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[Continued on next page]

(54) Title: METHODS FOR TREATING DIAGNOSING, AND MONITORING TREATMENT OF MUCOPOLYSACCHARIDOSIS

(57) Abstract: Provided herein are methods for diagnosing and treating mucopolysaccharidosis using spermine concentrations. Methods of treatment include use of specific poly amine inhibitors.

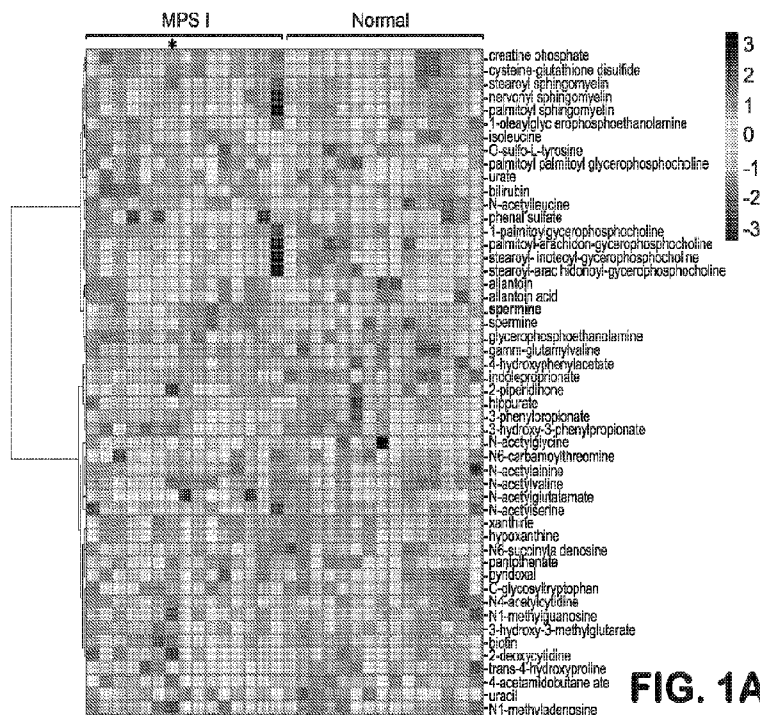


FIG. 1A



TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,
VN, ZA, ZM, ZW.

LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE,
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METHODS FOR TREATING, DIAGNOSING, AND MONITORING TREATMENT OF MUCOPOLYSACCHARIDOSES

5 STATEMENT OF FEDERALLY FUNDED RESEARCH

This application includes work which was supported in part by grant from the National Institutes of Health (NIH) number P30ES013508. The US government may have certain rights in this invention.

10 BACKGROUND OF THE INVENTION

The mucopolysaccharidoses (MPSs) are a family of genetic disorders caused by deficiency of enzymes required for lysosomal degradation of glycosaminoglycans (GAGs), leading to accumulation of GAGs in tissues and debilitating somatic disease. In a subset of these disorders that affect metabolism of the ubiquitous GAG heparan sulfate, patients also develop severe cognitive impairment. The etiology of cognitive dysfunction is not known, although MPS neurons have been shown to exhibit aberrant overgrowth of neurites and upregulation of GAP43, a key regulator of neurite growth, which may lead to defects in neural development and plasticity.

20 Mucopolysaccharidosis type I (MPS I, Hurler syndrome) is a recessive disorder caused by mutations in the gene encoding α -L-iduronidase (IDUA), a lysosomal enzyme responsible for the breakdown of complex polysaccharides called glycosaminoglycans (GAGs). MPS I is characterized by systemic accumulation of heparan and dermatan sulfate, two GAGs that are normally degraded by IDUA. MPS I patients present with a constellation of clinical findings, many of which are directly linked to mechanical consequences of GAG storage, such as cardiac valve thickening, liver and spleen enlargement, corneal clouding, and airway infiltration E. F. Neufeld, J. Muenzer, in The Online Metabolic and Molecular Bases of Inherited Disease, A. L. Beaudet, et al., Eds. (The McGraw-Hill Companies, Inc., New York, NY, 2014). J. These sequelae are common to many closely related lysosomal storage diseases—including MPS types II, III, IV, VI and VII—which are likewise caused by deficiency of lysosomal enzymes involved in GAG catabolism [(Neufeld, cited above)]. While there are clear phenotypic similarities among MPSs, not all are characterized by cognitive dysfunction; in MPS types I, II, III

and VII, patients exhibit a marked decline in cognitive development at 1-2 years of age [J. B. Holt, et al, , Natural Progression of Neurological Disease in Mucopolysaccharidosis Type II. *Pediatrics* 127, e1258-e1265 (2011); J. E. Wraith, et al,). *Pediatrics* 120, e37-46 (2007); published online EpubJul (10.1542/peds.2006-2156)]. In contrast, MPS types IV and VI show no cognitive involvement despite similar systemic signs of GAG storage [Neufeld, cited above]. This difference in phenotype correlates with heparan sulfate (HS) storage; all MPS types that involve accumulation of HS exhibit cognitive dysfunction, whereas MPS IV and VI, which are instead characterized by defects in keratan and dermatan sulfate catabolism, do not.

10 A mechanistic link between HS accumulation and cognitive impairment has not been established. Gangliosides, complex lipids involved in signal transduction, accumulate in neurons of MPS I patients and have been hypothesized to contribute to the neurological phenotype, given that disorders of ganglioside catabolism (e.g. Tay-Sachs and Sandhoff disease) have severe neurological manifestations [S. U. Walkley, M. T. Vanier, Secondary lipid accumulation in lysosomal disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1793, 726-736 (2009); published online Epub4// (<http://dx.doi.org/10.1016/j.bbamcr.2008.11.014>)]. However, ganglioside accumulation in neurons occurs to a similar degree in MPS VI, a disease without cognitive impairment, indicating that elevated brain gangliosides are unlikely to be related to CNS pathogenesis in MPS I, II, III, and VII [S. U. Walkley et al, Abnormal neuronal metabolism and storage in mucopolysaccharidosis type VI (Maroteaux-Lamy) disease. *Neuropathology and applied neurobiology* 31, 536-544 (2005); published online EpubOct (10.1111/j.1365-2990.2005.00675.x)]. Inflammation caused by HS-mediated activation of toll like receptors has also been hypothesized to play a role in MPS pathogenesis, though MPS III mice lacking toll like receptor 4 do not show improvements in disease progression despite attenuated activation of microglia, suggesting that inflammation does not drive CNS disease [J. Ausseil, et al, Early neurodegeneration progresses independently of microglial activation by heparan sulfate in the brain of mucopolysaccharidosis IIIB mice. *PLoS One* 3, e2296 (2008)].

30 Some evidence suggests that HS accumulation causes dysregulation of neurite growth. Murine models of MPS III show progressive overexpression of GAP43 in the brain, and cultured neurons from MPS mice likewise overexpress GAP43 and elaborate

longer neurites with more complex branching patterns than normal neurons [S. M. De la Monte, et al, GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. *Developmental Brain Research* 46, 161-168 (1989); published online Epub4/1/ ([http://dx.doi.org/10.1016/0165-3806\(89\)90279-4](http://dx.doi.org/10.1016/0165-3806(89)90279-4)); M. Hocquemiller, et al, GAP43overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. *Journal of neuroscience research* 88, 202-213 (2010); S. Vitry, et al, Enhanced degradation of synaptophysin by the proteasome in mucopolysaccharidosis type IIIB. *Molecular and Cellular Neuroscience* 41, 8-18 (2009); published online Epub5//. These findings indicate a defect in signaling pathways involved in neuron growth and plasticity [S.M. De La Monte, cited above]. Murine models of MPS III show progressive overexpression of GAP43 in the brain, and cultured neurons from MPS mice likewise overexpress GAP43 and elaborate longer neurites with more complex branching patterns than normal neurons [M. Hocquemiller, et al., J. M. Heard, GAP43 overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. *Journal of neuroscience research* 88, 202-213 (2010); S. Vitry, et al, Enhanced degradation of synaptophysin by the proteasome in mucopolysaccharidosis type IIIB. *Molecular and Cellular Neuroscience* 41, 8-18 (2009); published online Epub5// (<http://dx.doi.org/10.1016/j.mcn.2009.01.001>); H. H. Li, et al., Attenuated plasticity in neurons and astrocytes in the mouse model of Sanfilippo syndrome type B. *Journal of neuroscience research* 69, 30-38 (2002)]. These findings indicate a defect in signaling pathways involved in neuron growth and plasticity, which could explain the development of cognitive dysfunction in MPS patients in the absence of overt neurodegeneration [M. Hocquemiller, S. Vitry, S. Bigou, J. Bruyere, J. Ausseil, J. M. Heard, GAP43 overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. *Journal of neuroscience research* 88, 202-213 (2010); S. Vitry, et al, *Molecular and Cellular Neuroscience* 41, 8-18 (2009); published online Epub5//.

There remains a need for detecting mucopolysaccharidosis and for evaluating therapeutics.

SUMMARY OF THE INVENTION

Current methods for diagnosing and monitoring treatment of patients having MPS often involve obtaining cerebrospinal fluid (CSF) samples, which involves a procedure

many patients find unpleasant and which associated with not insignificant risk, particularly in young children. The present invention relies on a finding by the inventors that the polyamine spermine, which can be found in CSF and also in blood (plasma) samples, correlates with multiple mucopolysaccharidosis (MPS) disorders. The present invention allows for diagnosis and monitoring of treatment of MPS patients by assessing spermine levels in the patients.

The term “threshold spermine levels” refers to the spermine concentration as assessed in a suitable biological sample from a patient (e.g., cerebrospinal fluid (CSF)), which levels (i.e., concentrations) are at a predetermined value. Values above the threshold levels are indicative of a disease state. Such a predetermined value may be a value for a patient subpopulation (e.g., an MPS type or subtype, an age group, and/or a gender-based subgrouping) or may be determined on an individual basis to facilitate personalized treatment. In certain embodiments, these levels are determined in a quantitative manner, e.g., by measuring in a mass spectral apparatus. In the examples below, spermine concentrations in CSF of at least about 2 ng/mL, or greater than about 3 ng/mL, are threshold values indicative of MPS disease. In certain embodiments, spermine concentrations below 3 ng/mL, e.g., below about 2 ng/mL, or about 0.1 ng/mL to about 2 ng/mL, are considered normal levels. In certain embodiments, threshold spermine levels may be determined by values relative to normal subjects using other apparatus or methods. In the examples below in animal models, including one with inbred dogs, spermine levels up to 30 fold higher were observed in the CSF of animals having MPSI or MPSVII to normal levels. Similar trends are observed in the human population. Further, in human patients receiving treatment (illustrated in are enzyme replacement therapy, bone marrow transplant, and regimens involving combinations), significant reductions in spermine levels (e.g., a 2 to 3 fold decrease in spermine concentrations) have been observed. This significant reduction of spermine post-treatment is indicative of the ability of the method of invention to be used in monitoring patient treatment for the neurodegenerative component of MPS. Similar trends may be observed in other biological samples, such that spermine levels can be used to monitor the efficacy of systemic treatments. Such other samples may include, e.g., plasma, fibroblasts or urine.

In one aspect, a method for determining if a patient has a mucopolysaccharidosis disorder selected from type I, type II, type III or type VII is provided. In one embodiment, the method involves (a) obtaining a sample from a patient; and (b) detecting whether spermine is present in said sample above a threshold concentration, whereby these elevated concentrations (used interchangeably with the term “level”) correlate with a patient having an MPS disorder. Optionally, the method may further involve determining the type of MPS disorder the patient has. This may involve obtaining a sample from the patient diagnosed with an MPS disorder as provided herein and screening the sample for a deficiency in one or more of a human enzyme selected from:
10 α -L-iduronidase (IDUA), human iduronate sulfates (hIDS), and/or beta-glucuronidase.

For treatment and monitoring of the neurodegenerative aspects of an MPS condition, the sample is preferably obtained from the cerebrospinal fluid. However, the systemic effects of an MPS condition may be monitored using plasma, urine, or another suitable sample.

15 In another aspect, a method for monitoring a patient having a prior MPS diagnosis is provided, which involves (a) obtaining a sample from a patient; and (b) detecting whether spermine above a threshold level is present in said sample, whereby these elevated levels of spermine correlate to a patient having an MPS disorder. In another embodiment, efficacy of an MPS treatment can be monitored using the level of spermine, which when above a threshold level allows for a decision to be made regarding the
20 timing and dosing of further therapy in the patient, which may be enzyme replacement therapy (ERT), ERT co-therapy in a patient who is a gene therapy patient or candidate, and/or bone marrow transplant (BMT).

In a further aspect, provided herein is a viral vector expressing at least one
25 enzyme selected from alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-glucuronidase in treating a mucopolysaccharidosis in a patient having spermine levels above the threshold level.

In still another aspect, provided herein is an enzyme selected from one or more of:
30 alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase, for use in treating a

mucopolysaccharidosis in a patient, comprising: assaying a patient sample spermine levels above a threshold concentration; assaying a sample from the patient having spermine levels exceeding a threshold concentration for a deficiency in one or more of the enzymes; and administering a therapeutically effective amount of the enzyme in which
5 the patient is deficient.

In a further aspect, provided herein is a composition comprising at least one viral vector expressing at least one enzyme selected from one or more of: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or
10 beta-Glucuronidase for use in treating a mucopolysaccharidosis in a patient, comprising: assaying a sample from a patient for spermine levels above a threshold concentration; assaying a sample from the patient having spermine levels elevated above the threshold level for a deficiency in one or more of the enzymes; and administering a therapeutically effective amount of the composition expressing the enzyme in which the patient is
15 deficient.

In one aspect, a method of treating MPSI, II, III, or VII in a patient is provided which comprises administering an effective amount of a spermine or spermine synthase inhibitor to the patient. In one embodiment, the patient is a human.

In certain embodiments, a method of treating patient with a spermine synthase
20 inhibitor is provided, wherein the inhibitor is an anti-spermine synthase antibody. In certain embodiments, a method of treating a patient by delivering with a cell surface proteoglycan that blocks binding of spermine to heparin sulfate is provided. In certain embodiments, a method of treating a patient is provided which involves delivering a selective spermine synthase inhibitor selected from N-(3-aminopropyl)cyclohexylamine
25 (APCHA) and methylthiopropylamine (MTPA). In certain embodiments, a patient having MPSI, MPSII, III or VII is treated by delivering an an oligonucleotide inhibitor specific for spermine synthase.

In a further aspect, a method of treating cognitive dysfunction in a patient having MPSI, II, III, or VII in a patient is provided which comprises administering an effective
30 amount of an inhibitor of the activity of spermine synthase to the patient.

Still other advantages of the present invention will be apparent from the detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

- FIGS 1A-1B illustrate elevated CSF spermine in MPS I. A high throughput LC/MS and GC/MS metabolite screen was performed on CSF samples from MPS I dogs (n = 15) and normal controls (n = 15). FIG 1A shows a heatmap of the top 100 differentially detected metabolites (ANOVA) is shown. The youngest animal in the MPS I cohort (28 days of age) is indicated by an asterisk. FIG 1B is a graph showing spermine concentration was measured by a quantitative isotope dilution LC/MS assay in CSF samples from 6 infants with MPS I and 2 normal infants.
- FIGS 2A-2F illustrate spermine dependent aberrant neurite growth in MPS I neurons. Cortical neurons harvested from E18 wild-type or MPS I mouse embryos were treated with spermine (50 ng/mL) or the spermine synthase inhibitor APCHA 24 hours after plating. Neurite number, length and branching were quantified for 45-65 randomly selected neurons from duplicate cultures per treatment condition by a blinded reviewer. FIG 2A is a bar chart providing neurites for MPSI, MPSI + APCHA, or MPSI+APCHA+spermine, as compared to a wild-type. FIG 2B is a bar chart providing branch points for MPSI, MPSI + APCHA, or MPSI+APCHA+spermine, as compared to a wild-type. FIG 2C is a bar chart providing arbor length for MPSI, MPSI + APCHA, or MPSI+APCHA+spermine, as compared to a wild-type *** $p < 0.0001$ (ANOVA followed by Dunnett's test). FIG 2D is a bar chart comparing neurites/cell for wild-type treated with spermine as compared to wild-type. FIG 2E is a bar chart comparing branch points/cell for wild-type treated with spermine as compared to wild-type. FIG 2F is a bar chart comparing arbor length /cell for wild-type treated with spermine as compared to wild-type.
- FIGS 3A - 3C illustrate normalization of CSF spermine levels and brain GAP43 expression in MPS I dogs following gene therapy. Five MPS I dogs were treated with an intrathecal injection of an AAV9 vector expressing canine IDUA at one month of age. Two of the dogs (I-549, I-552) were tolerized to IDUA by liver directed gene therapy on postnatal day 1 in order to prevent the antibody response that is elicited to IDUA in some MPS I dogs. FIG 3A is a bar chart showing the results of IDUA activity measured in brain tissue six months after intrathecal vector injection. FIGS 3B and 3C are graphs showing results following measurement of GAP43 in cortical brain samples quantified relative to β -actin by densitometry. CSF spermine was measured at the time of sacrifice

by isotope dilution LC/MS. Untreated MPS I dogs (n = 3) and normal dogs (n = 2) served as controls. * p < 0.05 (Kruskal-Wallis test followed by Dunn's test).

FIGS 4A-4B are graphs which illustrate the use of spermine as a CSF biomarker for evaluation of CNS directed gene therapy in MPS I. Six MPS I dogs tolerized to human IDUA at birth were treated with intrathecal AAV9 expressing human IDUA (1012 GC/kg, n = 2, 1011 GC/kg, n = 2, 1010 GC/kg, n = 2) at one month of age. FIG 4A provides results following measurement of CSF spermine levels measured six months after treatment. Three MPS I cats were treated with intrathecal AAV9 expressing feline IDUA (1012 GC/kg). FIG 4B provides results following quantification of CSF spermine six months after treatment. Untreated MPS I dogs (n = 3) and normal dogs (n = 2) served as controls.

FIG 5 illustrates the mean decrease accuracy for metabolites identified by random forest analysis.

FIG 6 illustrates the expression of enzymes in the polyamine synthetic pathway in MPS I dog brain samples.

FIG 7 illustrates spermine concentration in MPS VII dog CSF.

FIG 8 illustrates that there is no impact of APCHA treatment on WT neuron growth.

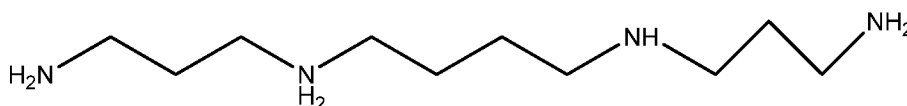
FIG 9 illustrates spermine levels in MPSI patients receiving: 1 intrathecal dose enzyme replacement therapy (ERT) + one dose iv ERT (no bone marrow transplant (BMT))(second point); 2 IT ERT and is 3 months post BMT (third point), and 3 IT ERT and is 6-months post-BMT (last point). This illustrates the usefulness of detecting spermine levels in patients undergoing various types of treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for treatment of the neurodegenerative and systemic symptoms and pathology of MPSI, MPSII, MPSIII and MPSVIII. As described herein, the inventors have found spermine accumulation is associated with mucopolysaccharidoses (MPS) type I, as well as other types of MPS associated with heparin accumulation, such as MPSII, MPSIII, and MPSVIII. Spermine levels may be used as a non-invasive biomarker for monitoring treatment of MPS and assessing pharmacodynamics of novel CNS-directed therapies for MPS. In addition, early studies

show that spermine accumulation may be age dependent. The potential for impaired HS metabolism to trigger accumulation of a metabolite that alters neuron growth could point to a novel connection between enzyme deficiencies and the abnormal neurite growth phenotype in MPS, which may explain the cognitive dysfunction associated with these disorders. Prevention of spermine accumulation may be useful in preventing or alleviating these cognitive issues.

Spermine is a polyamine. The sequence of human spermine synthase is provided in UniProtKB - P52788 (SPSY_Human) <http://www.uniprot.org/uniprot/P52788>, incorporated by reference; SEQ ID NO: 1. Isoform 2 of spermine synthase is reproduced in SEQ ID NO: 2. Spermine has the molecular formula: $C_{10}H_{26}N_4$ and is characterized by the structure:



The crystal structure of human spermine synthase has been described. Wu H, et al, Crystal structure of human spermine synthase: implications of substrate binding and catalytic mechanism. J Biol Chem. 2008 Jun 6;283(23):16135-46. doi: 10.1074/jbc.M710323200. Epub 2008 Mar 26.

The term “threshold spermine levels” refers to the spermine concentration as assessed in a suitable biological sample from a patient (e.g., cerebrospinal fluid (CSF)), which levels (i.e., concentrations) are at a predetermined value. Values above the threshold levels are indicative of a disease state. Such a predetermined value may be a value for a patient subpopulation (e.g., an MPS type or subtype, an age group, and/or a gender-based subgrouping) or may be determined on an individual basis to facilitate personalized treatment. In certain embodiments, these levels are determined in a quantitative manner, e.g., by measuring in a mass spectral apparatus. In the examples below, spermine concentrations in CSF of at least about 2 ng/mL, or greater than about 3 ng/mL, are threshold values indicative of MPS disease. In certain embodiments, spermine concentrations below 3 ng/mL, e.g., below about 2 ng/mL, or about 0.1 ng/mL to about 2 ng/mL, are considered normal levels. In certain embodiments, threshold spermine levels may be determined by values relative to normal subjects using other apparatus or methods. In the examples below in animal models, including one with inbred dogs, spermine levels up to 30 fold higher were observed in the CSF of animals

having MPSI or MPSVII to normal levels. Similar trends are observed in the human population. Further, in human patients receiving treatment (illustrated in are enzyme replacement therapy, bone marrow transplant, and regimens involving combinations), significant reductions in spermine levels (e.g., a 2 to 3 fold decrease in spermine concentrations) have been observed. This significant reduction of spermine post-treatment is indicative of the ability of the method of invention to be used in monitoring patient treatment for the neurodegenerative component of MPS. Similar trends may be observed in other biological samples, such that spermine levels can be used to monitor the efficacy of systemic treatments. Such other samples may include, e.g., plasma, fibroblasts or urine.

In certain embodiments, spermine concentrations in cerebrospinal fluid are used to monitor treatment or the disease progression of a patient having a neurodegenerative disorder, including one or more MPS disorder. In certain embodiments, personalized spermine concentrations for a selected patient may be used to monitor treatment post-diagnosis. Such diagnosis may be the methods described herein, or by conventional diagnostic methods. In these embodiments, an initial CSF spermine concentration may be obtained from a sample pre-treatment and one or more follow-up CSF spermine concentration may be obtained from a sample post-treatment. Such treatment may be enzyme replacement therapy, bone marrow transplant, or gene therapy, or a regimen involving a combination of one or more of these. In this embodiment, the threshold value is not restricted to the values defined above, as the subject has an existing diagnosis and to allow for the patient-specific spermine concentration levels.

Optionally, plasma or urine spermine levels may be monitored in conjunction with CSF, in order to monitor the peripheral organs which may be affected by an MPS disorder. In general, spermine levels in plasma may range from about 1 ng/mL to about 32 ng/mL; and are more typically in the range of about 10 ng/mL to about 12 ng/mL, and values within these ranges.

As used herein, e.g., for determining relative spermine levels, a “normal subject” refers to a subject (e.g., a human) who does not have a neurodegenerative disorder associated with storage diseases, e.g., an MPSI, MPSII, an MPSIII, or an MPS VII disorder.

As used herein a “spermine inhibitor” is any moiety which inhibits the activity of spermine, including its ability to bind to heparin sulfate. In certain embodiments, specific (or selective) spermine inhibitors are selected. Such selective spermine inhibitors are characterized by inhibiting spermine activity, but not substantially inhibiting the activity of spermidine or other polyamines.

As used herein an “inhibitor of spermine synthase” is a moiety which inhibits the ability of spermine synthase to catalyze the production of spermine from spermidine and decarboxylated S-adenosylmethionine (dcSAM). In certain embodiments, specific spermine synthase inhibitors are selected. Such spermine synthase inhibitors are characterized by inhibiting spermine synthesis, but not substantially inhibiting the activity of spermidine synthase or other polyamine synthetases.

Inhibitors may be selected from a variety of classes of compounds, including, e.g., polynucleotide sequences (e.g., antisense oligonucleotides, RNA), antibodies, peptides, proteins, or small molecules. Examples of suitable oligonucleotides may be those which prevent transcription of spermine synthase, e.g., antisense oligonucleotides, including antisense RNA, RNAi, and/or siRNA. In certain embodiments the inhibitor is an anti-spermine antibody. In still other embodiments the inhibitor may be another protein or a peptide that blocks binding of spermine to heparin sulfate, such as, e.g., a cell surface proteoglycan, e.g., a member of the syndecan family.

In still other embodiments, the inhibitor is a selective spermine synthase inhibitor. One examples of a selected synthase inhibitor is N-(3-aminopropyl)cyclohexylamine (APCHA).

In certain embodiments, an inhibitor of spermidine/spermidine synthase may be targeted, in order to prevent conversion to spermine. Examples of spermidine synthase inhibitors are adenosyl spermidine, Ado DATO, DCHA, trans-4-methylcyclohexylamine (4MCHA), cyclohexylamine, methylglyoxal bis-(cyclopentylamidino)hydrazine (MGMBP), 2-mercaptopropylamine, N-chlorosulfonyldicyclohexylamine, 5'-((3-aminopropyl) amino)-5'-deoxyadenosine, 1-aminooxyl-3-aminopropane, 5-(isobutylthio)adenosine, 5'-(methylthio)adenosine and any functional homologs and analogs thereof. See, e.g., US 6696454B2. However, such inhibitors may be non-specific for spermine and additional inhibitors for related polyamine pathways may be required for combination with these inhibitors.

As used herein, "inhibit" means to reduce spermine levels and/or spermine activity to below the elevated levels associated with mucopolysaccharidosis. In certain embodiments, levels are reduced by 5%, reduced by 10%, reduced by 15%, or reduced to within 10% of normal spermine levels in plasma and/or cerebrospinal fluid (CSF). In certain embodiments, levels are reduced to less than 2 ng/mL or less than 1 ng/mL in the CSF, or less than 10 to 12% ng/mL in plasma.

Spermine concentration may be determined using a suitable assay. For example the assay described in J Sanchez-Lopez, et al, "Underivatives polyamine analysis is plant samples by ion pair liquid chromatography coupled with electrospray tandem mass spectrometry," Plant Physiology and Biochemistry, 47 (2009): 592-598, avail online 28 Feb 2009; MR Hakkinen et al, "Analysis of underivatized polyamines by reversed phase liquid chromatography with electrospray tandem mass spectrometry", J Pharm Biomec Analysis, 44 (2007): 625-634, quantitative isotope dilution liquid chromatography (LC)/mass spectrometry (MS) assay. Other suitable assays may be used.

In certain embodiments, a method of diagnosing mucopolysaccharidoses (MPS) I, II, III or VII in a patient is possible. The method involves obtaining a cerebrospinal fluid or plasma sample in a human patient; detecting spermine concentration levels in the sample; and diagnosing the patient with a mucopolysaccharidosis selected from MPS I, II, III or VII in a patient having spermine concentrations in excess of normal levels.

Spermine concentrations may be determined in a quantitative manner, e.g., by measuring in a mass specification apparatus. In the examples below, threshold values are about 3 ng/mL or in certain embodiments, about 2 ng/mL. Thus, untreated patients having an MPS disorder as described herein have spermine concentrations above about 3 ng/mL, or in some embodiments, above 2 ng/mL are above threshold spermine values, wherein normal subjects, having values generally below 1 ng/mL to 2 ng/mL, and up to but not including 3 ng/mL. In other embodiments, significantly elevated spermine levels may be determined by values relative to normal subjects using other apparatus or methods.

In certain embodiments, the sample is a cerebrospinal fluid sample. In other embodiments, the sample is a plasma sample. The spermine concentration levels are measured by any suitable method, e.g., an isotope dilution liquid chromatography - mass spectrometry. Suitable instruments are commercially available. See, e.g., AB Sciex

QTrap 5500 mass spectrometer system [Metabolon]; Waters TQ-S Triple Quad mass spectrometer system; Thermo Scientific Q Exactive HF mass spectrometer system. Still other apparatus may be selected by one of skill in the art provided with the information in this specification.

5 In other embodiments, a method of diagnosing and treating mucopolysaccharidoses (MPS) I in a patient is provided. The method comprises: obtaining a cerebrospinal fluid or plasma sample in a human patient; detecting spermine concentration levels in the sample by performing an mass spectral analysis; diagnosing the patient with a mucopolysaccharidosis selected from MPS I in the patient having
10 statistically significantly elevated spermine concentrations; and delivering an effective amount of human alpha-L-iduronidase (hIDUA) to the diagnosed patient. In one embodiment, the hIDUA is delivered via a viral vector. Optionally, the hIDUA is delivered intrathecally. In other embodiments, the hIDUA is delivered to the liver. Suitable vector constructs and methods have been described, *e.g.*, US Published
15 Application No. 2016/0000887; US Provisional Application Nos. 62/367,798, filed July 28, 2016, US Provisional Patent Application No. 62/337,178, filed May 16, 2016, 62/323,271, filed April 16, 2016 and US Provisional Patent Application No. 62/290,547, filed February 3, 2016, filed Feb 3, 2016, each entitled "Gene Therapy for Treating Mucopolysaccharidosis Type I". In certain other embodiments, the hIDUA is delivered
20 via enzyme replacement therapy (ERT). Such ERT may involve administering a fusion protein comprising the hIDUA.

 In certain embodiments, a method of diagnosing and/or treating MPSI in a patient, or monitoring treatment, is provided. The method involves obtaining a suitable sample from a human patient suspected of having MPSI; detecting spermine concentration levels
25 in the sample; diagnosing the patient with a mucopolysaccharidosis selected from MPS I in the patient having significantly elevated spermine concentrations; and delivering an effective amount of human IDUA to the diagnosed patient as provided herein.

 The amino acid sequence of human IDUA is reproduced in SEQ ID NO: 10. This enzyme may be used to deliver functional IDUA to a patient for enzyme replacement
30 therapy. Alternatively, the coding sequence for this enzyme may be used to construct a viral vector expressing this enzyme. An example of a suitable enzyme may include laronidase (available as Aldurazyme™ (Genzyme)).

In another aspect, the method involves monitoring and adjusting MPSII therapy. Such method involves obtaining a sample from a human patient undergoing therapy for MPSII; detecting spermine concentration levels in the sample by performing a mass spectral analysis; adjusting dosing levels of the MPSII therapeutic if spermine concentrations above the threshold level are detected. For example, patients having untreated MPSII may have spermine concentration levels of greater than 2 ng/mL, more commonly above 3 ng/mL and up to about 100 ng/mL. If a patient has levels approaching normal levels, dosing may be lowered. Conversely, if a patient has higher than desired MPSII levels, higher doses, or an additional therapy, e.g., ERT may be provided to the patient.

Human IDS includes the protein encoded by the published gene coding sequence of Genbank NP000193.1 [SEQ ID NO:8]. In another embodiment, the expression cassette contains a hIDS gene characterized by having the nucleotide sequence at least about 80% identical to SEQ ID NO: 8 and encodes a functional human iduronate-2-sulfatase. In another embodiment, the sequence is at least about 85% identity to SEQ ID NO: 8 or at least about 90% identical to SEQ ID NO:8 and encodes a functional human iduronate-2-sulfatase. In another embodiment, a functional human iduronate-2-sulfatase may include a synthetic amino acid sequence in which all or a portion of the first 25 amino acids of the preproprotein SEQ ID NO:7, which correspond to the leader (signal) peptide, are replaced with a heterologous leader peptide. This leader peptide, e.g., such as the leader peptides from interleukin-2 (IL-2) or oncostatin, can improve transport of the enzyme out of the cell through its secretory pathway into the circulation and consequently facilitate re-uptake by untransduced cells in addition to normal trafficking of the enzyme to the intracellular lysosomal compartment. Suitable leader peptides are preferably, although not necessarily of human original. Suitable leader peptides may be chosen from <http://proline.bic.nus.edu.sg/spdb/zhang270.htm>, which is incorporated by reference herein, or may be determined using a variety of computational programs for determining the leader (signal) peptide in a selected protein. Although not limited, such sequences may be from about 15 to about 50 amino acids in length, or about 19 to about 28 amino acids in length, or may be larger or smaller as required. In addition, at least one in vitro assay has been described as being useful to assess the enzymatic activity of an IDS enzyme [see, e.g., Dean et al, Clinical Chemistry, 2006 Apr; 52(4): 643-649]. In addition to removal of all or a portion of the preproprotein (amino acids 1 to 25 of SEQ ID NO: 7), all or a portion of the proprotein (amino acids 26 to 33 of SEQ ID NO: 7) may be

removed and optionally replaced with a heterologous mature peptide. In another embodiment, human IDS variant 2 [SEQ ID NO: 9], or a sequence encoding same, may be used to express the protein.

In one embodiment, the hIDS is delivered via a viral vector. Optionally, the hIDS is delivered intrathecally. In other embodiments, the hIDS is delivered to the liver. Suitable vector constructs and methods have been described, *e.g.*, US Published Application No. 2016/0000887; US Provisional Patent Application Nos. 62/367,780, filed July 28, 2016, 62/337,163, filed May 16, 2016, US Provisional Application No. 62/330,938, filed May 3, 2016, US Provisional Application No. 62/323,194, filed April 15, 2016, each entitled "Gene Therapy for Treating Mucopolysaccharidosis Type II". In certain other embodiments, the hIDS is delivered via enzyme replacement therapy (ERT) (*e.g.*, using Elaprase® or another suitable composition). Such ERT may involve administering a fusion protein comprising the hIDS. Optionally, the enzyme is delivered intrathecally. In other embodiments, the enzyme is delivered to the liver.

In certain embodiments, a method of diagnosing and treating a MPS III in a patient is provided. The method comprises: obtaining a sample from a human patient; detecting spermine concentration levels in the sample; diagnosing the patient with a mucopolysaccharidosis selected from a MPS III in a patient having spermine concentrations above the threshold level; and (d) delivering an effective amount of a therapeutic gene to the diagnosed patient. In certain circumstances, the patient may be diagnosed with Sanfilippo Syndrome, of which there are four types: (A): heparin N-sulfatase (gene location 17q25.3), MPSIII-B (N-acetyl-alpha-D-glucosaminadase) (gene location 17q21), MPIII-C (acetyl-CoA; alpha-glucosaminide acetyltransferase (gene location 8p11-q13); MPSIII-D: N-acetylglucosamine-G-sulfate sulfatase (gene location 12q14). The different subtypes of MPS III are treated using different enzymes. For example, MPS IIIA may be treated using N-sulfoglucosamine sulfohydrolase (SGSH) [UniProtKB - P51688; signal peptide (aa 1-20) and mature chain (aa 21-502), SEQ ID NO: 3] or heparan N-sulfatase (Sanfilippo syndrome); MPS IIIB may be treated using N-acetyl-alpha-D-glucosaminadase [UniProtKB - P54802; signal peptide (aa 1-23); 82kDa chain (aa 24-743); 77 kDa chain (aa 59-743); SEQ ID NO:4]; MPS IIIC may be treated using acetyl-CoA:alpha-glucosaminidase [UniProtKB - Q68CP4; SEQ ID NO:5; and MPS IIID may be treated using N-acetylglucosamine-6-sulfate sulfatase [*see*, UnitProtKB

- P15586; enzyme SEQ ID NO:6]. The enzyme may be expressed from a viral vector delivered according to gene therapy methods. In certain other embodiments, the gene is delivered via enzyme replacement therapy (ERT). Such ERT may involve administering a fusion protein comprising the enzyme. Optionally, the enzyme is delivered
5 intrathecally. In other embodiments, the enzyme is delivered to the liver.

In another aspect, the method involves monitoring and adjusting MPSIII therapy. Such method involves obtaining a sample from a human patient undergoing therapy for MPSIII; detecting spermine concentration levels in the sample by performing a mass spectral analysis; adjusting dosing levels of the MPS III therapeutic if spermine
10 concentrations above a threshold value are detected. For example, certain patients having an untreated MPSIII may have spermine concentration levels of greater than 2 ng/mL, more generally 3 ng/mL and up to about 100 ng/mL. In a monitoring embodiment of the invention, if a patient has levels approaching normal levels, dosing may be lowered. Conversely, if a patient has higher than desired MPSIII levels, higher doses, or an
15 additional therapy, e.g., ERT may be provided to the patient.

In certain embodiments, a method of diagnosing and treating a MPS VII patient (Sly syndrome) is provided. The method comprises: obtaining a cerebrospinal fluid or plasma sample in a human patient; detecting spermine concentration levels in the sample by performing a mass spectral analysis; diagnosing the patient with a
20 mucopolysaccharidosis selected from MPS VII in a patient having spermine concentrations above a threshold value; and delivering an effective amount of a therapeutic gene to the diagnosed patient. Optionally, the gene is delivered intrathecally. In other embodiments, the gene is delivered to the liver. In certain other embodiments, the gene is delivered via enzyme replacement therapy (ERT). Such ERT may involve
25 administering a fusion protein comprising the enzyme. In one embodiment, the treatment involves delivering the enzyme β -glucuronidase (GUS). The enzyme may be expressed from a viral vector delivered according to gene therapy methods. In other embodiments, ERT is used. An example of a suitable enzyme for therapy is ABO-102 [Abeona Therapeutics].

30 In another aspect, the method involves monitoring and adjusting MPS VII (Sly syndrome) therapy. Such method involves obtaining a sample from a human patient undergoing therapy for MPS VII; detecting spermine concentration levels in the sample

by performing a mass spectral analysis; adjusting dosing levels of the MPS VII therapeutic if spermine levels above a threshold level are detected. For example, in certain embodiments, patients having untreated MPS VII may have spermine concentration levels of greater than 2 ng/mL, and the levels may be from 3 ng/mL and up to about 100 ng/mL. If a patient has levels approaching normal levels, dosing may be lowered. Conversely, if a patient has higher than desired MPS VII levels, higher doses, or an additional therapy, e.g., ERT may be provided to the patient.

The amino acid sequence of human GUSB variant 1 is reproduced in SEQ ID NO: 11. This enzyme may be used to deliver functional GUSB A to a patient for enzyme replacement therapy. Alternatively, the coding sequence for this enzyme may be used to construct a viral vector expressing this enzyme. An example of a suitable enzyme includes Naglazyme® (galsulfase; BioMarin) and UX003 (Ultragenyx Pharmaceutical),

A variety of vectors may be selected for use in delivering one or more enzymes useful in treating an MPS disorder. In one embodiment, the viral vector is an adeno-associated viral (AAV) vector. One desirable AAV is a replication-defective AAV having an AAV9 capsid. As used herein, “AAV9 capsid” refers to the AAV9 having the amino acid sequence of GenBank accession:AAS99264, which is incorporated by reference herein. The sequence is reproduced in SEQ ID NO: 12. Such AAV may include, e.g., natural isolates (e.g., hu31 or hu32), or variants of AAV9 having amino acid substitutions, deletions or additions, e.g., including but not limited to amino acid substitutions selected from alternate residues “recruited” from the corresponding position in any other AAV capsid aligned with the AAV9 capsid; e.g., such as described in US 9,102,949, US 8,927,514, US2015/349911; and WO 2016/049230A1. Some variation from this encoded sequence is encompassed by certain embodiments, which may include sequences having about 99% identity to the referenced amino acid sequence in GenBank accession:AAS99264 and US7906111 (also WO 2005/033321) (*i.e.*, less than about 1% variation from the referenced sequence. However, in other embodiments, other variants of AAV9, or AAV9 capsids having at least about 95% identity to the above-referenced sequences may be selected. See, e.g., US 2015/0079038. Methods of generating the capsid, coding sequences therefore, and methods for production of rAAV viral vectors have been described. See, e.g., Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

The term "AAV9 intermediate" or "AAV9 vector intermediate" refers to an assembled rAAV capsid which lacks the desired genomic sequences packaged therein. These may also be termed an "empty" capsid. Such a capsid may contain no detectable genomic sequences of an expression cassette, or only partially packaged genomic sequences which are insufficient to achieve expression of the gene product. These empty capsids are non-functional to transfer the gene of interest to a host cell.

However, other AAV capsids may be selected or generated. For example, the sequences of AAV8 and methods of generating vectors based on the AAV8 capsid are described in US Patent 7,282,199 B2, US 7,790,449, and US 8,318,480, which are incorporated herein by reference. The sequences of a number of such AAV are provided in the above-cited US Patent 7,282,199 B2, US 7,790,449, US 8,318,480, and US Patent 7,906,111, and/or are available from GenBank. The sequences of any of the AAV capsids can be readily generated synthetically or using a variety of molecular biology and genetic engineering techniques. Suitable production techniques are well known to those of skill in the art. See, *e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (Cold Spring Harbor, NY). Alternatively, oligonucleotides encoding peptides (*e.g.*, CDRs) or the peptides themselves can generated synthetically, *e.g.*, by the well-known solid phase peptide synthesis methods (Merrifield, (1962) *J. Am. Chem. Soc.*, **85**:2149; Stewart and Young, *Solid Phase Peptide Synthesis* (Freeman, San Francisco, 1969) pp. 27-62). These and other suitable production methods are within the knowledge of those of skill in the art and are not a limitation of the present invention.

Replication-defective recombinant AAV (rAAV) may be generated using techniques which are known. See, *e.g.*, WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid; a functional rep gene; an expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into the AAV capsid protein.

The term "identity" or "percent sequence identity" may be readily determined for amino acid sequences, over the full-length of a protein, a subunit, or a fragment thereof. Suitably, a fragment is at least about 8 amino acids in length, and may be up to about 700 amino acids. Examples of suitable fragments are described herein. Generally, when

referring to “identity”, “homology”, or “similarity” between two different adeno-associated viruses, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments” refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Examples of such programs include, “Clustal W”, “CAP Sequence Assembly”, “MAP”, and “MEME”, which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art.

Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference. Multiple sequence alignment programs are also available for amino acid sequences, *e.g.*, the “Clustal X”, “MAP”, “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. *See, e.g.*, J. D. Thomson *et al*, Nucl. Acids Res., “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

In certain embodiments, a composition is provided which provides a combination or co-therapy useful in treating a patient having an MPS disorder. Such compositions may comprise one or more viral vector stocks, each stock expressing at least one functional enzyme and optionally, a second active component. For example, a patient having an MPS disorder may have more than one of MPSI, MPSII, MPSIII, or MPSVII deficiencies, or may have a second condition requiring therapy. Such active component

may include enzyme replacement therapy which is the same or different from the enzyme expressed from the viral vector. In other embodiments, another component may include an immune modulator (e.g., an immunosuppressant), or another active component.

As used herein, the singular form of the articles “a,” “an,” and “the” includes
5 plural references unless indicated otherwise. For example, the phrase “a virus particle” includes one or more virus particles.

As used herein, the terms "comprise", "comprising", "contain", "containing", and their variants are open claim language, i.e., are permissive of additional elements. In contrast, the terms "consists", "consisting", and its variants are closed claim language, i.e.,
10 exclusive additional elements.

Unless otherwise specified “about” refers to a variability of $\pm 10\%$ from a given value. In certain embodiments, the variability may be 1%, 5%, 10%, or values there between.

In one aspect, a method for determining if a patient has a mucopolysaccharidosis
15 disorder selected from type I, type II, type III or type VII is provided. The method involves (a) obtaining a plasma or CSF sample from a patient; and (b) detecting whether spermine is present in said sample above a threshold value, whereby an amount of spermine in the sample above the threshold correlates to a patient having an MPS disorder. Optionally, the method may further involve determining the type of MPS
20 disorder the patient has. This may involve obtaining a sample from the patient diagnosed with an MPS disorder as provided herein and screening the sample for a deficiency in one or more of a human enzyme selected from: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-glucuronidase.

25 In another aspect, a method for monitoring a patient having a prior MPS diagnosis is provided, which involves (a) obtaining a plasma or CSF sample from a patient; and (b) detecting whether spermine is present above the threshold amount in said sample. An evaluation of the level of spermine allows for a decision to be made regarding the timing and dosing of further therapy in the patient, which may be enzyme replacement therapy
30 (ERT), or ERT co-therapy in a patient who is a gene therapy patient or candidate.

In a further aspect, provided herein is a viral vector expressing at least one enzyme selected from alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase,

alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-glucuronidase in treating a mucopolysaccharidosis in a patient having significantly elevated spermine levels.

In still another aspect, provided herein is an enzyme selected from one or more of:

5 alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase, for use in treating a mucopolysaccharidosis in a patient, comprising: assaying a plasma or CSF sample in a patient for the presence of spermine above a threshold amount; assaying a sample from

10 the patient having spermine above the threshold for a deficiency in one or more of the enzymes: and administering a therapeutically effective amount of the enzyme in which the patient is deficient.

In a further aspect, provided herein is a composition comprising at least one viral vector expressing at least one enzyme selected from one or more of: alpha-L-

15 Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase for use in treating a mucopolysaccharidosis in a patient, comprising: assaying a CSF sample in a patient for the presence of spermine in an amount in excess of a threshold value; assaying a sample from the patient having spermine in excess of the

20 threshold value in CSF for a deficiency in one or more of the enzymes: and administering a therapeutically effective amount of the composition expressing the enzyme in which the patient is deficient.

In one aspect, a method of treating MPSI, II, III, or VII in a patient is provided which comprises administering an effective amount of a spermine or spermine synthase

25 inhibitor to the patient. In one embodiment, the patient is a human.

In certain embodiments, a method of treating patient with a spermine synthase inhibitor is provided, wherein the inhibitor is an anti-spermine antibody. In certain embodiments, a method of treating a patient by delivering with a cell surface

30 proteoglycan that blocks binding of spermine to heparin sulfate is provided. In certain embodiments, a method of treating a patient is provided which involves delivering a selective spermine synthase inhibitor selected from N-(3-aminopropyl)cyclohexylamine (APCHA) and methylthiopropylamine (MTPA). In certain embodiments, a patient

having MPSI, MPSII, III or VII is treated by delivering an oligonucleotide inhibitor specific for spermine synthase.

In a further aspect, a method of treating cognitive dysfunction in a patient having MPSI, II, III, or VII in a patient is provided which comprises administering an effective
5 amount of an inhibitor of the activity of spermine synthase/spermine synthase to the patient.

The examples describe studies which show that a significant elevation in the polyamine spermine correlates with MPSI, MPSII and MPSVII. Using a high throughput LC/MS and GC/MS screen, a marked elevation in the polyamine spermine in animal
10 models and patients having different types of mucopolysaccharidosis (MPS). However, it will be understood that other types of assays may be used to assess the significant polyamine spermine levels, as compared to a normal baseline. It will be further understood that these other methods may result in the determination of different threshold values. The following examples are illustrative of the present invention.

15

EXAMPLES

As described in the examples below, metabolite profiling of CSF samples from MPS I dogs was performed, which revealed substantial disease related alterations in the CSF metabolome. The most striking difference was an over 30-fold elevation in
20 spermine levels compared to normal controls. This finding was confirmed in MPS I patient samples, as well as in a feline model of MPS I and a canine model of MPS VII. Spermine binds to and is transported by heparin sulfate (HS), and stimulated neurite growth. Cellular uptake of spermine is dependent on this interaction [M. Belting, et al., Journal of Biological Chemistry 278, 47181-47189 (2003); M. Belting, et al, Biochemical
25 Journal 338, 317-323 (1999)]. Cell surface proteoglycans such as glypican-1 can bind spermine through their HS moieties, and after endocytosis of the glypican protein, intracellular cleavage of the HS chain releases bound spermine into the cell [H. H. Li, et al., Journal of neuroscience research 69, 30-38 (2002); C. Hinderer, et al., Molecular
Genetics and Metabolism, <http://dx.doi.org/10.1016/j.ymgme.2016.06.006>]. Thus, intact
30 HS recycling is essential for spermine uptake. In MPS I, extracellular spermine accumulation could occur through inhibition of this uptake mechanism due to inefficient HS recycling, or through simple binding of spermine to the extracellular GAGs that

accumulate in MPS, shifting the spermine binding equilibrium to favor extracellular distribution. Future studies should address the relative contribution of these mechanisms to spermine accumulation in MPS I CSF.

A number of studies have implicated spermine in neurite outgrowth; following axon injury, the rate-limiting enzymes for the synthesis of spermine and its precursors putrescine and spermidine are elevated, allowing for enhanced neurite outgrowth even in the presence of inhibitory signals from myelin [J. E. Welch, et al., International journal of oncology 32, 749-756 (2008); D. Cai, et al, Neuron 35, 711-719 (2002); published online Epub Aug 15; K. Deng, et al, The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 9545-9552 (2009); published online Epub Jul 29 (10.1523/jneurosci.1175-09.2009); Y. Gao, et al., Neuron 44, 609-621 (2004); published online Epub Nov 18 (10.1016/j.neuron.2004.10.030)]. Further, treatment of neurons with putrescine induces neurite growth when injected directly into CSF, an effect that is blocked by inhibitors of spermine synthesis [D. Cai, 2002, cited above]. The mechanism by which polyamines exert their effect on neurite growth is not known. One potential target is the NMDA receptor, activation of which is potentiated by spermine binding [C. Hinderer, et al., Molecular therapy : the journal of the American Society of Gene Therapy 22, 2018-2027 (2014); published online Epub Dec (10.1038/mt.2014.135)]. NMDA signaling induces neurite outgrowth, and the spermine sensitive subunit of the receptor is highly expressed during development [K. Ding, et al, The Journal of biological chemistry 276, 46779-46791 (2001); published online Epub Dec 14 (10.1074/jbc.M105419200); N. Volpi, et al., Journal of chromatography. B, Biomedical applications 685, 27-34 (1996); published online Epub Oct 11; J. Lerma, et al., Neuron 8, 343-352 (1992); published online Epub 2// ([http://dx.doi.org/10.1016/0896-6273\(92\)90300-3](http://dx.doi.org/10.1016/0896-6273(92)90300-3))].

The studies described herein showed that inhibitors of spermine synthesis blocked excess neurite growth in MPS neurons, and that neurite growth could be induced in WT neurons by spermine concentrations similar to those found in patient CSF. Gene therapy in the dog model of MPS I reversed spermine accumulation and normalized expression of GAP43, suggesting that the same pathway was impacted *in vivo*. While our *in vitro* findings support a role for spermine in aberrant neurite growth in MPS I, it is important to note that inhibiting spermine synthesis did not completely reverse the phenotype, and spermine addition to normal neurons did not increase neurite growth to the level of MPS I

neurons. The effects of spermine modulation may have been limited by the relatively short period of treatment. It is also possible that spermine accumulation is not the sole mediator contributing to neurite outgrowth in MPS I. Notably many neurotrophic factors bind through HS modified receptors, and interactions with HS in extracellular matrix can influence neurite growth [D. Georgiev, et al., A critical importance of polyamine site in NMDA receptors for neurite outgrowth and fasciculation at early stages of P19 neuronal differentiation. Experimental cell research 314, 2603-2617 (2008); published online EpubAug 15 (10.1016/j.yexcr.2008.06.009)]. Spermine accumulation may therefore be one of several factors promoting abnormal neurite growth in MPS I. Of the 15 MPS I dog CSF samples screened, only one fell within the normal range of spermine concentration. At 28 days of age, this was the youngest animal included in the study. This finding indicates that spermine accumulation may be age dependent. Future studies should evaluate CSF spermine levels longitudinally in MPS patients. If spermine increases with age in MPS patients, this could explain the kinetics of cognitive decline, as most patients experience 1-2 years of normal development before the onset of developmental delays.

The potential for impaired HS metabolism to trigger accumulation of a metabolite that alters neuron growth could point to a novel connection between enzyme deficiencies and the abnormal neurite growth phenotype in MPS, which may explain the cognitive dysfunction associated with these disorders. These findings also indicate that CSF spermine is a useful as a noninvasive biomarker for assessing pharmacodynamics of novel CNS-directed therapies for MPS.

Materials and Methods:

Experimental design: This study was initially designed to detect metabolites that were present at significantly different levels in MPS I patient CSF samples compared to samples from healthy controls. Due to the limited availability of CSF samples from children with MPS IH and healthy controls, the initial screen was performed using CSF samples from MPS I dogs, for which greater numbers were available, with the intention of subsequently evaluating candidate biomarkers in human samples. A total of 15 CSF samples from individual untreated MPS I dogs were available for analysis, and an additional 15 samples were obtained from healthy controls. Following identification of elevated spermine in MPS I dog CSF in the prospective metabolite screen, spermine was

retrospectively measured in CSF samples from previous studies of MPS I dogs and cats treated with gene therapy, as well as patient samples. The number of subjects included in each group for these analyses was limited by sample availability and was not based on statistical considerations; therefore in some cases numbers are insufficient for statistical comparisons. For studies of *in vitro* neurite growth, the number of cells quantified for each condition was based on pilot experiments which indicated that >30 cells per condition was required to detect a 20% difference in arbor length, neurite number or neurite branches per cell. After cells were plated and treated with the designated drug, the wells were coded and the acquisition of cell images and the manual quantification of neurite length and branching were performed by a blinded reviewer. The comparison of wildtype and MPS mouse neurons was repeated using a different substrate [poly-L-lysine (Sigma) coated tissue culture plates rather than chamber slides (Sigma S6815)] with similar results. The comparison of wildtype neurons with and without spermine addition was performed four times using both substrates with similar results. CSF metabolite profiling: CSF metabolite profiling was performed by Metabolon.

Samples were stored at -80 ° C until processing. Samples were prepared using the MicroLab STAR® system (Hamilton Company). A recovery standard was added prior to the first step in the extraction process for QC purposes. Proteins were precipitated with methanol under vigorous shaking for 2 min followed by centrifugation. The resulting extract was divided into five fractions: one for analysis by reverse phase (RP)UPLC-MS/MS with positive ion mode electrospray ionization, one for analysis by RP/UPLC-MS/MS with negative ion mode electrospray ionization, one for analysis by hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode electrospray ionization, one for analysis by GC- MS, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. For LC, the samples were stored overnight under nitrogen before preparation for analysis. For GC, each sample was dried under vacuum overnight before preparation for analysis.

The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The

sample extract was dried then reconstituted in solvents compatible to each of the LC/MS methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency For RP chromatography, one aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions Each method utilized separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 μ m). The extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid. The basic extracts were similarly eluted using methanol and water, however with 6.5mM ammonium bicarbonate. The third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10mM ammonium formate. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 80-1000 m/z.

The samples destined for analysis by GC-MS were dried under vacuum for a minimum of 18 h prior to being derivatized under dried nitrogen using bistrimethylsilyltrifluoroacetamide. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 μ m film thickness) with helium as carrier gas and a temperature ramp from 60° to 340°C in a 17.5 min period. Samples were analyzed on a Thermo- Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. The scan range was from 50–750 m/z.

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of

the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon. Identification of known chemical entities was based on comparison to metabolomics library entries of purified standards. Peaks were quantified using area-under-the-curve measurements. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences by the median value for each run-day, therefore, setting the medians to 1.0 for each run. This preserved variation between samples but allowed metabolites of widely different raw peak areas to be compared on a similar graphical scale. Missing values were imputed with the observed minimum after normalization.

Quantitative MS assay: CSF samples (50 μ L) were mixed with a spermine-d8 internal standard (IsoSciences). Samples were deproteinized by mixing with a 4-fold excess of methanol and centrifuging at 12,000 $\times$ g at 4°C. The supernatant was dried under a stream of nitrogen, and then resuspended in 50 μ L of water. An aliquot of 5 μ L was subjected to LC-MS analysis. The LC separations were carried out using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) equipped with an Xbridge® C18 column (3.5 μ m, 150 $\times$ 2.1 mm). The flow-rate was 0.15 mL/min, solvent A was 0.1% formic acid and solvent B was 98/2 acetonitrile/H₂O (v/v) with 0.1% formic acid. The elution conditions were as follows: 2% B at 0 min, 2% B at 2 min, 60% B at 5 min, 80% B at 10 min, 98% B at 11 min, 98% B at 16 min, 2% B at 17 min, 2% B at 22 min, with the column temperature being 35 °C. A Finnigan TSQ Quantum Ultra spectrometer (Thermo Fisher, San Jose, CA) was used to conduct MS/MS analysis in positive ion mode with the following parameters: spray voltage at 4000 V, capillary temperature at 270 °C, sheath gas pressure at 35 arbitrary units, ion sweep gas pressure at 2 arbitrary units, auxiliary gas pressure at 10 arbitrary units, vaporizer temperature at 200 °C, tube lens offset at 50, capillary offset at 35 and skimmer offset at 0. The following transitions were monitored: 203.1/112.1 (spermine); 211.1/120.1 (spermine-d8) with scan width of 0.002 m/z , and scan time being 0.15 s.

Animal procedures: All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. For CSF metabolite screening, samples were collected by suboccipital puncture in normal dogs at 3-26 months of age, and in MPS I dogs at 1-18 months of age. Gene transfer studies in MPS I dogs and cats were performed as previously described [C. Hinderer, et al, Neonatal Systemic AAV Induces Tolerance to CNS Gene Therapy in MPS I Dogs and Nonhuman Primates. *Molecular therapy : the journal of the American Society of Gene Therapy* 23, 1298-1307 (2015); published online EpubAug (10.1038/mt.2015.99); C. Hinderer, et al, Intrathecal gene therapy corrects CNS pathology in a feline model of mucopolysaccharidosis I. *Molecular therapy : the journal of the American Society of Gene Therapy* 22, 2018-2027 (2014); published online Epub Dec (10.1038/mt.2014.135)]. CSF samples were collected 6-8 months after vector administration. For mouse cortical neuron experiments, primary cortical neuron cultures were prepared from E18 IDUA^{-/-} or IDUA^{+/+} embryos.

Patient samples: Informed consent was obtained from each subject's parent or legal guardian. The protocol was approved by the Institutional Review Board of the University of Minnesota. CSF was collected by lumbar puncture. All MPS I patients had a diagnosis of Hurler syndrome and had not received enzyme replacement therapy or hematopoietic stem cell transplantation prior to sample collection. MPS I patients were 6-26 months of age. The healthy controls were 36 and 48 months of age.

Statistical analysis: The random forest analysis and heat map generation were performed using MetaboAnalyst 3.0(27-29). Raw peak data were log transformed and normalized to the mean of normal sample values. All other statistical analyses were performed with GraphPad Prism 6. Cultured neuron arbor length, neurite number, and branching were compared by ANOVA followed by Dunnett's test. CSF spermine and cortical GAP43 were compared by Kruskal-Wallis test followed by Dunn's test.

GAP43 western: Samples of frontal cortex were homogenized in 0.2% triton X-100 using a Qiagen Tissuelyser at 30 Hz for 5 min. Samples were clarified by centrifugation at 4° C. Protein concentration was determined in supernatants by BCA assay. Samples were incubated in NuPAGE LDS buffer with DTT (Thermo Fisher Scientific) at 70° C for 1 hr and separated on a Bis-Tris 4-12% polyacrylamide gel in MOPS buffer. Protein was transferred to a PVDF membrane, and blocked for 1 hr in 5%

nonfat dry milk. The membrane was probed with rabbit polyclonal anti-GAP43 antibody (Abcam) diluted to 1 µg/mL in 5% nonfat dry milk followed by an HRP conjugated polyclonal anti-rabbit antibody (Thermo Fisher Scientific) diluted 1:10,000 in 5% nonfat dry milk. Bands were detected using SuperSignal West Pico substrate (Thermo Fisher Scientific). Densitometry was performed using Image Lab 5.1 (Bio-Rad).

Neurite growth assay: Day 18 embryonic cortical neurons were harvested as described above, and plated at a concentration of 100,000 cells / mL on chamber slides (Sigma S6815) or poly-L-lysine (Sigma) coated tissue culture plates in serum-free Neurobasal medium (Gibco) supplemented by B27 (Gibco). Treatments were applied to duplicate wells 24 hours after plating (day 1). Phase-contrast images for quantification were taken on a Nikon Eclipse Ti at 20X using a 600 ms manual exposure and 1.70x gain on high contrast. An individual blind to treatment conditions captured 10-20 images per well and coded them. Images were converted to 8-bit format in ImageJ (NIH) and traced in NeuronJ [E. Meijering, et al., Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 58, 167-176 (2004); published online Epub Apr (10.1002/cyto.a.20022)] by a blinded reviewer. Soma diameter, neurite number, branch points, and arbor length were traced manually. Images traced in NeuronJ were converted to micrometers using a conversion factor based on image size; 2560x1920 pixel images were converted to micrometers using a conversion factor of 0.17 micrometers / pixel.

Histology: Brain tissue processing and LIMP2 immunofluorescence were performed as previously described [C. Hinderer, et al., Intrathecal gene therapy corrects CNS pathology in a feline model of mucopolysaccharidosis I. *Molecular therapy : the journal of the American Society of Gene Therapy* 22, 2018-2027 (2014); published online Epub Dec (10.1038/mt.2014.135)].

RT-PCR: Samples of frontal cortex from 3 normal dogs and 5 MPS dogs were immediately frozen on dry ice at necropsy. RNA was extracted with TRIzol reagent (Thermo Fisher Scientific), treated with DNase I (Roche) for 20 min at room temperature, and purified using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Purified RNA (500 ng) was reverse transcribed using the High Capacity cDNA Synthesis Kit (Applied Biosystems) with random hexamer primers. Transcripts for

arginase, ornithine decarboxylase, spermine synthase, spermidine synthase, spermine-spermidine acetyltransferase and glyceraldehyde phosphate dehydrogenase were quantified by Sybr green PCR using an Applied Biosystems 7500.

Real-Time PCR System. A standard curve was generated for each target gene using four-fold dilutions of a pooled standard comprised of all individual samples. The highest standard was assigned an arbitrary transcript number, and Ct values for individual samples were converted to transcript numbers based on the standard curve. Values are expressed relative to the GAPDH control.

Statistical analysis: Random forest analysis and heat map generation were performed using MetaboAnalyst 3.0 [J. Xia, et al, (2012); published online Epub May 2, 2012 (10.1093/nar/gks374); J. Xia, et al, MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Research 37, W652-W660 (2009); published online Epub July 1, 2009 (10.1093/nar/gkp356); J. Xia et al, MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Research, (2015); published online Epub April 20, 2015 (10.1093/nar/gkv380)]. Undetectable values in the metabolite screen were imputed with the minimum values observed in the data set. Raw peak data were normalized to the mean of normal sample values and log transformed. All other statistical analyses were performed with GraphPad Prism 6. Cultured neuron arbor length, neurite number, and branching were compared by ANOVA followed by Dunnett's test. CSF spermine and cortical GAP43 were compared by Kruskal-Wallis test followed by Dunn's test.

Results

1. **Identification of elevated CSF spermine through metabolite profiling**

An initial screen of CSF metabolites was carried out using a canine model of MPS I. These animals carry a splice site mutation in the *IDUA* gene, resulting in complete loss of enzyme expression and development of clinical and histological features analogous to those of MPS I patients [K. P. Menon, et al, Architecture of the canine IDUA gene and mutation underlying canine mucopolysaccharidosis I. Genomics 14, 763-768 (1992). R. Shull, et al, Morphologic and biochemical studies of canine mucopolysaccharidosis I. The American journal of pathology 114, 487 (1984)]. CSF samples were collected from 15 normal dogs and 15 MPS I dogs. CSF samples were

evaluated for relative quantities of metabolites by LC and GC-MS. A total of 281 metabolites could be positively identified in CSF samples by mass spectrometry. Of these, 47 (17%) were significantly elevated in MPS I dogs relative to controls, and 88 (31%) were decreased relative to controls. A heat map of the 50 metabolites most different between groups is shown in Figure 1. Metabolite profiling identified marked differences in polyamine, sphingolipid, acetylated amino acid, and nucleotide metabolism between MPS I and normal dogs. Random forest clustering analysis identified the polyamine spermine as the largest contributor to the metabolite differences between MPS I and normal dogs (FIG 5). On average spermine was more than 30-fold elevated in MPS I dogs, with the exception of one MPS I dog that was under 1 month of age at the time of sample collection. A stable isotope dilution (SID)-LC-MS/MS assay was developed to quantitatively measure spermine in CSF. Samples were screened from 6 children with Hurler syndrome (ages 6-26 months), as well as 2 healthy controls (ages 36 and 48 months). Both healthy controls had CSF spermine levels below the limit of quantification (1 ng/mL) of the assay, whereas CSF samples from MPS I patients were on average 10-fold above the limit of quantification (Figure 1). Spermine elevation in MPS IH patients appeared consistent with the known role of HS in spermine binding and uptake (11-13); increased synthesis appeared unlikely as a cause of elevated CSF spermine, as normal and MPS I dog brain samples had similar mRNA expression levels for transcriptionally regulated enzymes in the polyamine synthetic pathway (FIG 6). To determine whether spermine elevation was a general property of heparan sulfate storage diseases or specific to MPS I, spermine was measured in a CSF sample from a canine model of MPS VII, which exhibited a similar elevation (FIG 7).

2. Role of spermine in abnormal neurite growth associated with MPS

Following axon injury neurons upregulate polyamine synthesis, which promotes neurite outgrowth [D. Cai, et al, Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. Neuron 35, 711-719 (2002); published online Epub Aug 15; K. Deng, et al, Increased synthesis of spermidine as a result of upregulation of arginase I promotes axonal regeneration in culture and in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 9545-9552 (2009); published online Epub Jul 29

(10.1523/jneurosci.1175-09.2009); Y. Gao, et al, Neuron 44, 609-621 (2004); published online Epub Nov 18 (10.1016/j.neuron.2004.10.030); R. C. Schreiber, et al, Polyamines increase in sympathetic neurons and non-neuronal cells after axotomy and enhance neurite outgrowth in nerve growth factor-primed PC12 cells. Neuroscience 128, 741-749 (2004) [10.1016/j.neuroscience.2004.07.014]. We therefore evaluated the role of spermine in the abnormal neurite overgrowth phenotype that has been described in MPS neurons [M. Hocquemiller, et al, GAP43 overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. Journal of neuroscience research 88, 202-213 (2010)]. Cultures of E18 cortical neurons from MPS I mice exhibited greater neurite number, branching, and total arbor length after 4 days in culture than neurons derived from wild type mice from the colony (Figure 2). Treatment of MPS neurons with APCA, an inhibitor of spermine synthesis, significantly reduced neurite growth and branching. The effect was reversible by replacing spermine (Figure 2). The same APCA concentration did not affect the growth of normal neurons (FIG 8). Addition of spermine to wild type neuron cultures at concentrations similar to those identified *in vivo* resulted in significant increases in neurite growth and branching (Figure 2).

3. Impact of gene therapy on CSF spermine and GAP43 expression

GAP43, a central regulator of neurite growth, is overexpressed by MPS III mouse neurons both *in vitro* and *in vivo*, suggesting that the same neurite growth pathway aberrantly activated in neuron cultures is also active *in vivo* [M. Hocquemiller, et al, GAP43 overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. Journal of neuroscience research 88, 202-213 (2010); S. Vitry, et al, Enhanced degradation of synaptophysin by the proteasome in mucopolysaccharidosis type IIIB. Molecular and Cellular Neuroscience 41, 8-18 (2009); published online Epub5// (<http://dx.doi.org/10.1016/j.mcn.2009.01.001>); H. H. Li et al, , Attenuated plasticity in neurons and astrocytes in the mouse model of Sanfilippo syndrome type B. Journal of neuroscience research 69, 30-38 (2002)]. In order to evaluate the effect of IDUA deficiency on GAP43 expression and spermine accumulation *in vivo*, we measured CSF spermine and brain GAP43 levels in untreated MPS I dogs as well as those treated with CNS directed gene therapy. We previously described five MPS

I dogs that were treated with an intrathecal injection of an adeno-associated virus serotype 9 vector carrying the canine IDUA transgene C. Hinderer, et al, Neonatal Systemic AAV Induces Tolerance to CNS Gene Therapy in MPS I Dogs and Nonhuman Primates. Molecular therapy : the journal of the American Society of Gene Therapy 23, 1298-1307 (2015); published online EpubAug (10.1038/mt.2015.99]. MPS I dogs can develop antibodies to the normal IDUA enzyme, so two of the dogs were pre-treated as newborns with hepatic IDUA gene transfer to induce immunological tolerance to the protein. Both tolerized dogs exhibited brain IDUA activity well above normal following AAV9 treatment. The three non-tolerized dogs exhibited varying levels of expression, with one animal reaching levels greater than normal and the other two exhibiting expression near normal (Figure 3A). CSF spermine reduction was inversely proportional to brain IDUA activity, with a 3-fold reduction relative to untreated animals in the two dogs with the lowest IDUA expression, and more than 20-fold reduction in the animal with the highest expression (Figure 3). GAP43 was upregulated in frontal cortex of MPS I dogs, and expression was normalized in all vector treated animals (Figure 3).

The relationship between CSF spermine levels and IDUA reconstitution in MPS I dogs treated with a range of vector doses was further evaluated. MPS I dogs previously tolerized to human IDUA by neonatal hepatic gene transfer were treated with intrathecal injection of an AAV9 vector expressing human IDUA at one of 3 doses (10^{10} , 10^{11} , 10^{12} GC/kg, n = 2 per dose) [C. Hinderer, et al, Neonatal tolerance induction enables accurate evaluation of gene therapy for MPS I in a canine model. Molecular Genetics and Metabolism, <http://dx.doi.org/10.1016/j.ymgme.2016.06.006>]. CSF spermine was evaluated 6 months after injection (Figure 4). Reduction of CSF spermine was dose dependent, with animals at the mid and high vector doses reaching the normal range, whereas CSF spermine was only partially reduced in the low dose animals. For independent verification of the connection between IDUA deficiency and CSF spermine accumulation, we evaluated CSF spermine levels in a feline model of MPS I. Using CSF samples from our previously reported gene therapy studies, we found that untreated MPS I cats exhibited elevated CSF spermine (Figure 4) (22). Intrathecal administration of a high dose of an AAV9 vector expressing feline IDUA normalized CSF spermine levels (Figure 4).

C. Discussion

In the present study metabolite profiling of CSF samples from MPS I dogs was performed, which revealed substantial disease related alterations in the CSF metabolome. The most striking difference was an over 30-fold elevation in spermine levels compared to normal controls. This finding was confirmed in MPS I patient samples, as well as in a feline model of MPS I and a canine model of MPS VII. Spermine binds directly to HS with high affinity, and cellular uptake of spermine is dependent on this interaction [M. Belting, et al., Proteoglycan involvement in polyamine uptake. *Biochemical Journal* 338, 317-323 (1999); J. E. Welch, et al, Single chain fragment anti-heparan sulfate antibody targets the polyamine transport system and attenuates polyamine-dependent cell proliferation. *International journal of oncology* 32, 749-756 (2008)]. Cell surface proteoglycans such as glypican-1 can bind spermine through their HS moieties, and after endocytosis of the glypican protein, intracellular cleavage of the HS chain releases bound spermine into the cell [M. Belting, K et al, . *Journal of Biological Chemistry* 278, 47181-47189 (2003); K. Ding, et al, , Modulations of glypican-1 heparan sulfate structure by inhibition of endogenous polyamine synthesis. Mapping of spermine-binding sites and heparanase, heparin lyase, and nitric oxide/nitrite cleavage sites. *The Journal of biological chemistry* 276, 46779-46791 (2001); published online EpubDec 14]. Thus, intact HS recycling is essential for spermine uptake. Inefficient HS recycling due to IDUA deficiency could inhibit this spermine uptake mechanism, leading to extracellular spermine accumulation. Alternatively, extracellular GAGs may sequester spermine, shifting the equilibrium to favor extracellular distribution. The methanol deproteinization step employed for LC-MS sample preparation in this study also precipitates soluble HS, suggesting that the spermine detected in CSF is unbound, and therefore that uptake inhibition rather than GAG binding is responsible for extracellular spermine accumulation [N. Volpi, Purification of heparin, dermatan sulfate and chondroitin sulfate from mixtures by sequential precipitation with various organic solvents. *Journal of chromatography. B, Biomedical applications* 685, 27-34 (1996); published online EpubOct 11]. Formation and maintenance of functional neural networks requires precise control of neurite growth and synapse formation. During development, the CNS environment becomes increasingly inhibitory to neurite formation, with myelin associated proteins largely blocking neurite growth in the adult brain. This developmental shift toward decreased neurite growth is paralleled by a decrease in GAP43 expression [S.

- M. De la Monte, H. J. Federoff, S.-C. Ng, E. Grabczyk, M. C. Fishman, GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. *Developmental Brain Research* 46, 161-168 (1989); published online Epub4/1/ ([http://dx.doi.org/10.1016/0165-3806\(89\)90279-4](http://dx.doi.org/10.1016/0165-3806(89)90279-4)]. The persistent
- 5 GAP43 expression and exaggerated neurite outgrowth exhibited by MPS neurons may interfere with this normal balance of inhibitory and growth promoting signals, resulting in abnormal connectivity and impaired cognition [M. Hocquemiller, et al, GAP43 overexpression and enhanced neurite outgrowth in mucopolysaccharidosis type IIIB cortical neuron cultures. *Journal of neuroscience research* 88, 202-213 (2010)]. How HS
- 10 storage leads to this increase in neurite growth has not been established. A number of studies have implicated polyamines in neurite outgrowth; following axon injury, the rate-limiting enzymes for the synthesis of spermine and its precursors putrescine and spermidine are elevated, allowing for enhanced neurite outgrowth even in the presence of inhibitory signals from myelin [D. Cai, et al, *Neuron* 35, 711-719 (2002); published
- 15 online Epub Aug 15; K. Deng, et al, . *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 9545-9552 (2009); published online EpubJul 29; Y. Gao, et al, . *Neuron* 44, 609-621 (2004); published online Epub Nov 18; R. C. Schreiber, et al., Polyamines increase in sympathetic neurons and non-neuronal cells after axotomy and enhance neurite outgrowth in nerve growth factor-primed PC12 cells. *Neuroscience*
- 20 128, 741-749 (2004)]. Further, treatment of neurons with putrescine induces neurite growth when injected directly into CSF, an effect that is blocked by inhibitors of spermine synthesis [K. Deng, 2009, cited above]. The mechanism by which polyamines exert their effect on neurite growth is not known. One potential target is the NMDA receptor, activation of which is potentiated by spermine binding [J. Lerma, Spermine
- 25 regulates N-methyl-d-aspartate receptor desensitization. *Neuron* 8, 343-352 (1992); published online Epub2// ([http://dx.doi.org/10.1016/0896-6273\(92\)90300-3](http://dx.doi.org/10.1016/0896-6273(92)90300-3)]. NMDA signaling induces neurite outgrowth, and the spermine sensitive subunit of the receptor is highly expressed during development [D. Georgiev, et al., *Experimental cell research* 314, 2603-2617 (2008); published online EpubAug 15 (10.1016/j.yexcr.2008.06.009); R.
- 30 G. Kalb, Regulation of motor neuron dendrite growth by NMDA receptor activation. *Development* 120, 3063-3071 (1994); J. Zhong, et al, Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. *Journal of*

neurochemistry 64, 531-539 (1995)]. We found that inhibitors of spermine synthesis blocked excess neurite growth in MPS neurons, and that neurite growth could be induced in WT neurons by spermine concentrations similar to those found in patient CSF. Gene therapy in the dog model of MPS I reversed spermine accumulation and normalized expression of GAP43, suggesting that the same pathway was impacted *in vivo*. We could not directly evaluate the impact of spermine synthesis inhibition *in vivo*, as available inhibitors do not cross the blood-brain barrier, and chronic direct CNS administration is not feasible in our animal models. While our *in vitro* findings support a role for spermine in aberrant neurite growth in MPS I, it is important to note that inhibiting spermine synthesis did not completely reverse the phenotype, and spermine addition to normal neurons did not increase neurite growth to the level of MPS I neurons. The effects of spermine modulation may have been limited by the relatively short period of treatment. It is also possible that spermine accumulation is not the sole mediator contributing to neurite outgrowth in MPS I. Notably many neurotrophic factors bind through HS modified receptors, and interactions with HS in extracellular matrix can influence neurite growth [D. Van Vactor, et al., Current opinion in neurobiology 16, 40-51 (2006); published online Epub Feb (10.1016/j.conb.2006.01.011)].

Spermine accumulation may therefore be one of several factors promoting abnormal neurite growth in MPS I. Of the 15 MPS I dog CSF samples screened, only one fell within the normal range of spermine concentration. At 28 days of age, this was the youngest animal included in the study. This finding indicates that spermine accumulation may be age dependent, although this study demonstrates that it is already elevated by 6 months of age in infants with Hurler syndrome. A further study (data not shown here) indicates an age-related effect on spermine levels in MPSI dogs, tracked at 1 month and 1 year. If spermine increases with age in MPS patients, this could explain the kinetics of cognitive decline, as most patients experience 1-2 years of normal development before the onset of developmental delays. The potential for impaired HS metabolism to trigger accumulation of a metabolite that alters neuron growth could point to a novel connection between enzyme deficiencies and the abnormal neurite growth phenotype in MPS, which may explain the cognitive dysfunction associated with these disorders.

These findings also indicate that CSF spermine may be useful as a noninvasive biomarker for assessing pharmacodynamics of novel CNS-directed therapies for MPS.

Table
(Sequence Listing Free Text)

- 5 The following information is provided for sequences containing free text under
numeric identifier <223>.

SEQ ID NO	Free Text under <223>
12	<223> AAV9 vp1 capsid protein

- 10 All publications and references to GenBank and other sequences cited in this
specification are incorporated herein by reference. US Provisional Application Nos.
62/367,770, filed July 28, 2016 and 62/337,186, filed May 16, 2016 and the attached
sequence listing is hereby incorporated by reference, as are US Provisional Patent
Application No. 62/452,560, filed January 31, 2017, US Provisional Patent Application
No. 62/367,798, filed July 28, 2016, US Provisional Patent Application No. 62/337,178,
15 filed May 16, 2016, US Patent Application No. 62/323,271, filed April 16, 2016 and US
Provisional Patent Application No. 62/290,547, filed February 3, 2016. While the
invention has been described with reference to particularly preferred embodiments, it will
be appreciated that modifications can be made without departing from the spirit of the
invention.

20

CLAIMS:

1. A method of determining if a patient has a mucopolysaccharidosis disorder selected from type I, type II, type III or type VII, said method comprising:
 - (a) obtaining a sample from a patient; and
 - (b) detecting whether spermine is present in said sample above a threshold level in the sample, whereby a concentration of spermine in the sample above the threshold level correlates to the patient having an MPS disorder.
2. The method according to claim 1, further comprising obtaining a sample from the patient diagnosed with an MPS disorder according to step (b) is obtained and screening the sample for a deficiency in one or more of a human enzyme selected from: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-glucuronidase.
3. The method according to claim 1 or claim 2, wherein the threshold level corresponds to about 2 ng/mL to about 3 ng/mL spermine in the sample, wherein the sample is cerebrospinal fluid.
4. The method according to claim 2, wherein the patient sample is a blood sample which is assessed for two more enzyme deficiencies.
5. The method according to claim 2, wherein the patient sample is skin or fibroblasts assessed for deficient IDUA levels and/or deficient beta-glucuronidase levels.
6. The method according to claim 2, wherein the patient has a deficiency in IDUA and is treated with the deficiency IDUA.

7. The method according to claim 2, wherein the patient has a deficiency in IDS and is treated with IDS.
8. The method according to claim 2, wherein the patient has a deficiency in beta-glucuronidase and is treated with beta-glucuronidase.
9. The method according to claim 2, wherein a viral vector expressing the deficient enzyme is administered to the patient.
10. The method according to claim 2, wherein the patient receives enzyme replacement therapy and/or a bone marrow transplant.
11. The method according to 2, wherein a viral vector expressing the enzyme is administered to the patient.
12. The method according 2, wherein the patient receives enzyme replacement therapy and/or a bone marrow transplant.
13. A viral vector expressing at least one enzyme selected from alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-glucuronidase in treating a mucopolysaccharidosis in a patient having significantly elevated spermine levels.
14. The viral vector according to claim 13, wherein the patient having an MPS disorder has a concentration of spermine above about 2 ng/mL to about 3 ng/mL, wherein the sample is cerebrospinal fluid.
15. An enzyme selected from one or more of: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase, for use in treating a mucopolysaccharidosis in a patient, comprising:

assaying a sample from a patient for a spermine concentration above a threshold level;

assaying a sample from the patient having the elevated spermine concentration for a deficiency in one or more of the enzymes: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase,

administering a therapeutically effective amount of the enzyme in which the patient is deficient.

16. The enzyme according to claim 15, wherein the sample from a patient having an MPS disorder has a spermine concentration greater than about 2 ng/mL to about 3 ng/mL spermine.

17. A composition comprising at least one viral vector expressing at least one enzyme selected from one or more of: alpha-L-Iduronidase, iduronate sulfatase, heparan N-sulfatase, alpha-N-acetylglucosaminidase, Acetyl CoA: alpha-glycosaminide acetyltransferase, N-acetylglucosamine 6-sulfatase, or beta-Glucuronidase for use in treating a mucopolysaccharidosis in a patient, comprising:

assaying a sample from a patient for the presence of a spermine concentration above a threshold level;

assaying a sample from the patient having the spermine concentration above the threshold for a deficiency in one or more of the enzymes:

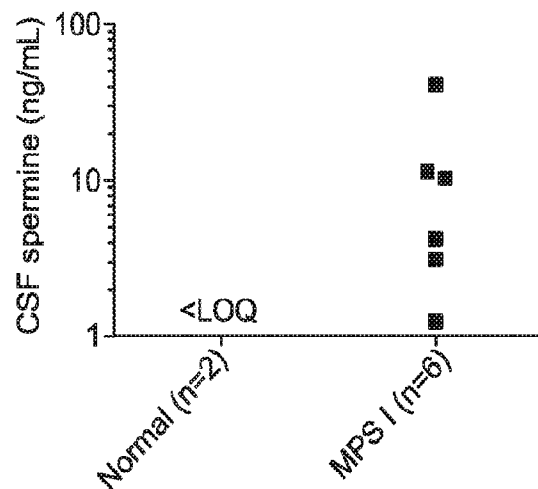
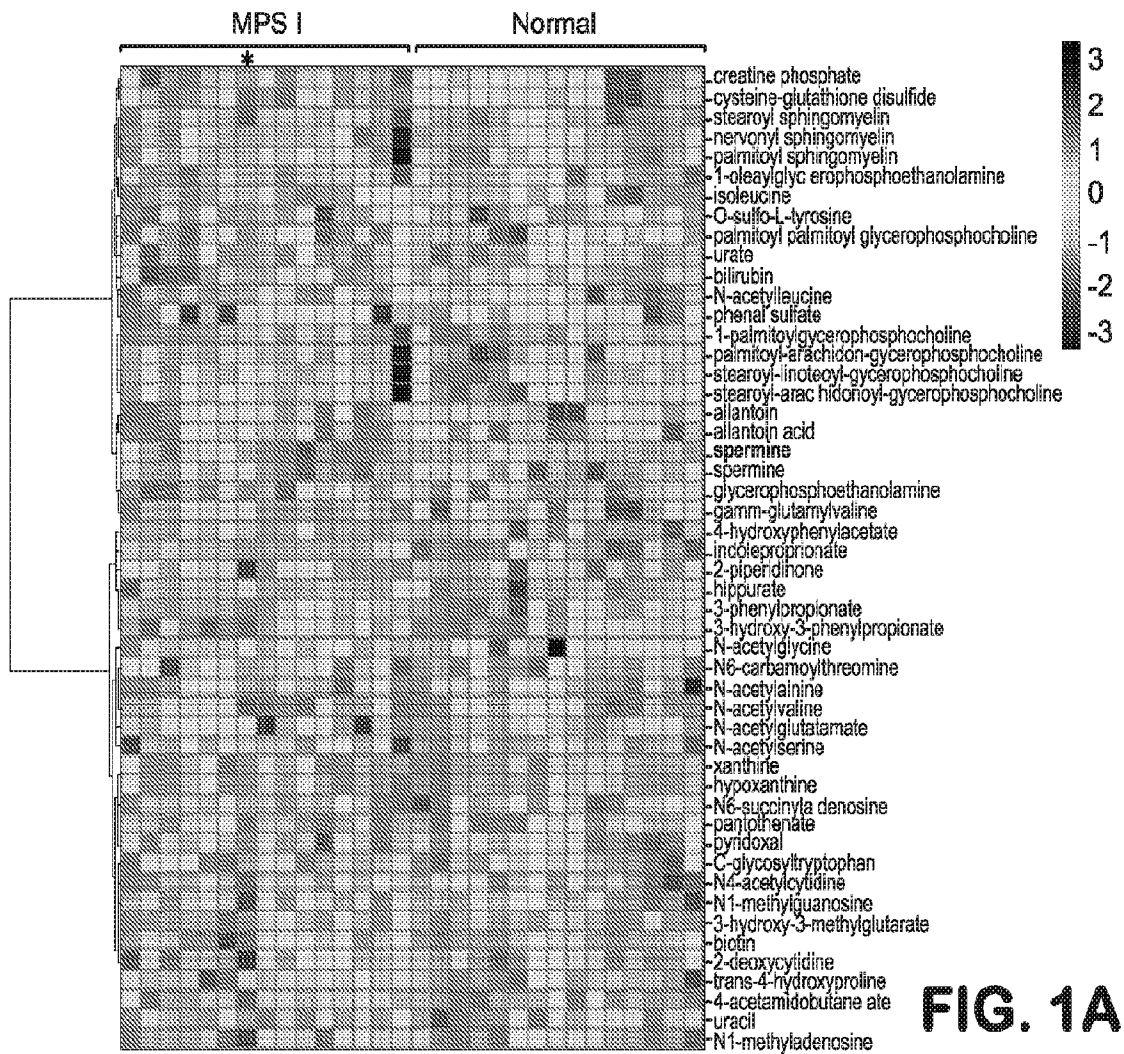
administering a therapeutically effective amount of the composition expressing the enzyme in which the patient is deficient.

18. The composition according to claim 17, wherein the sample from the patient having an MPS disorder has a spermine concentration greater than about 2 ng/mL to about 3 ng/mL spermine.

19. A method of treating MPSI, II, III, or VII in a patient, said method comprising administering an effective amount of a spermine or spermine synthase inhibitor to the patient.

20. The method according to claim 19, wherein a spermine synthase inhibitor is an anti-spermine antibody.
21. The method according to claim 19, wherein the spermine inhibitor comprises a cell surface proteoglycan that blocks binding of spermine to heparin sulfate.
22. The method according to claim 20, wherein the inhibitor is a selective spermine synthase inhibitor selected from N-(3-aminopropyl)cyclohexylamine (APCHA) and methylthiopropylamine (MTPA).
23. The method according to claim 20, wherein the patient is administered an oligonucleotide inhibitor specific for spermine synthase.
24. A method of treating cognitive dysfunction in a patient having MPSI, II, III, or VII in a patient, said method comprising administering an effective amount of an inhibitor of the activity of spermine synthase/spermine synthase to the patient.
25. The method according to claim 24, wherein a spermine synthase inhibitor is an anti-spermine antibody.
26. The method according to claim 28, wherein the spermine inhibitor comprises a cell surface proteoglycan that blocks binding of spermine to heparin sulfate.
27. The method according to claim 26, wherein the inhibitor is a selective spermine synthase inhibitor selected from N-(3-aminopropyl)cyclohexylamine (APCHA) and methylthiopropylamine (MTPA).
28. The method according to claim 27, wherein the patient is administered an oligonucleotide inhibitor specific for spermine synthase.

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**FIG. 1B**

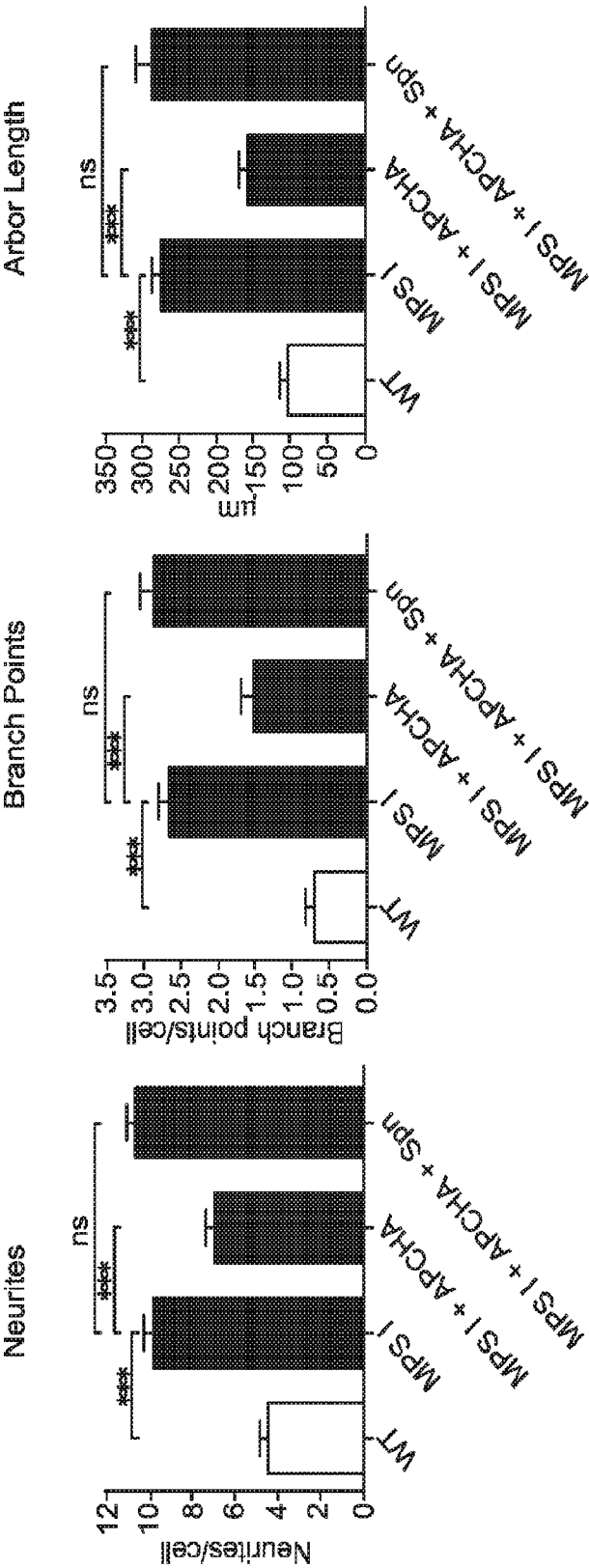


FIG. 2A

FIG. 2B

FIG. 2C

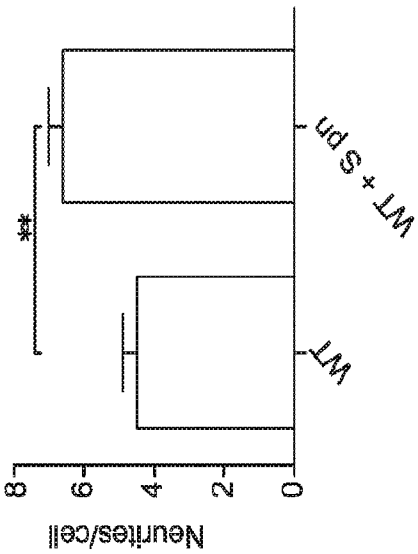


FIG. 2D

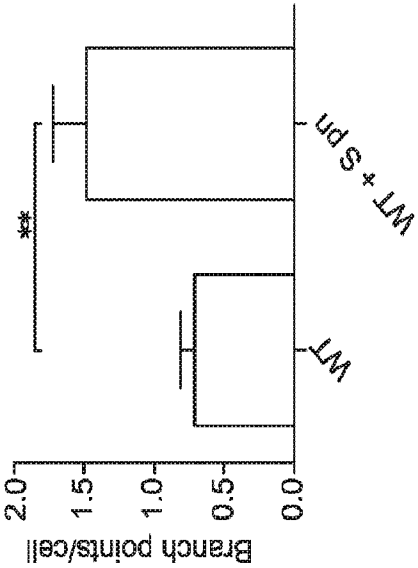


FIG. 2E

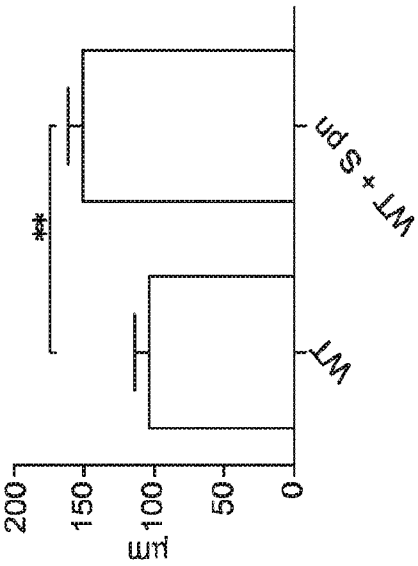
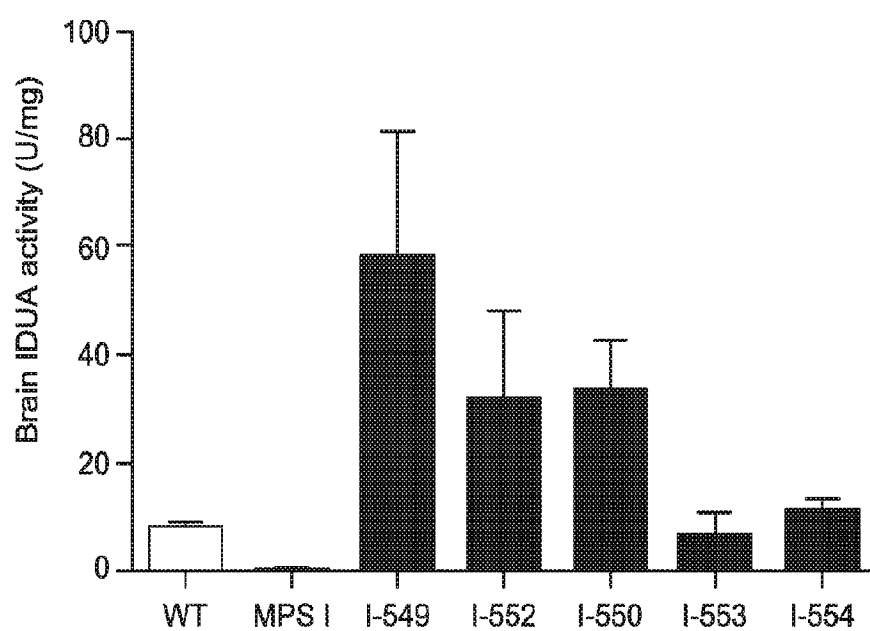
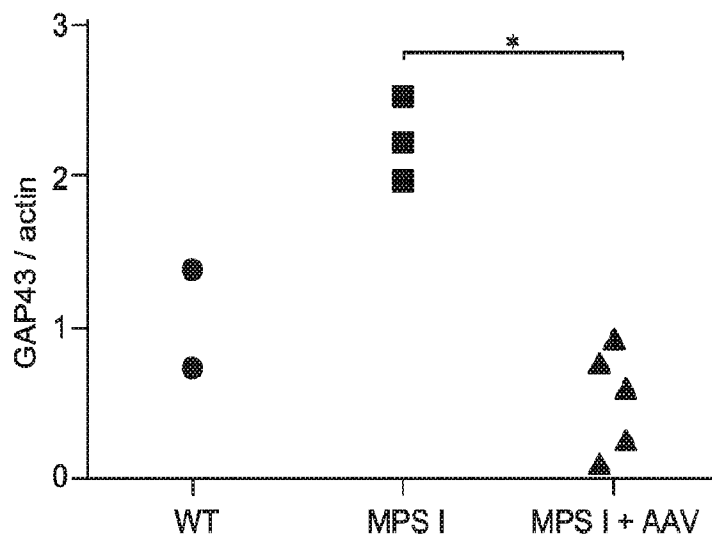
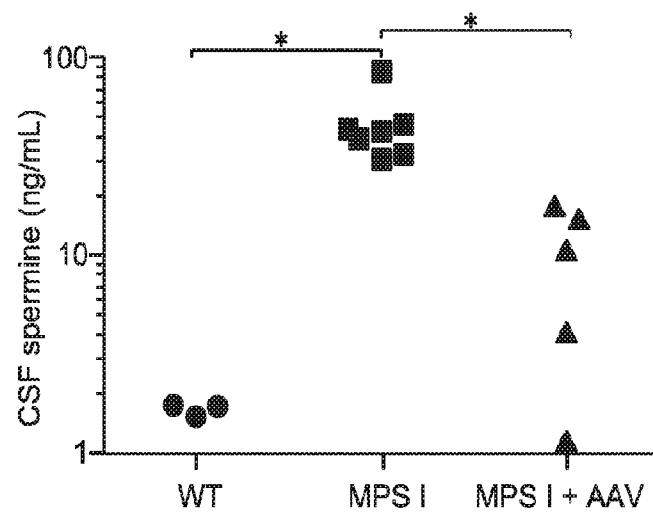


FIG. 2F

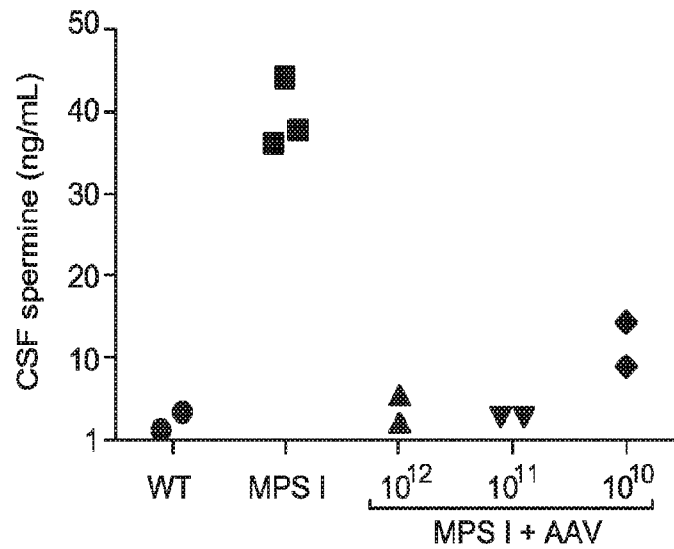
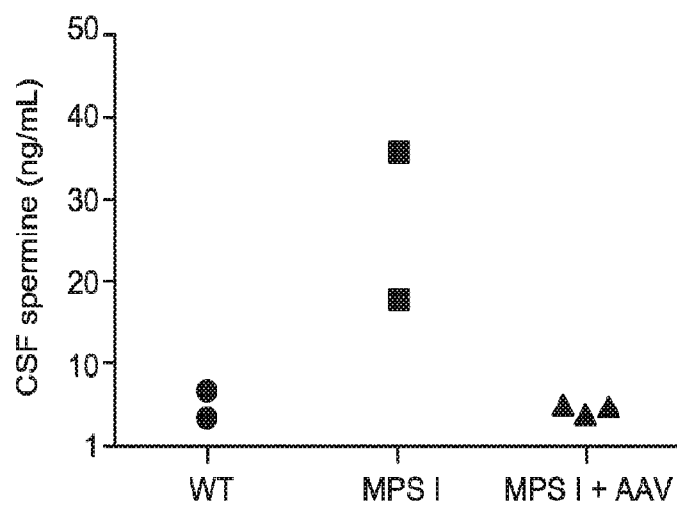
4/10

**FIG. 3A**

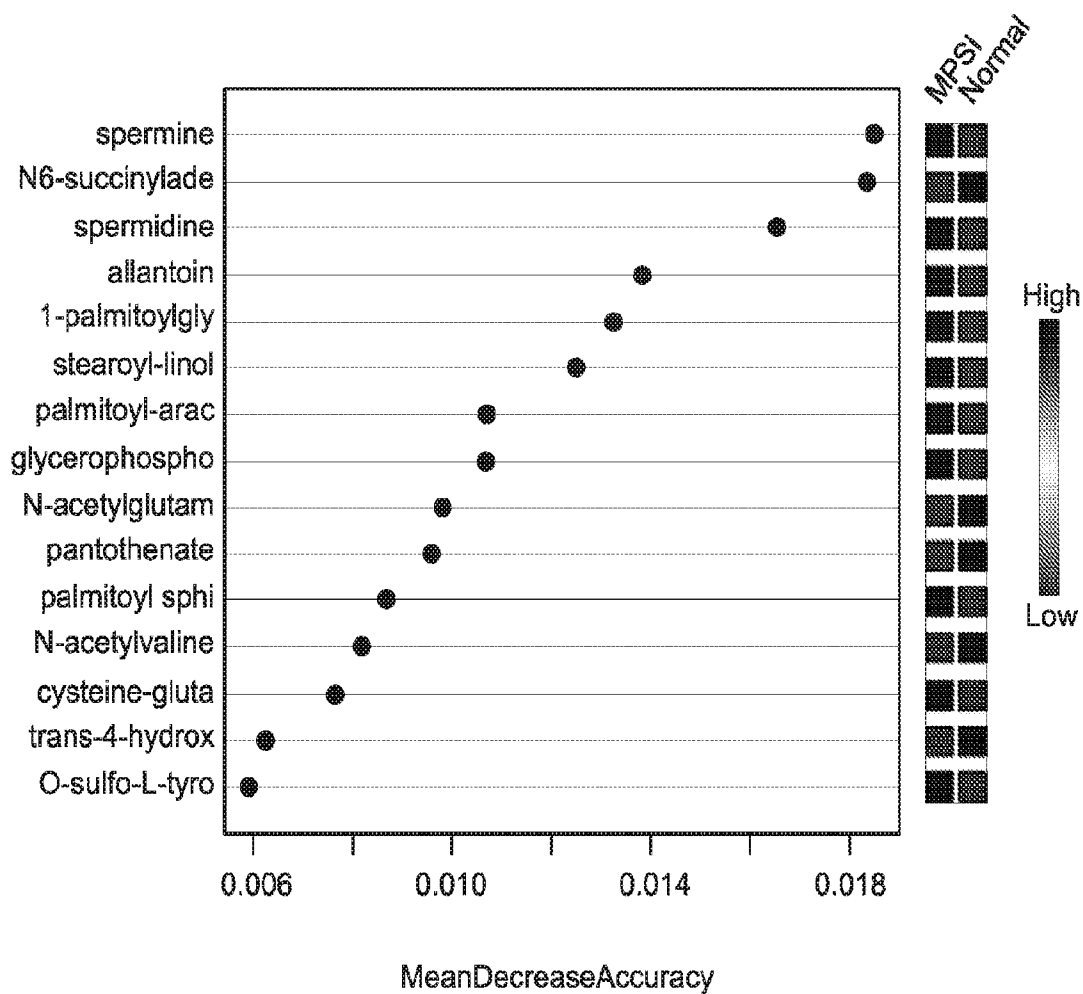
5/10

**FIG. 3B****FIG. 3C**

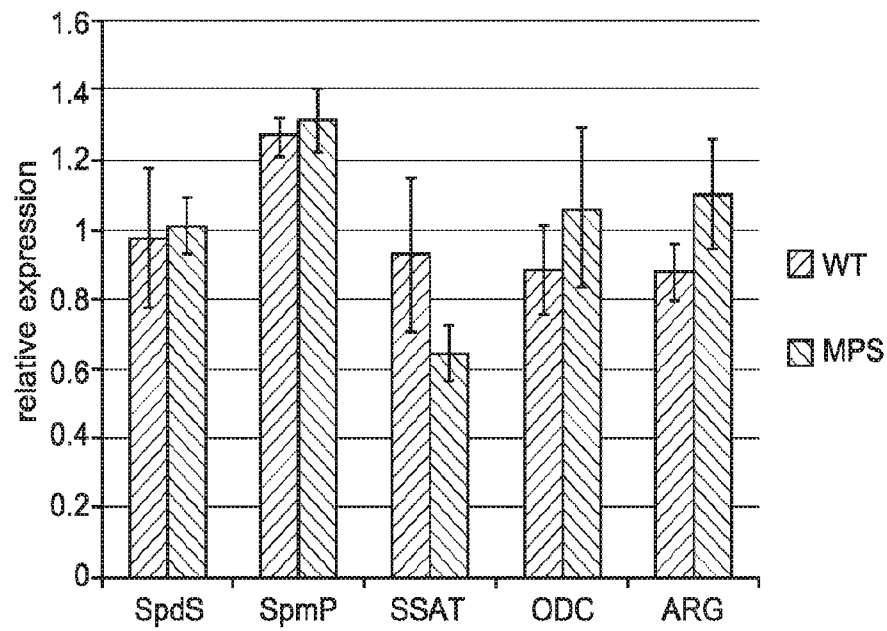
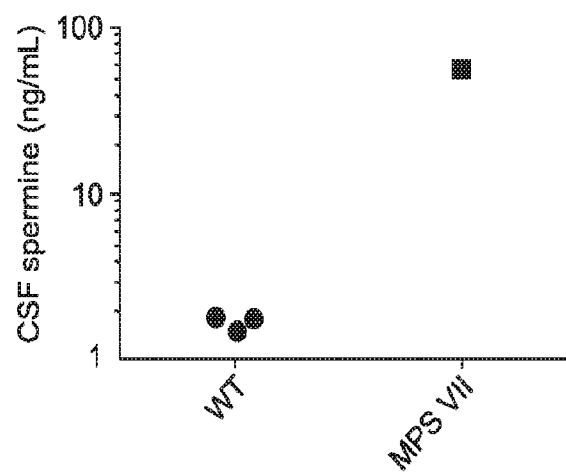
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**FIG. 4A****FIG. 4B**

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**FIG. 5**

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**FIG. 6****FIG. 7**

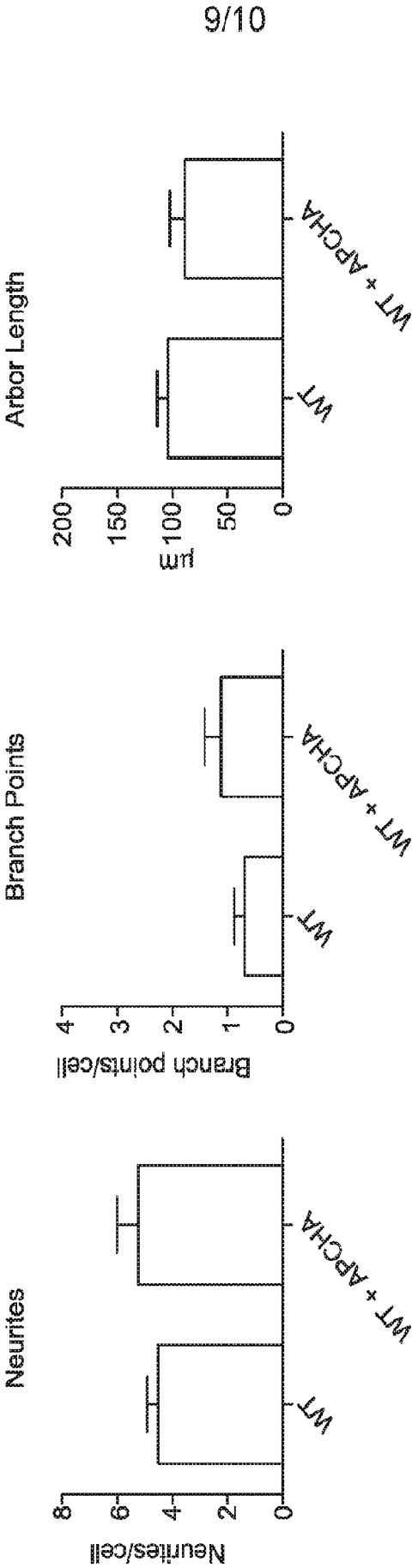
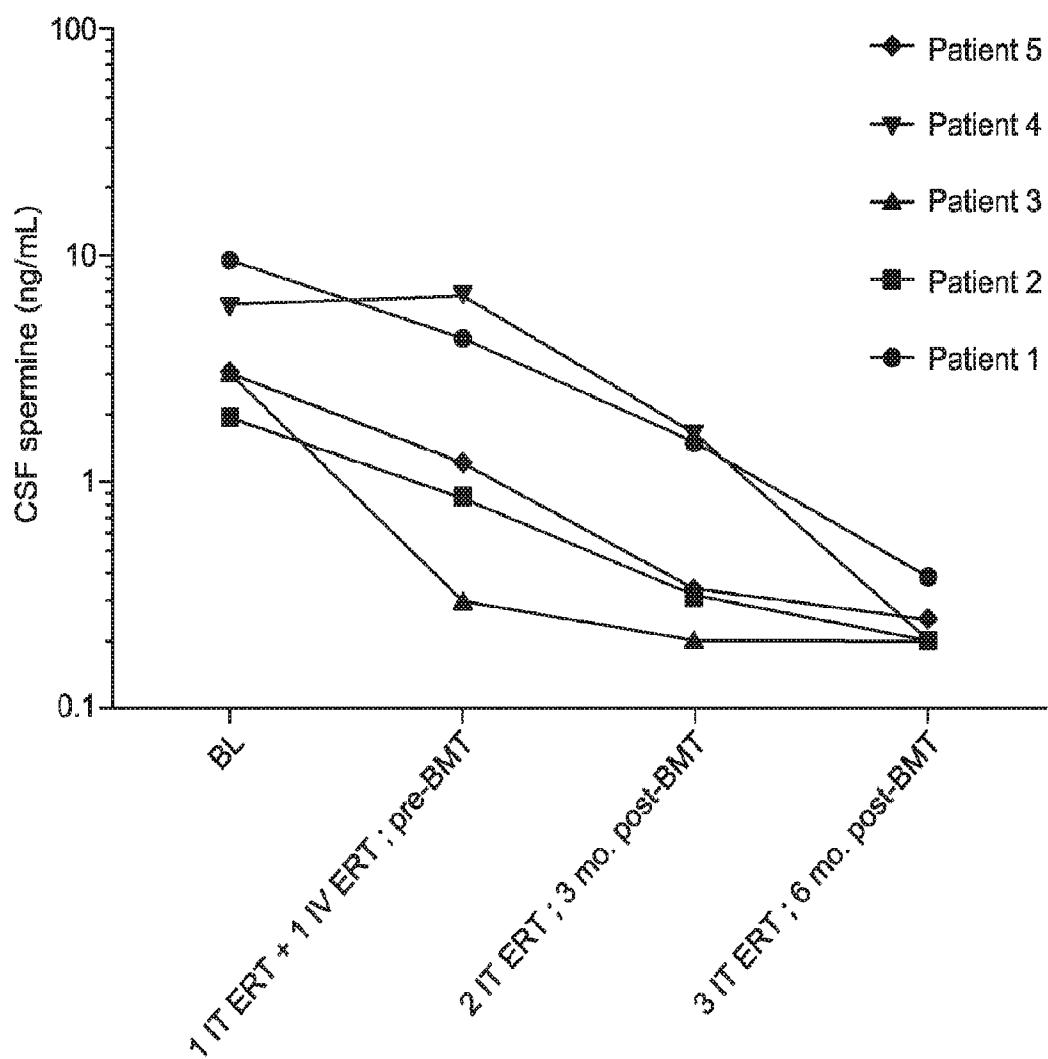


FIG. 8C

FIG. 8B

FIG. 8A

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**FIG. 9**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/16188

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 9/00, 35/76, 38/47, 48/00; C12N 7/00, 9/00, 9/24, 15/86; C12Q 1/68 (2017.1)
 CPC - A61K 9/0019, 38/47, 35/76, 48/00, 48/0058; C12N 7/00, 9/00, 15/86; C12Y 302/00, 302/01076;
 C12Q 1/68

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	(COBOS, PN et al.) Dried Blood Spots Allow Targeted Screening to Diagnose Mucopolysaccharidosis and Mucopolipidosis. Journal of Inherited Metabolic Disease. 06 May 2014, Vol. 15, pages 123-132; page 123, 1st column, 1st – 2nd paragraphs; page 123; 2nd column, 2nd paragraph; DOI 10.1007/8904_2014_308	1-2, 3/1-2, 4-11
A	US 2013/0345526 A1 (FLINT HILLS SCIENTIFIC, LLC) 26 December 2013; paragraph [0046], [0054]	1-2, 3/1-2, 4-11
A	(PARK, MH et al.) Polyamines and Their Metabolites as Diagnostic Markers of Human Diseases; Biomolecules and Therapeutics; January 2013; Vol. 21, No. 1, pages 1-9; http://dx.doi.org/10.4002/biomolther.2012.097 ; page 5, 2nd column, 3rd paragraph; abstract	1-2, 3/1-2, 4-11
A	US 2011/0008810 A1 (TOMATSU, S et al.) 13 January 2011; paragraph [0001], [0007], [0075]	1-2, 3/1-2, 4-11
A	US 2016/0000887 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 07 January 2016; paragraph [0007], [0009], [0011], [0060], [0072]; claim 263	13-28
A	US 2006/0057114 A1 (WHITLEY, CB et al.) 16 March 2006; paragraph [0009]-[0010], [0017], [0021]	13-28
A	(GURDA, BL et al.) Evaluation of AAV-mediated Gene Therapy for Central Nervous System Disease in Canine Mucopolysaccharidosis VII; Molecular Therapy; February 2016; Vol. 24, No. 2, pages 206-216; doi:10.1038/mt.2015.189; page 206, 1st column, 1st paragraph; page 206, 2	13-18



Further documents are listed in the continuation of Box C.



See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"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

22 March 2017 (22.03.2017)

Date of mailing of the international search report

18 APR 2017

Name and mailing address of the ISA/

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Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/16188

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	(HINDERER, C et al.) Intrathecal Gene Therapy Corrects CNS Pathology in a Feline Model of Mucopolysaccharidosis I; Molecular Therapy; December 2014; Vol. 22, No. 12, pages 2018-2027; doi:10.1038/mt.2014.135; page 2018; 1st column, 1st paragraph; page 2018, 2nd column, 2nd paragraph; page 2019, 2nd column, 2nd paragraph	13-18
A	WO 00/06546 (THE PICOWER INSTITUTE FOR MEDICAL RESEARCH) 31 October 2002; page 1, line 6; page 5, line 12	19-28
A	US 2002/0137797 A1 (MEYSKENS, FL et al.) 26 September 2002; paragraph [0024]	19-28