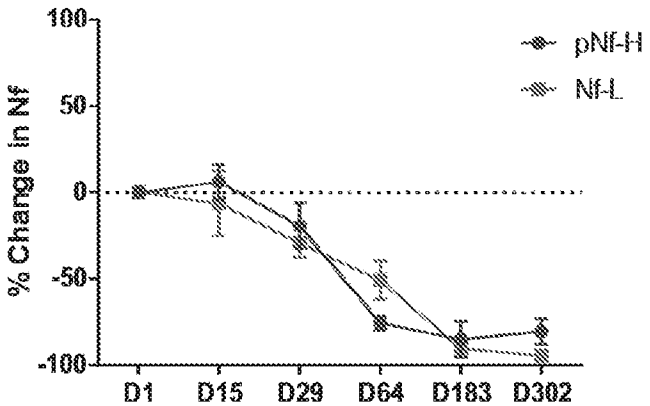


FIG. 41B

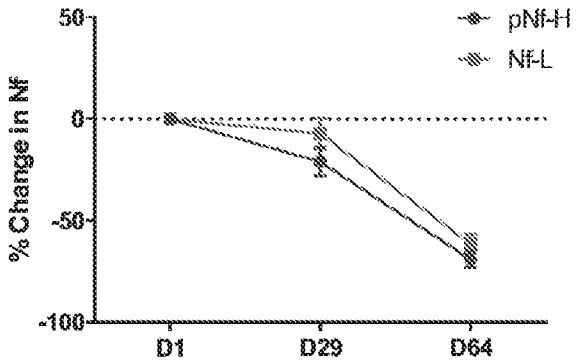


FIG. 41C

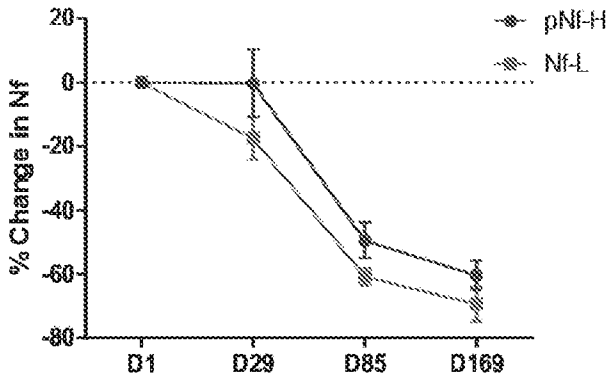


FIG. 41D

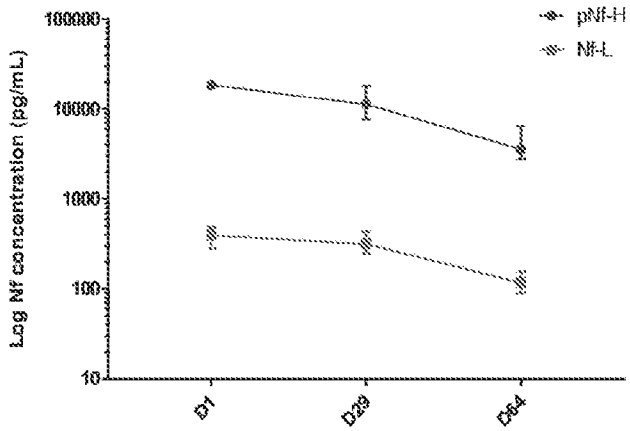


FIG. 41E

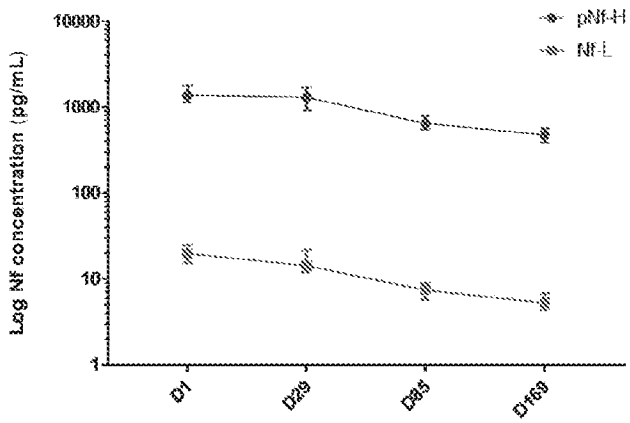


FIG. 41F

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/015185

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/087212 A1 (PASSINI MARCO A [US] ET AL) 30 March 2017 (2017-03-30) par 26, 68, 195, claims 1-19;	1-46
X	US 2010/267073 A1 (PRZEDBORSKI SERGE [US] ET AL) 21 October 2010 (2010-10-21) par 10, 34, 35, 53-57, claims 1-18;	1-46
X	US 2015/285822 A1 (ZHANG SU-CHUN [US] ET AL) 8 October 2015 (2015-10-08)	41-46
Y	claims;	1-40
Y	US 2017/363643 A1 (RIGO FRANK [US] ET AL) 21 December 2017 (2017-12-21) claims	1-40

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2019	10/04/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilch, Bartosz

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/015185

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