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(54) Title: MITOCHONDRIAL AUGMENTATION THERAPY OF BRAIN DISEASES

(57) Abstract: The present invention provides stem cells enriched with healthy functional mitochondria, pharmaceutical compositions comprising these cells and methods of use thereof for treating brain diseases, disorders and symptoms thereof where the disease may or may not be associated with acquired mitochondrial dysfunction.



WO 2020/021538 A1

MITOCHONDRIAL AUGMENTATION THERAPY OF BRAIN DISEASES

FIELD OF THE INVENTION

The present invention provides stem cells enriched with functional mitochondria and
5 methods of using mitochondrial augmentation therapy for treating CNS-associated diseases,
disorders and conditions in humans.

BACKGROUND OF THE INVENTION

Central nervous system (CNS) diseases, also known as central nervous system
disorders, are a group of neurological disorders that affect the structure and/or function of the
10 brain or spinal cord, which collectively form the central nervous system.

Being one of the most sensitive organs in the human body, the brain is prone to a wide
variety of diseases, including e.g. traumas and injuries to the brain or spinal cord, neurologic
infectious diseases, genetic disease, degeneration, auto-immune disorders and interruptions of
oxygen and blood supply to the brain.

15 Mitochondrial diseases are a genetically heterogeneous group of disorders caused by
mutations in mitochondrial DNA (mtDNA) displaying a wide range of severity and
phenotypes (Wallace, D. C. and Chalkia, D. Cold Spring Harb. Perspect. Biol.
2013;5:a021220). The prevalence of mtDNA-related disease is about 1 in 8500 in the
population (Elliott, H. R. et al., American Journal of Human Genetics, 83, 254-260, 2008),
20 yet to date, apart from supportive therapy there is no effective treatment for the majority of
mitochondrial diseases. A variety of treatments have been evaluated in clinical trials but none
has delivered breakthrough results (Kanabus, M. et al., British journal of pharmacology, 171,
1798-1817, 2014).

WO 2013/035101 to the present inventors relates to mitochondrial compositions and
25 therapeutic methods of using same, and discloses compositions of partially purified
functional mitochondria and methods of using the compositions to treat conditions which
benefit from increased mitochondrial function by administering the compositions to a subject
in need thereof.

WO 2016/008937 relates to methods for the intercellular transfer of mitochondria
30 isolated from a population of donor cells into a population of recipient cells. The methods
show improved efficacy of transfer of an amount mitochondria.

US 2012/0107285 is directed to mitochondrial enhancement of cells. Certain embodiments include, but are not limited to, methods of modifying stem cells, or methods of administering modified stem cells to at least one biological tissue.

WO 2016/135723 to the present inventors relates to human bone-marrow cells enriched by at least 50% with functional mitochondria, methods for their production, and therapeutic methods utilizing such cells.

There is a long-felt need in the field of therapy of brain diseases, e.g. those unrelated to primary mitochondrial diseases, for effective and long term therapies.

10 SUMMARY OF THE INVENTION

According to the principles of the present invention, human stem cells enriched with healthy and functional mitochondria are introduced into a subject afflicted with a brain disease or disorder. This process, generally referred to as “mitochondria augmentation therapy”, increases the number of healthy and functional mitochondria within these stem cells. Stem cells enriched with healthy and functional mitochondria are administered to patients suffering from brain-related diseases and disorders to provide alleviation of symptoms associated with the brain-related diseases or disorders. In certain embodiments, the disease or disorder is associated with acquired mitochondrial dysfunction. In other embodiments, the disease or disorder is not associated with acquired mitochondrial dysfunction.

The present invention is based in part on the findings that a single round of treatment of juveniles with Pearson Syndrome (PS), a congenital disease caused by a mutation in mitochondrial DNA, and a patient with Kearns-Sayre Syndrome (KSS) with stem cells enriched with healthy functional mitochondria was sufficient to ameliorate significantly a variety of adverse conditions and symptoms relating to brain function.

Without being restricted to any theory or mechanism, it is hypothesized that the systemic administration of mitochondrially-augmented human stem cells resulted in the enhancement of mitochondrial activity in the patient’s CNS, and that this enhancement ameliorated the severity of symptoms of Pearson Syndrome and KSS, including brain-related symptoms deriving directly or indirectly from the mitochondrial DNA mutation.

The present invention provides, in one aspect, a method for treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, , the method

comprising the step of administering parenterally a pharmaceutical composition to the patient, the pharmaceutical composition comprising at least about 5×10^5 to 5×10^9 human stem cells, wherein the human stem cells are enriched with frozen-thawed healthy functional human exogenous mitochondria, wherein the brain disease or disorder is not a mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule.

In another aspect, the present invention provides a pharmaceutical composition for use in treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the composition comprising at least 10^5 to 2×10^7 human stem cells per kilogram bodyweight of the patient in a pharmaceutically acceptable liquid medium capable of supporting the viability of the cells, wherein the human stem cells are enriched with frozen thawed healthy functional human exogenous mitochondria, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial protein.

In some embodiments, the enrichment comprises introducing into the stem cells a dose of mitochondria of at least 0.088 up to 176 milliunits of CS activity per million cells.

In further embodiments, the enrichment comprises contacting the stem cells with a dose of mitochondria of 0.88 up to 17.6 milliunits of CS activity per million cells.

In some embodiments, the dose of isolated mitochondria is added to the recipient cells at the desired concentration. The ratio of the number of mitochondria donor cells versus the number of mitochondria recipient cells is a ratio above 2:1 (donor cells vs. recipient cells). In typical embodiments, the ratio is at least 5, alternatively at least 10 or higher. In specific embodiments, the ratio of donor cells from which mitochondria are collected to recipient cells is at least 20, 50, 100 or possibly even higher. Each possibility is a separate embodiment.

In certain embodiments, the healthy functional human exogenous mitochondria are syngeneic or allogeneic. In certain embodiments, the healthy functional human exogenous mitochondria are syngeneic. In certain embodiments, the healthy functional human exogenous mitochondria are autologous, i.e., of the same maternal bloodline. In certain embodiments, the healthy functional human exogenous mitochondria are allogeneic.

In certain embodiments, the disease or disorder is associated with acquired mitochondrial dysfunction. In other embodiments, the disease or disorder is not associated with acquired mitochondrial dysfunction.

In certain embodiments, the disease or disorder is selected from the group consisting of Parkinson Disease (PD), Alzheimer Disease (AD), stroke, epilepsy, multiple sclerosis (MS), dementia, adrenoleukodystrophy (ALD), Huntington Disease (HD), Guillain Barre Syndrome (GBS), autism and Friedreich's ataxia. Each possibility represents a separate
5 embodiment of the present invention.

In certain embodiments the disease or disorder is a psychiatric disorder. In specific embodiments the psychiatric disorder is selected from schizophrenia including schizophrenia related disorders and psychotic disorders, mood disorders including bipolar disorder, obsessive compulsive disorder, depressive disorders, as well as personality disorders.

10 In certain embodiments, the symptom is selected from the group consisting of impaired ability to execute commands, impaired ability to speak, impaired response time, impaired motor skills, impaired learning, impaired ability to walk, impaired ability to climb stairs, impaired EEG (Electroencephalogram), convulsions or seizures, impaired ability to draw, impaired language skills and impaired memory. Each possibility represents a separate
15 embodiment of the present invention.

In certain embodiments, the pharmaceutical composition is administered by systemic administration. According to some embodiments the administration is intravenous. In certain embodiments, the pharmaceutical composition is administered directly to the central nervous system (CNS). In certain embodiments, the pharmaceutical composition is administered
20 intrathecally. In certain embodiments, the pharmaceutical composition is administered directly to the brain. In certain embodiments, the pharmaceutical composition is administered directly to the spinal cord.

In certain embodiments, the pharmaceutical composition comprises about 10^6 mitochondrially-enriched human stem cells per kilogram body weight of the patient. In certain
25 embodiments, the pharmaceutical composition comprises about total of 5×10^5 to 5×10^9 human stem cells enriched with human mitochondria.

In certain embodiments, the mitochondrially-enriched human stem cells have at least one of: (i) an increased mitochondrial DNA content; (ii) an increased level of CS activity; (iii) an increased content of at least one mitochondrial protein selected from SDHA and COX1;
30 (iv) an increased rate of O_2 consumption; (v) an increased rate of ATP production; or (vi) any combination thereof, relative to the corresponding level in the stem cells prior to mitochondrial enrichment. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, the human stem cells are obtained or derived from the patient before enrichment with the exogenous mitochondria.

In certain embodiments, the human stem cells are obtained or derived from a donor different than the patient before enrichment with the exogenous mitochondria. In certain
5 embodiments, the donor of the stem cells is at least partly HLA-matched with the patient. In certain embodiments, the method described above further comprises a step of administering to the patient an agent which prevents, delays, minimizes or abolishes an adverse immunogenic reaction between the patient and the mitochondrially-enriched human stem cells. In certain
embodiments, the adverse immunogenic reaction is a graft-versus-host disease (GvHD).

10 In certain embodiments, the human stem cells are CD34⁺. In certain embodiments, the human stem cells are hematopoietic stem cells. In certain embodiments, the human stem cells are mesenchymal stem cells. In certain embodiments, the human stem cells are pluripotent stem cells (PSCs) or induced pluripotent stem cells (iPSCs).

In certain embodiments, the method described above further comprises the preceding
15 steps of isolating, deriving or obtaining human stem cells, and introducing healthy functional human exogenous mitochondria into the human stem cells, thus producing the mitochondrially-enriched human stem cells. In certain embodiments, the method comprises (a) freezing the human stem cells, (b) thawing the human stem cells, and (c) introducing healthy functional human exogenous mitochondria into the human stem cells. In certain
20 embodiments, the human stem cells are isolated, derived or obtained from cells of the bone marrow. In other embodiments, the human stem cells are isolated, derived or obtained from adipose tissue, oral mucosa, skin fibroblasts, blood or umbilical cord blood. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, the human stem cells have undergone at least one freeze-thaw
25 cycle prior to introducing healthy functional human exogenous mitochondria into said human stem cells.

In certain embodiments, the method comprises (a) freezing the healthy functional human exogenous mitochondria, (b) thawing the healthy functional human exogenous mitochondria, and (c) introducing the healthy functional human exogenous mitochondria into
30 the human stem cells. In certain embodiments, the human stem cells are isolated, derived or obtained from cells of the bone marrow, adipose tissue, oral mucosa, skin fibroblasts, blood or umbilical cord blood. In certain embodiments, the healthy functional human exogenous

mitochondria are isolated or obtained from placenta, placental cells grown in culture or blood cells. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, the human stem cells have undergone at least one freeze-thaw cycle after enrichment with the healthy functional human exogenous mitochondria. In certain
5 embodiments, the method further comprises the preceding steps of (a) freezing the human stem cells enriched with healthy functional human exogenous mitochondria, and (b) thawing the human stem cells enriched with healthy functional human exogenous mitochondria, prior to administering the human stem cells enriched with healthy functional human exogenous mitochondria to the patient.

10 In certain embodiments, the healthy functional human exogenous mitochondria constitute at least 3% of the total mitochondria in the mitochondrially enriched human stem cells. In certain embodiments, the healthy functional human exogenous mitochondria constitute at least 10% of the total mitochondria in the mitochondrially enriched human stem cells.

15 In certain embodiments, the pharmaceutical composition further comprises non-enriched stem cells, megakaryocytes, erythrocytes, mast cells, myeloblasts, basophils, neutrophils, eosinophils, monocytes, macrophages, natural killer (NK) cells, small lymphocytes, T lymphocytes, B lymphocytes, plasma cells, reticular cells, or any combination thereof. Each possibility represents a separate embodiment of the present invention.

20 The present invention further provides, in another aspect, a plurality of human stem cells enriched with healthy functional human exogenous mitochondria, for use in treating brain diseases or disorders.

The present invention further provides, in another aspect, a pharmaceutical composition comprising a therapeutically-effective amount of a plurality of human stem cells
25 enriched with healthy functional human exogenous mitochondria, for use in treating brain diseases or disorders, as described above.

In certain embodiments, the pharmaceutical composition described above is for use in treating a brain disease or a brain disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation
30 in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule, such as, for example, a protein or a peptide.

The present invention further provides, in another aspect, a method for treating a brain disease or a brain disorder or a symptom thereof in a patient in need thereof, comprising

administering to the patient the pharmaceutical composition described above, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule, such as, for example, a protein or a peptide.

5 Further embodiments and the full scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those
10 skilled in the art from this detailed description. Further limitations and disadvantages of conventional and traditional approaches will become apparent to one of skill in the art, though comparison of such systems with some aspects of the present invention as set forth in the remainder of the present application with reference to the drawings.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing a comparison of FVB/N ATP8 mutated mtDNA levels in the brains of FVB/N mice 1 month post administration of stem cells enriched with healthy functional wild type mitochondria (isolated from liver of C57BL mice), in untreated FVB/N mice (Naive), FVB/N mice administered with stem cells enriched with C57BL healthy liver
20 mitochondria (C57BL Mito), FVB/N mice administered with stem cells enriched with C57BL healthy mitochondria and were subjected to total body irradiation (TBI) prior to stem cells administration (TBI C57BL Mito) and FVB/N mice administered with stem cells enriched with C57BL healthy mitochondria and were subjected to Busulfan chemotherapeutic agent prior to stem cell administration (Busulfan C57BL Mito).

25 **Figure 2** is a graph bar illustrating the biodistribution of bone marrow cells enriched with mitochondria by the amount of C57BL mtDNA in the brains of mice up to 3 months after MAT. White bars and associated dots indicate augmented bone marrow samples, grey bars are controls.

Figure 3A is a scheme of the different stages of treatment of a Pearson Syndrome (PS)
30 patient, as provided by the present invention.

Figure 3B is a line graph illustrating the standard deviation score of the weight and height of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

Figure 3C is a line graph illustrating the alkaline phosphatase level of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

Figure 3D is a bar graph illustrating the level of lactate in the blood of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

5 **Figure 4** is a line graph illustrating the normal mtDNA content in 3 PS patients (Pt.1, Pt.2 and Pt.3) treated by the methods provided in the present invention as a function of time before and after therapy, as measured by digital PCR for the deleted region (in each patient) compared to the 18S genomic DNA representing number of normal mtDNA per cell, and normalized per baseline.

10 **Figure 5A** is another scheme of the different stages of treatment of a Pearson Syndrome (PS) patient, as further provided by the present invention.

Figure 5B is a bar graph illustrating the level of lactate in the blood of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

15 **Figure 5C** is a bar graph illustrating the ATP level in lymphocytes of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

Figure 6A is yet another scheme of the different stages of treatment of a Pearson Syndrome (PS) patient, as further provided by the present invention.

Figure 6B is a bar graph illustrating the level of lactate in the blood of a PS patient treated by the methods provided in the present invention as a function of time before and after therapy.

20 **Figures 7A-7C** show line graphs illustrating open field behavioral test performance of 12-month old C57BL/6J mice treated with: mitochondria-enriched BM cells (MNV-BM-PLC, 1×10^6 cells, n=24), bone marrow cells (BM control, 1×10^6 cells, n=23) or a control vehicle solution (control, 4.5% Albumin in 0.9% w/v NaCl, n=23), before treatment and 9 months post treatment. **Figure 7A** shows quantification of the distance moved during the open field test. **Figure 7B-** shows center duration (time (s) or % change from baseline); **Figure 7C-** wall duration (time(s) or % change from baseline).

30 **Figures 8A-8B** show bar graphs illustrating Rotarod test of 12-month old C57BL/6J mice administered treated with either mitochondria-enhanced bone marrow (BM) cells (MNV-BM-PLC, 1×10^6 cells, n=24), bone marrow cells (BM, 1×10^6 cells, n=23) or a control vehicle solution (VEHICLE, 4.5% Albumin in 0.9% w/v NaCl, n=23). The results presented are before treatment and 1 and 3 months after treatment. **Figure 8A-** Rotarod score (in seconds (s)), of the various treated test groups at the indicated time points. **Figure 8B-** Rotarod score

(presented as percentage from baseline), of the various treated test groups at the indicated time points.

DETAILED DESCRIPTION OF THE INVENTION

5 It has now been shown for the first time that human stem cells loaded with healthy functional exogenous mitochondria can achieve *in-vivo* systemic delivery of healthy functional mitochondria to organs, tissues and cells in patients suffering from diseases and disorders of diversified etiologies.

10 Without being limited to any theory or mechanism, it is now hypothesized that functional exogenous mitochondria can enter human stem cells, and thereby increase their mitochondrial activity and energy production. Such cells may hypothetically reach distal organs through circulation, and transfer at least part of their functional mitochondria to cells in other organs.

15 Again without being limited to any theory or mechanism, it is now further hypothesized that augmented stem cells as described below are recruited to damaged or diseased organs and improve their function either by e.g. mitochondrial transfer, secretion of different factors, differentiation, etc.

20 More specifically, it has been surprisingly found that a single round of treatment of young Pearson Syndrome (PS) patients as well as a Kearns Sayre Syndrome (KSS) patient by autologous stem cells which underwent mitochondria augmentation therapy was sufficient to significantly ameliorate a wide variety of adverse PS-related and PS-independent symptoms.

25 The present invention provides, in one aspect, a method for treating a brain disease, disorder or a symptom thereof in a subject in need of such treatment, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule, the method comprising the step of administering a pharmaceutical composition comprising a plurality of stem cells to the patient, wherein the stem cells are enriched with healthy functional exogenous mitochondria. In some embodiments, the subject is a mammalian subject and the stem cells are mammalian stem cells. In certain
30 embodiments, the subject is a human subject and the stem cells are human stem cells.

 In some embodiments, the present invention provides a method for treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, wherein

the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule, the method comprising the step of administering a pharmaceutical composition comprising a plurality of human stem cells to the patient, wherein the human stem cells are enriched with healthy functional human exogenous mitochondria.

As used herein and in the claims, the terms “mitochondrial disease” and “primary mitochondrial disease” may be interchangeably used. The terms “mitochondrial disease” and “primary mitochondrial disease” refer to a congenital mitochondrial disease which is diagnosed by a known or indisputably pathogenic mutation in the mitochondrial DNA, or by mutations in genes of the nuclear DNA, whose gene products are imported into the mitochondria. The phrase “the brain disease or disorder is not a primary mitochondrial disease” means that the brain disease or disorder is not primarily diagnosed by a known or indisputably pathogenic mutation in the mitochondrial DNA, or by mutations in genes of the nuclear DNA whose gene products are imported into the mitochondria. The brain disease or disorder which is the object of treatment of the present invention is not necessarily operably linked to a mutation, or to a group of mutations, in a coding region in mitochondrial or nuclear DNA, coding for a mitochondrial molecule.

In some embodiments, the brain disease or disorder is associated with acquired mitochondrial dysfunction. In other embodiments, the disease or disorder is not associated with acquired mitochondrial dysfunction. In some embodiments, the brain disease or disorder is associated with a secondary mitochondrial dysfunction. In other embodiments, the brain disease or disorder is not associated with a secondary mitochondrial dysfunction.

As used herein, the term “secondary mitochondrial dysfunction” and “acquired mitochondrial dysfunction” are used interchangeably and refer to an acquired mitochondrial dysfunction that can accompany many non-primary mitochondrial diseases and may be caused by genes encoding neither function nor production of the oxidative phosphorylation (OXPHOS) proteins. Secondary mitochondrial dysfunction can also be caused by environmental factors which can cause oxidative stress.

In another aspect, the present invention provides a pharmaceutical composition for use in treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the composition comprising a plurality of human stem cells in a pharmaceutically acceptable liquid medium capable of supporting the viability of the cells, wherein the human stem cells are enriched with frozen thawed healthy functional human

exogenous mitochondria., wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial protein.

In some embodiments, the pharmaceutical composition comprises at least 10^5 to 4×10^7 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. In some
5 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. In some embodiments, the pharmaceutical composition comprises at least 10^5 to 2×10^7 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. In some embodiments, the pharmaceutical composition comprises at least 5×10^5 to 1.5×10^7 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. In some
10 embodiments, the pharmaceutical composition comprises at least 10^6 to 10^7 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. In other embodiments, the pharmaceutical composition comprises at least 10^5 or at least 10^6 mitochondrially-enriched human stem cells per kilogram bodyweight of the patient. Each possibility represents a separate embodiment of the present invention. In some embodiments, the pharmaceutical
15 composition comprises a total of at least 5×10^5 up to 5×10^9 mitochondrially-enriched human stem cells. In some embodiments, the pharmaceutical composition comprises a total of at least 10^6 up to 10^9 mitochondrially-enriched human stem cells. In other embodiments, the pharmaceutical composition comprises a total of at least 2×10^6 up to 5×10^8 mitochondrially-enriched human stem cells.

20 In certain embodiments, the healthy functional human mitochondria are autologous or allogeneic. In certain embodiments, the healthy functional human mitochondria are autologous. In certain embodiments, the healthy functional human mitochondria are allogeneic.

In certain embodiments, the method is for treating a brain disease, disorder or a
25 symptom thereof in a human patient in need of such treatment, the method comprising the step of administering a pharmaceutical composition comprising a plurality of human stem cells to the patient, wherein the human stem cells are enriched with healthy functional autologous or allogeneic mitochondria without a pathogenic mutation in mitochondrial DNA and without a mutated mitochondrial protein encoded by nuclear DNA, and wherein the brain
30 disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule (such as a protein, peptide, nucleic acid, and the like).

In some embodiments, there is provided a method for treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the method comprising

the step of administering a pharmaceutical composition comprising a plurality of human stem cells to the patient, wherein the human stem cells are enriched with healthy functional exogenous mitochondria without a pathogenic mutation in mitochondrial DNA, and wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA.

The term “method” as used herein generally refers to manners, means, techniques and procedures for accomplishing a given task, including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

The term “treating” as used herein includes the diminishment, alleviation, or amelioration of at least one symptom associated or induced by a disease or condition. The term “treating” as used herein also includes preventative (e.g., prophylactic), palliative and curative treatment.

The term “pharmaceutical composition” as used herein refers to any composition comprising at least one biologically active agent. As used herein, the term “pharmaceutical composition” further refers to a composition comprising an active pharmaceutical ingredient to be delivered to a subject, for example, for therapeutic, prophylactic, diagnostic, preventative or prognostic effect. The term “pharmaceutical composition” as used herein further refers to any composition comprising human stem cells, optionally further comprising a medium or carrier in which the cells are maintained in a viable state. In certain embodiments, the pharmaceutical composition comprises the active pharmaceutical ingredient and a pharmaceutically acceptable carrier. As used herein, the term “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In certain embodiments, the pharmaceutical composition is frozen. In certain embodiments, the pharmaceutical composition is thawed. In certain embodiments, the pharmaceutical composition is thawed prior to being administered. In certain embodiments, the pharmaceutical composition is thawed up to 24 hours prior to being administered. In certain embodiments, the enriched human stem cells are the only active ingredient in the pharmaceutical composition.

The term “biologically active agent” as used herein refers to any molecule capable of eliciting a response in a biological system such as, for example, living cell(s), tissue(s), organ(s), and being(s). Non-limiting examples of biologically active agents according to the present inventions include cells, intact mitochondria, mitochondrial DNA, and a mitochondrial protein. According to the principles of the present invention, a plurality of human stem cells enriched with healthy functional human mitochondria without a pathogenic mutation in mitochondrial DNA is a biologically active agent.

The phrase “a brain disease or a brain disorder” as used herein refers to damage to, or a disease of, the brain.

It is to be understood explicitly that for diseases associated or caused by genetic abnormalities the methods and compositions of the present invention will be useful to alleviate the symptoms of the disease rather than to treat the underlying pathology.

The term “therapeutically-effective amount” or “an effective amount” refers to the amount of an active agent or composition that is required to confer a therapeutic effect on the treated patient. Effective doses will vary, as recognized by those skilled in the art, e.g. depending on route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

The term “stem cells” as used herein generally refers to any human stem cells. Stem cells are undifferentiated cells that can differentiate into other types of cells and can divide to produce more of the same type of stem cells. Stem cells can be either totipotent or pluripotent. The term “human stem cells” as used herein generally refers to all stem cells naturally found in humans, and to all stem cells produced or derived ex-vivo and are compatible with humans. A “progenitor cell”, like a stem cell, has a tendency to differentiate into a specific type of cell, but is already more specific than a stem cell and is pushed to differentiate into its “target” cell. The most important difference between stem cells and progenitor cells is that stem cells can replicate indefinitely, whereas progenitor cells can divide only a limited number of times. The term “human stem cells” as used herein further includes “progenitor cells” and “non-fully-differentiated stem cells”.

According to the principles of the present invention, stem cells are enriched with healthy functional human exogenous mitochondria prior to being administered to a patient in need in order to increase the number and/or function of mitochondria in them. Without being limited to any theory or mechanism, the increased number and/or function of mitochondria in the administered stem cells is responsible for the various therapeutic effects exemplified

herein for the first time in human patients. The term “enriching” as used herein refers to any action designed to increase the mitochondrial content, e.g. the number of intact mitochondria, or the functionality of mitochondria of a mammalian cell. In particular, stem cells enriched with functional mitochondria will show enhanced mitochondrial function compared to the same stem cells prior to enrichment. The term “enriching” as used herein further refers to any action performed ex vivo, which increases the mitochondrial content, e.g. the number of intact, functional, healthy, mitochondria, of a human cell. According to the principles of the present invention, healthy functional human exogenous mitochondria are introduced into human stem cells, thus enriching these cells with healthy functional human mitochondria. It should be understood that such enrichment changes the mitochondrial content of the human stem cells: while naïve human stem cells substantially have one population of host/autologous/endogenous mitochondria, human stem cells enriched with exogenous mitochondria substantially have two populations of mitochondria - one population of host/autologous/endogenous mitochondria and another population of the introduced mitochondria (i.e., the exogenous mitochondria). Thus, the term “enriched” relates to the state of the cells after receiving/incorporation exogenous mitochondria. Determining the number and/or ratio between the two populations of mitochondria is straightforward, as the two populations may differ in several aspects e.g. in their mitochondrial DNA. Therefore, the phrase “human stem cells enriched with healthy functional human mitochondria” is equivalent to the phrase “human stem cells comprising endogenous mitochondria and healthy functional exogenous mitochondria”. For example, human stem cells which comprise 1% and 33% healthy functional exogenous mitochondria of the total mitochondria, are considered comprising host/autologous/endogenous mitochondria and healthy functional exogenous mitochondria in a ratio of 99:1 and a ratio 67:33, respectively. For example, “3% of the total mitochondria” means that after enrichment the original (endogenous) mitochondrial content is 97% of the total mitochondria and the introduced (exogenous) mitochondria is 3% of the total mitochondria - this is equivalent to $(3/97=)$ 3.1% enrichment. Another example - “33% of the total mitochondria” means that after enrichment, the original (endogenous) mitochondrial content is 67% of the total mitochondria and the introduced (exogenous) mitochondria is 33% of the total mitochondria - this is equivalent to $(33/67=)$ 49.2% enrichment.

It should be understood that the phrase “human stem cells enriched with healthy functional exogenous mitochondria” as used herein refers to human stem cells comprising healthy functional mitochondria, wherein the healthy functional mitochondria are of a different origin than the human stem cells, i.e. these mitochondria are

obtained/derived/isolated from an exogenous source. The presence of “exogenous”, “foreign” or “non-original” healthy functional mitochondria within human stem cells serves as evidence that these cells are enriched with said mitochondria. A person of average skill in the art would know how to determine that human stem cells comprise exogenous allogeneic mitochondria from different origins based on well-known methods in the art (see e.g. Zander J. et al., Forensic Sci. Int. Genet., 2017, Vol. 29, pages 242-249). Such methods can be based e.g. on genetic differences between different mitochondria populations within a human stem cell or within a plurality of human stem cells. For example, in humans, the mitochondrial DNA encodes 37 genes (Nature. 290 (5806): 457–65), thus by sequencing the mtDNA one can easily determine the existence of 1, 2 or more different populations of mtDNA in a human stem cell or in a plurality of human stem cells.

In some embodiments, enrichment of the stem cells with healthy functional human exogenous mitochondria comprises washing the mitochondrially-enriched stem cells after incubation of the human stem cells with said healthy functional human exogenous mitochondria. This step provides a composition of the mitochondrially-enriched stem cells substantially devoid of cell debris or mitochondrial membrane remnants and mitochondria that did not enter the stem cells. In some embodiments, washing comprises centrifugation of the mitochondrially-enriched stem cells after incubation of the human stem cells with said healthy functional human exogenous mitochondria. According to some embodiments, the pharmaceutical composition comprising the mitochondrially-enriched human stem cells is separated from free mitochondria, i.e., mitochondria that did not enter the stem cells, or other cell debris. According to some embodiments, the pharmaceutical composition comprising the mitochondrially-enriched human stem cells does not comprise a detectable amount of free mitochondria.

The terms “healthy functional mitochondria”, “healthy functional human mitochondria”, “healthy functional exogenous mitochondria”, “healthy functional human exogenous mitochondria”, “healthy functional human exogenous mitochondria without a pathogenic mutation in mitochondrial DNA or in a mitochondrial protein” and “healthy functional human exogenous mitochondria without a pathogenic mutation in mitochondrial DNA or in a mitochondrial molecule” may interchangeably be used and refer to mitochondria displaying normal, non-pathologic levels of activity. The activity of mitochondria can be measured by a variety of methods well known in the art, such as Tetramethylrhodamine Ethyl Ester, Perchlorate (TMRE) staining, O₂ consumption, ATP production, and CS activity level.

In certain embodiments, the human stem cells enriched with healthy functional exogenous mitochondria comprise a mixture of healthy functional mitochondria of different origins. In certain embodiments, one of the origins of the mixture of healthy functional mitochondria is the same as the origin of the human stem cells. In certain embodiments, none
5 of the origins of the mixture of healthy functional mitochondria is the origin of the human stem cells. In certain embodiments, the human stem cells enriched with healthy functional mitochondria comprise healthy functional mitochondria of a single origin which is different than the origin of the human stem cells.

As the introduction of healthy functional exogenous mitochondria to human stem cells
10 may increase the total number/content of healthy functional mitochondria in these cells, it should further be understood that the phrase “human stem cells enriched with healthy functional mitochondria” as used herein may, in certain embodiments, refer to human stem cells comprising increased amounts of healthy functional mitochondria, either, endogenous, from the stem cells or exogenous, from a different source or origin.

The term “healthy mitochondria” or “functional mitochondria” refers to normally-
15 functioning mitochondria. The term “healthy mitochondrial DNA” or “normal mitochondrial DNA” refers to mitochondrial DNA which does not include a mutation which affects the normal function of the mitochondria. The term “functional mitochondria” as used herein refers to mitochondria displaying normal, non-pathologic levels of activity. The activity of
20 mitochondria can be measured by a variety of methods well known in the art, such as membrane potential, O₂ consumption, ATP production, and CS activity level. The term “functional mitochondria” and “healthy mitochondria” are used interchangeably, and further refer to mitochondria that exhibit parameters indicative of normal mtDNA, normal levels of oxygen consumption and ATP production.

The term “associated with” in connection with the relationship between a mutation in
25 mitochondrial DNA and a disease or disorder generally means that the mutation in mitochondrial DNA is at least partly responsible to at least one of the symptoms of the disease or disorder, either directly or indirectly, either alone or in combination with other factors, by any biological mechanism. The term “mutation” as used herein refers to a deletion, an
30 insertion or a point mutation which affects the structure and/or function of a molecule, e.g. an RNA molecule or a protein molecule, encoded by DNA.

In certain embodiments, the pathogenic mutation in mitochondrial DNA or the pathogenic mutation in nuclear DNA is not in a gene encoding a mitochondrial molecule. In certain embodiments, the pathogenic mutation in mitochondrial DNA or the pathogenic

mutation in nuclear DNA is not in a gene encoding a mitochondrial protein. In certain embodiments, the pathogenic mutation in mitochondrial DNA or the pathogenic mutation in nuclear DNA is not in a gene encoding a mitochondrial enzyme. In certain embodiments, the pathogenic mutation in mitochondrial DNA or the pathogenic mutation in nuclear DNA is not in a gene encoding a mitochondrial peptide. In certain embodiments, the pathogenic mutation in mitochondrial DNA or the pathogenic mutation in nuclear DNA is not in a gene encoding a mitochondrial RNA molecule.

In certain embodiments, the disease or disorder is selected from the group consisting of Parkinson Disease (PD), Alzheimer Disease (AD), stroke, multiple sclerosis (MS), dementia, adrenoleukodystrophy (ALD), amyotrophic lateral sclerosis (ALS), Huntington Disease (HD), Guillain Barre Syndrome (GBS), Autism and Friedreich's ataxia. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, the symptom is selected from the group consisting of inability to gain weight, low blood alkaline phosphatase levels, high blood lactate levels, low ATP content in lymphocytes, impaired ability to walk, impaired ability to climb stairs, impaired ability to using scissors, impaired ability to draw, impaired ability to execute commands, impaired response time, impaired motor skills, impaired learning, impaired reaction time, impaired language skills, stuttering and impaired memory. Each possibility represents a separate embodiment of the present invention. In certain embodiments, the symptom is a plurality of symptoms. It should be understood that defining symptoms as "high" and "low" correspond to "detectably higher than normal" and "detectably lower than normal", respectively, wherein the normal level is the corresponding level in a healthy subject or a plurality of healthy subjects.

In certain embodiments, the stem cells substantially comprise mitochondrial DNA of a single origin. In certain embodiments, the stem cells substantially comprise mitochondria of a single mitochondrial DNA haplogroup. In human genetics, the term "a human mitochondrial DNA haplogroup" is used to refer to a haplogroup defined by differences in human mitochondrial DNA. The term "haplogroup" as used herein further refers to a genetic population group of people who share a common ancestor on the matriline. Mitochondrial haplogroup is determined by sequencing.

In certain embodiments, the stem cells comprise mitochondrial DNA of two or more origins. In certain embodiments, the stem cells comprise mitochondria of two or more mitochondrial DNA haplogroups. In certain embodiments, the stem cells comprise functional

mitochondria of a mitochondrial DNA haplogroup selected from the group consisting of haplogroup J and haplogroup V.

In certain embodiments, the pharmaceutical composition is administered directly to the central nervous system (CNS). In certain embodiments, the pharmaceutical composition is administered directly to the brain. In certain embodiments, the pharmaceutical composition is administered directly to the spinal cord.

In certain embodiments, the pharmaceutical composition comprises at least $1*10^4$, at least $1*10^5$, at least $1*10^6$, or at least $1*10^7$ mitochondrially-enriched human stem cells. Each possibility represents a separate embodiment of the present invention. . In certain 10 embodiments, the pharmaceutical composition comprises at least 10^4 mitochondrially-enriched human stem cells. In certain embodiments, the pharmaceutical composition comprises about $1*10^4$ to about $1*10^8$ mitochondrially-enriched human stem cells. In certain embodiments, the pharmaceutical composition comprises $1*10^4$ to $1*10^9$, $1*10^5$ to $1*10^9$, $1*10^6$ to $1*10^9$, or $1*10^7$ to $1*10^9$ mitochondrially-enriched human stem cells. Each 15 possibility represents a separate embodiment of the present invention. In certain embodiments, the pharmaceutical composition comprises at least 10^5 mitochondrially-enriched human stem cells. In certain embodiments, the pharmaceutical composition comprises at least about 10^5 mitochondrially-enriched human stem cells per kilogram body weight of the patient.

In certain embodiments, the pharmaceutical composition is administered by parenteral administration. In certain embodiments, the pharmaceutical composition is administered by systemic administration. In certain embodiments, the pharmaceutical composition is administered intravenously to the patient. In certain embodiments, the pharmaceutical composition is administered by intravenous infusion. In certain embodiments, the 20 pharmaceutical composition comprises at least $1*10^5$, at least $1*10^6$, or at least $1*10^7$ mitochondrially-enriched human stem cells. In certain embodiments, the pharmaceutical composition comprises about $1*10^6$ to about $1*10^8$ mitochondrially-enriched human stem cells. In certain embodiments, the pharmaceutical composition comprises at least about $1*10^5$, at least $1*10^6$, or at least $1*10^7$ mitochondrially-enriched human stem cells per kilogram body 25 weight of the patient. In certain embodiments, the pharmaceutical composition comprises about $1*10^5$ to about $1*10^7$ mitochondrially-enriched human stem cells per kilogram body weight of the patient. 30

In certain embodiments, the level of mitochondrial enrichment in the mitochondrially-enriched human stem cells is determined by: (i) the levels of host (endogenous) mitochondrial

DNA and exogenous mitochondrial DNA; (ii) the level of citrate synthase or the level of citrate synthase activity; or (iii) both (i) and (ii). Each possibility represents a separate embodiment of the present invention.

In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of at least about $1 \cdot 10^4$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of at least about $1 \cdot 10^5$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of at least about $1 \cdot 10^6$ stem cells per kilogram body weight of the patient.

In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $1 \cdot 10^4$ to about $1 \cdot 10^8$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $1 \cdot 10^5$ to about $1 \cdot 10^7$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $5 \cdot 10^4$ to about $5 \cdot 10^6$ stem cells per kilogram body weight of the patient.

In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $1 \cdot 10^4$ to about $4 \cdot 10^8$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $1 \cdot 10^5$ to about $4 \cdot 10^7$ stem cells per kilogram body weight of the patient. In certain embodiments, the method comprises administering to the patient a pharmaceutical composition comprising a therapeutically-effective amount of about $1 \cdot 10^6$ to about $4 \cdot 10^6$ stem cells per kilogram body weight of the patient.

In certain embodiments, the mitochondrially-enriched human stem cells are obtained or derived from the patient himself, before enrichment with the exogenous functional mitochondria.

In certain embodiments, the mitochondrially-enriched human stem cells are obtained or derived from a donor different than the patient before enrichment of the cells with exogenous mitochondria. In certain embodiments, the donor is at least partly human leukocyte

antigen (HLA)-matched with the patient. In certain embodiments, the method described above further comprises a step of administering to the patient an agent which prevents, delays, minimizes or abolishes an adverse immunogenic reaction between the patient and the mitochondrially-enriched human stem cells. In certain embodiments, the adverse immunogenic reaction is a graft-versus-host disease (GvHD).

In certain embodiments, the stem cells are pluripotent stem cells (PSCs). In certain embodiments, the stem cells are induced pluripotent stem cells (iPSCs). As used herein the term "pluripotent stem cells (PSCs)" refers to cells that can propagate indefinitely, as well as give rise to a plurality of cell types in the body, for example neuronal cells. Totipotent stem cells are cells that can give rise to every other cell type in the body. Embryonic stem cells (ESCs) are totipotent stem cells and induced pluripotent stem cells (iPSCs) are pluripotent stem cells. As used herein the term "induced pluripotent stem cells (iPSCs)" refers to a type of pluripotent stem cell that can be generated from human adult somatic cells. In some embodiments, the PSCs are non-embryonic stem cells. According to some embodiments embryonic stem cells are explicitly excluded from the scope of the invention. As used herein the term "embryonic stem cells (ESC)" refers to a type of totipotent stem cell derived from the inner cell mass of a blastocyst.

In certain embodiments, the stem cells are mesenchymal stem cells. In certain embodiments, the stem cells are CD34⁺ cells. The term "CD34⁺ cells" as used herein refers to stem cells characterized as being CD34-positive, regardless of their origin. The term further refers to hematopoietic stem cells characterized as being CD34-positive that are obtained from stem cells or mobilized from bone marrow or obtained from umbilical cord blood. As used herein, the term "CD34⁺ cells" denotes cells that express the surface marker protein CD34. Expression of CD34 can be determined by immunofluorescence analysis or FACS analysis using an antibody directed against CD34. Hematopoietic progenitor cell antigen CD34, also known as CD34 antigen, is a protein that in humans is encoded by the CD34 gene.

In certain embodiments, the CD34⁺ cells are umbilical cord cells. In certain embodiments, the CD34⁺ cells are bone marrow cells. In certain embodiments, the CD34⁺ cells are hematopoietic cells. In certain embodiments, the CD34⁺ cells are mesenchymal stem cells. In certain embodiments, the CD34⁺ cells are endothelial progenitor cells. In certain embodiments, the CD34⁺ cells are endothelial cells of blood vessels. In certain embodiments, the CD34⁺ cells are mast cells. In certain embodiments, the CD34⁺ cells are a sub-population dendritic cells (which are factor XIIIa-negative). In certain embodiments, the CD34⁺ cells are

Long-Term Hematopoietic Stem Cells (LT-HSCs). In certain embodiments, the CD34⁺ cells are human HSCs cells. In certain embodiments, the CD34⁺ cells are HLA-matched to the patient. In certain embodiments, the CD34⁺ cells are HLA-matched with the patient. In certain embodiments, the CD34⁺ cells are autologous to the patient.

5 In certain embodiments, the stem cells are derived from adipose tissue, oral mucosa, peripheral blood or umbilical cord blood. Each possibility represents a separate embodiment of the present invention. In certain embodiments, the stem cells are derived from bone marrow cells. The term “bone marrow cells” as used herein generally refers to all human cells naturally found in the bone marrow of humans, and to all cell populations naturally found in
10 the bone marrow of humans. The term “bone marrow stem cells” refers to the stem cell population derived from the bone marrow.

 The term “myelopoietic cells” as used herein refers to cells involved in myelopoiesis, e.g. in the production of bone-marrow and of all cells that arise from it, namely, all blood cells.

15 The term “erythropoietic cells” as used herein refers to cells involved in erythropoiesis, e.g. in the production of red blood cells (erythrocytes).

 The term “multi-potential hematopoietic stem cells” or “hemocytoblasts” as used herein refers to the stem cells that give rise to all the other blood cells through the process of hematopoiesis.

20 The term “common myeloid progenitor” as used herein refers to the cells that generate myeloid cells. The term “common lymphoid progenitor” as used herein refers to the cells that generate lymphocytes.

 The term “mesenchymal stem cells” as used herein refers to multipotent stromal cells that can differentiate into a variety of cell types, including neuronal cells, osteoblasts (bone
25 cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells).

 In certain embodiments, the pharmaceutical composition may further comprise non-enriched stem cells, megakaryocytes, erythrocytes, mast cells, myeloblasts, basophils, neutrophils, eosinophils, monocytes, macrophages, natural killer (NK) cells, small lymphocytes, T lymphocytes, B lymphocytes, plasma cells, reticular cells, or any combination
30 thereof. Each possibility represents a separate embodiment of the present invention.

 In certain embodiments, the method described above further comprises the preceding steps of isolating, deriving or obtaining human stem cells, and introducing healthy functional

human exogenous mitochondria into the human stem cells, thus producing the mitochondrially-enriched human stem cells. In certain embodiments, the method comprises (a) freezing the human stem cells, (b) thawing the human stem cells, and (c) introducing healthy functional human exogenous mitochondria into the human stem cells. In certain
5 embodiments, the human stem cells are isolated, derived or obtained from cells of the bone marrow, adipose tissue, oral mucosa, skin fibroblasts, blood or umbilical cord blood. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the method described above further comprises the step of selection of CD34 positive cells from the human stem cells prior to introducing the healthy
10 functional exogenous mitochondria into the cells. Selection of CD34 positive cells can be done by methods known in the art including but not limited to the CliniMACS or Prodigy systems (Miltenyi).

In certain embodiments, the method described above further comprises the preceding steps of isolating or obtaining healthy functional human exogenous mitochondria from a
15 suitable source, and introducing the healthy functional human exogenous mitochondria into human stem cells, thus producing the mitochondrially-enriched human stem cells. In certain embodiments, the method may include the steps of: (a) freezing the healthy functional human exogenous mitochondria, (b) thawing the healthy functional human exogenous mitochondria, and (c) introducing the healthy functional human exogenous mitochondria into the human
20 stem cells. In certain embodiments, the healthy functional human exogenous mitochondria are isolated or obtained from a suitable source, including, but not limited to: placenta, placental cells grown in culture or blood cells. Each possibility represents a separate embodiment of the present invention.

According to the principles of the present invention, the possibility to freeze healthy
25 functional exogenous mitochondria before enriching the human stem cells is crucial for mitochondrial augmentation therapy process as it e.g. provides sufficient time to test functionality and/or certain attributes of the healthy functional exogenous mitochondria, as well as increases the shelf-life of the healthy functional exogenous mitochondria and/or allows the healthy functional exogenous mitochondria to be easily distributed, before
30 enriching the human stem cells.

Without wishing to be bound by any theory or mechanism, mitochondria that have undergone a freeze-thaw cycle demonstrate a comparable oxygen consumption rate following thawing, as compared to control mitochondria that have not undergone a freeze-thaw cycle.

According to some embodiments, the freeze-thaw cycle comprises freezing said functional mitochondria for at least 24 hours prior to thawing. According to other embodiments, the freeze-thaw cycle comprises freezing said functional mitochondria for at least 1 month prior to thawing, several months prior to thawing or longer. Each possibility represents a separate embodiment of the present invention. According to another embodiment, the oxygen consumption of the functional mitochondria after the freeze-thaw cycle is equal or higher than the oxygen consumption of the functional mitochondria prior to the freeze-thaw cycle.

As used herein, the term “freeze-thaw cycle” refers to freezing of the functional mitochondria to a temperature below 0 °C, maintaining the mitochondria in a temperature below 0 °C for a defined period of time and thawing the mitochondria to room temperature or body temperature or any temperature above 0 °C which enables treatment of the stem cells with the mitochondria. Each possibility represents a separate embodiment of the present invention. The term “room temperature”, as used herein typically refers to a temperature of between 18 °C and 25 °C. The term “body temperature”, as used herein, refers to a temperature of between 35.5 °C and 37.5 °C, preferably 37°C. In another embodiment, mitochondria that have undergone a freeze-thaw cycle are functional mitochondria.

In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen at a temperature of -70° C or lower. In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen at a temperature of -20 °C or lower. In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen at a temperature of -4 °C or lower. According to another embodiment, freezing of the mitochondria is gradual. According to some embodiment, freezing of mitochondria is through flash-freezing. As used herein, the term “flash-freezing” refers to rapidly freezing the mitochondria by subjecting them to cryogenic temperatures.

In another embodiment, the mitochondria that underwent a freeze-thaw cycle were frozen for at least 30 minutes prior to thawing. According to another embodiment, the freeze-thaw cycle comprises freezing the functional mitochondria for at least 30, 60, 90, 120, 180, 210 minutes prior to thawing. Each possibility represents a separate embodiment of the present invention. In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24, 48, 72, 96, or 120 hours prior to thawing. Each freezing time presents a separate embodiment of the present invention. In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen for at least 4, 5, 6, 7, 30, 60, 120, 365 days prior to thawing. Each freezing time

presents a separate embodiment of the present invention. According to another embodiment, the freeze-thaw cycle comprises freezing the functional mitochondria for at least 1, 2, 3 weeks prior to thawing. Each possibility represents a separate embodiment of the present invention. According to another embodiment, the freeze-thaw cycle comprises freezing the functional mitochondria for at least 1, 2, 3, 4, 5, 6 months prior to thawing. Each possibility represents a separate embodiment of the present invention.

In another embodiment, the mitochondria that have undergone a freeze-thaw cycle were frozen at -70°C for at least 30 minutes prior to thawing. Without wishing to be bound by any theory or mechanism, the possibility to freeze mitochondria and thaw them after a long period enables easy storage and use of the mitochondria with reproducible results even after a long period of storage.

According to one embodiment, thawing is at room temperature. In another embodiment, thawing is at body temperature. According to another embodiment, thawing is at a temperature which enables administering the mitochondria according to the methods of the invention. According to another embodiment, thawing is performed gradually.

According to another embodiment, the mitochondria that underwent a freeze-thaw cycle were frozen within a freezing buffer. According to another embodiment, the mitochondria that underwent a freeze-thaw cycle were frozen within the isolation buffer. As used herein, the term "isolation buffer" refers to a buffer in which the mitochondria of the invention have been isolated. In a non-limiting example, the isolation buffer is a sucrose buffer. Without wishing to be bound by any mechanism or theory, freezing mitochondria within the isolation buffer saves time and isolation steps, as there is no need to replace the isolation buffer with a freezing buffer prior to freezing or to replace the freezing buffer upon thawing.

According to another embodiment, the freezing buffer comprises a cryoprotectant. According to some embodiments, the cryoprotectant is a saccharide, an oligosaccharide or a polysaccharide. Each possibility represents a separate embodiment of the present invention. According to another embodiment, the saccharide concentration in the freezing buffer is a sufficient saccharide concentration which acts to preserve mitochondrial function. According to another embodiment, the isolation buffer comprises a saccharide. According to another embodiment, the saccharide concentration in the isolation buffer is a sufficient saccharide concentration which acts to preserve mitochondrial function. According to another embodiment, the saccharide is sucrose.

In certain embodiments, the method further comprises the preceding steps of (a) freezing the human stem cells enriched with healthy functional human exogenous mitochondria, (b) thawing the human stem cells enriched with healthy functional human exogenous mitochondria, and (c) administering the human stem cells enriched with healthy functional human exogenous mitochondria to the patient.

In certain embodiments, the healthy functional exogenous mitochondria constitute at least 3% of the total mitochondria in the mitochondrially-enriched cell. In certain embodiments, the healthy functional exogenous mitochondria constitute at least 10% of the total mitochondria in the mitochondrially-enriched cell. In some embodiments, the healthy functional exogenous mitochondria constitute at least about 3%, 5%, 10%, 15%, 20%, 25% or 30% of the total mitochondria in the mitochondrially-enriched cell. Each possibility represents a separate embodiment of the present invention.

The extent of enrichment of the stem cells with functional mitochondria may be determined by functional and/or enzymatic assays, including but not limited to rate of oxygen (O_2) consumption, content or activity level of citrate synthase, rate of adenosine triphosphate (ATP) production. In the alternative the enrichment of the stem cells with healthy donor mitochondria may be confirmed by the detection of mitochondrial DNA of the donor. According to some embodiments, the extent of enrichment of the stem cells with functional mitochondria may be determined by the level of change in heteroplasmy and/or by the copy number of mtDNA per cell. Each possibility represents a separate embodiment of the present invention.

TMRM (tetramethylrhodamine methyl ester) or the related TMRE (tetramethylrhodamine ethyl ester) are cell-permeant fluorogenic dyes commonly used to assess mitochondrial function in living cells, by identifying changes in mitochondrial membrane potential. According to some embodiments, the level of enrichment can be determined by staining with TMRE or TMRM.

According to another embodiment, the intactness of a mitochondrial membrane may be determined by any method known in the art. In a non-limiting example, intactness of a mitochondrial membrane is measured using the tetramethylrhodamine methyl ester (TMRM) or the tetramethylrhodamine ethyl ester (TMRE) fluorescent probes. Each possibility represents a separate embodiment of the present invention. Mitochondria that were observed under a microscope and show TMRM or TMRE staining have an intact mitochondrial outer membrane. As used herein, the term "a mitochondrial membrane" refers to a mitochondrial

membrane selected from the group consisting of the mitochondrial inner membrane, the mitochondrial outer membrane, and both.

In certain embodiments, the level of mitochondrial enrichment in the mitochondrially-enriched human stem cells is determined by sequencing at least a statistically-representative portion of total mitochondrial DNA in the cells and determining the relative levels of host/endogenous mitochondrial DNA and exogenous mitochondrial DNA. In certain
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embodiments, the level of mitochondrial enrichment in the mitochondrially-enriched human stem cells is determined by single nucleotide polymorphism (SNP) analysis. In certain
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embodiments, the largest mitochondrial population and/or the largest mitochondrial DNA population is the host/endogenous mitochondrial population and/or the host/endogenous
mitochondrial DNA population; and/or the second-largest mitochondrial population and/or
the second-largest mitochondrial DNA population is the exogenous mitochondrial population
and/or the exogenous mitochondrial DNA population. Each possibility represents a separate
embodiment of the invention.

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According to certain embodiments, the enrichment of the stem cells with healthy functional mitochondria may be determined by conventional assays that are recognized in the art. In
certain embodiments, the level of mitochondrial enrichment in the mitochondrially-enriched
human stem cells is determined by (i) the levels of host/endogenous mitochondrial DNA and
exogenous mitochondrial DNA; (ii) the level of mitochondrial proteins selected from the
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group consisting of citrate synthase (CS), cytochrome C oxidase (COX1), succinate
dehydrogenase complex flavoprotein subunit A (SDHA) and any combination thereof; (iii)
the level of CS activity; or (iv) any combination of (i), (ii) and (iii). Each possibility
represents a separate embodiment of the invention.

In certain embodiments, the level of mitochondrial enrichment in the mitochondrially-
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enriched human stem cells is determined by at least one of: (i) the levels of host mitochondrial
DNA and exogenous mitochondrial DNA in case of allogeneic mitochondria; (ii) the level of
citrate synthase activity; (iii) the level of succinate dehydrogenase complex flavoprotein
subunit A (SDHA) or cytochrome C oxidase (COX1); (iv) the rate of oxygen (O₂)
consumption; (v) the rate of adenosine triphosphate (ATP) production or (vi) any combination
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thereof Each possibility represents a separate embodiment of the present invention. Methods
for measuring these various parameters are well known in the art.

In certain embodiments, the pharmaceutical composition may further include non-
enriched stem cells, megakaryocytes, erythrocytes, mast cells, myeloblasts, basophils,
neutrophils, eosinophils, monocytes, macrophages, natural killer (NK) cells, small

lymphocytes, T lymphocytes, B lymphocytes, plasma cells, reticular cells, or any combination thereof.

In some embodiments, there is provided a method for treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial protein, the method comprising the step of administering a pharmaceutical composition comprising a plurality of mitochondrially enriched human stem cells to the patient, said stem cells are enriched with healthy functional human exogenous mitochondria.

In some embodiments, there is provided a method for treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the method comprising the step of administering a pharmaceutical composition comprising a plurality of human stem cells to the patient, wherein the human stem cells are enriched with healthy functional exogenous mitochondria without a pathogenic mutation in mitochondrial DNA, and wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or with a pathogenic mutation in nuclear DNA.

The present invention further provides, in another aspect, a pharmaceutical composition comprising a plurality of human stem cells enriched with healthy functional human exogenous mitochondria, for use in a method of treating a brain disease, disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule.

In certain embodiments, the method comprises (a) thawing a frozen pharmaceutical composition comprising a therapeutically-effective amount of human stem cells enriched with healthy functional exogenous mitochondria, and (b) administering the thawed pharmaceutical composition to the patient.

In some embodiments, there is provided a pharmaceutical composition comprising a plurality of human stem cells enriched with healthy functional mitochondria without a pathogenic mutation in mitochondrial DNA, for use in a method of treating a brain disease, disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or with a pathogenic mutation in nuclear DNA encoding a mitochondrial protein.

The present invention further provides, in another aspect, a pharmaceutical composition comprising a plurality of human stem cells enriched with healthy functional human mitochondria, for use in a method of treating a brain disease, disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule.

The present invention further provides, in another aspect, an *ex-vivo* method for enriching human stem cells with healthy functional human exogenous mitochondria, the method comprising the steps of: (i) providing a first composition, comprising a plurality of isolated or partially purified human stem cells from a patient afflicted with a brain disease or a brain disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or with a pathogenic mutation in nuclear DNA encoding a mitochondrial molecule, or plurality of isolated or partially purified human stem cells from a healthy donor; (ii) providing a second composition, comprising a plurality of isolated healthy functional human exogenous mitochondria obtained from a donor without a pathogenic mutation in mitochondrial DNA or in a mitochondrial molecule (such as a protein); (iii) contacting the human stem cells of the first composition with the healthy functional human exogenous mitochondria of the second composition, thus providing a third composition; and (iv) incubating the third composition under conditions allowing the healthy functional human exogenous mitochondria to enter the human stem cells, thereby enriching said human stem cells with said healthy functional human exogenous mitochondria, thus providing a fourth composition comprising human stem cells enriched with healthy functional human exogenous mitochondria without a pathogenic mutation in mitochondrial DNA or in a mitochondrial molecule; wherein the enriched human stem cells of step (iv) have a detectably higher total content of healthy functional human mitochondria compared to the human stem cells in step (i).

The term “*ex-vivo* method” as used herein refers to a method comprising steps performed exclusively outside the human body. In particular, an *ex vivo* method comprises manipulation of cells outside the body that are subsequently reintroduced or transplanted into the subject to be treated.

The term “healthy donor” and “healthy subject” are used interchangeably, and refer to a subject not suffering from the disease or condition which is being treated.

The term “contacting” refers to bringing the composition of mitochondria and cells into sufficient proximity to promote entry of the mitochondria into the cells. The term

“introducing” mitochondria into the stem cells is used interchangeably with the term contacting.

The term “isolated human healthy functional mitochondria” as used herein refers to intact mitochondria isolated, obtain or derived from cells obtained from a healthy subject, not afflicted with a primary mitochondrial disease. In some embodiments, such mitochondria are exogenous mitochondria.

The term “isolated” as used herein and in the claims in the context of mitochondria includes mitochondria that were purified, at least partially, from other components found in said source. In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-80%, 20-70%, 40-70%, 20-40%, or 20-30% of the total amount of cellular proteins within the sample. Each possibility represents a separate embodiment of the present invention. In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-80% of the total amount of cellular proteins within the sample. In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-80% of the combined weight of the mitochondria and other sub-cellular fractions. In other embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is above 80% of the combined weight of the mitochondria and other sub-cellular fractions.

In some embodiments, the methods described above in various embodiments thereof further comprises expanding the stem cells of the first composition by culturing said stem cells in a culture or proliferation medium capable of expanding stem cells. In other embodiments, the method further comprises expanding the mitochondrially-enriched stem cells of the fourth composition by culturing said cells in a culture or proliferation medium capable of expanding stem cells. As used throughout this application, the term "culture or proliferation medium" is a fluid medium such as cell culture media, cell growth media, buffer which provides sustenance to the cells. As used throughout this application, and in the claims the term “pharmaceutical composition” comprises a fluid carrier such as cell culture media, cell growth media, buffer which provides sustenance to the cells.

In additional embodiments, the human stem cells are expanded before or after mitochondrial augmentation.

According to some embodiments, the method for enriching human stem cells with healthy functional exogenous mitochondria does not comprise measuring the membrane potential of the cell.

In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.044 up to 176 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.088 up to 176 milliunits of CS activity per million cells. In other embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.2 up to 150 milliunits of CS activity per million cells. In other embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.4 up to 100 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.6 up to 80 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.7 up to 50 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.8 up to 20 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.88 up to 17.6 milliunits of CS activity per million cells. In some embodiments, the enrichment of the stem cells with healthy functional exogenous mitochondria comprises introducing into the stem cells a dose of mitochondria of at least 0.44 up to 17.6 milliunits of CS activity per million cells.

Mitochondrial dose can be expressed in terms of units of CS activity or mtDNA copy number of other quantifiable measurements of the amount of healthy functional mitochondria as explained herein. A “unit of CS activity” is defined as the amount that enables conversion of one micromole substrate in 1 minute in 1mL reaction volume.

The present invention further provides, in another aspect, a plurality of human stem cells enriched with healthy functional human exogenous mitochondria, obtained by the method described above.

The present invention further provides, in another aspect, a pharmaceutical composition comprising a therapeutically-effective amount of a plurality of human stem cells enriched with healthy functional human exogenous mitochondria, as described above.

5 In certain embodiments, the pharmaceutical composition described above is for use in a method for treating a brain disease or a brain disorder or a symptom thereof, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or with a pathogenic mutation in nuclear DNA.

10 The present invention further provides, in another aspect, a method for treating a brain disease or a brain disorder or a symptom thereof in a patient in need thereof, comprising administering to the patient the pharmaceutical composition described above, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or with a pathogenic mutation in nuclear DNA.

15 In certain embodiments, the first composition is fresh. In certain embodiments, the first composition was frozen and then thawed prior to incubation. In certain embodiments, the second composition is fresh. In certain embodiments, the second composition was frozen and then thawed prior to incubation. In certain embodiments, the fourth composition is fresh. In certain embodiments, the fourth composition was frozen and then thawed prior to administration.

20 In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-80% of the total amount of cellular proteins within the sample. In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-70%, 20%-60% or 30%-50% of the total amount of cellular proteins within the sample.

25 Each possibility represents a separate embodiment of the present invention. In certain embodiments, the total amount of mitochondrial proteins in the second composition comprising the plurality of isolated healthy functional exogenous mitochondria, is between 20%-80% of the combined weight of the mitochondria and other sub-cellular fractions. In other embodiments, the total amount of mitochondrial proteins in the second composition

30 comprising the plurality of isolated healthy functional exogenous mitochondria, is above 80% of the combined weight of the mitochondria and other sub-cellular fractions.

Eukaryotic NADPH-cytochrome C reductase (cytochrome C reductase) is a flavoprotein localized to the endoplasmic reticulum. It transfers electrons from NADPH to

several oxygenases, the most important of which are the cytochrome P450 family of enzymes, responsible for xenobiotic detoxification. Cytochrome C reductase is widely used as an endoplasmic reticulum marker. In certain embodiments, the second composition is substantially free from cytochrome C reductase or cytochrome C reductase activity. In certain
5 embodiments, the fourth composition is not enriched with cytochrome C reductase or cytochrome C reductase activity compared to the first composition.

In some embodiments, the stem cells in the fourth composition have at least one of (i) an increased mitochondrial DNA content; (ii) an increased content of at least one mitochondrial protein selected from the group consisting of CS, COX1 and SDHA; (iii) an
10 increased rate of oxygen (O₂) consumption; (ii) an increased activity level of citrate synthase; (iii) an increased rate of adenosine triphosphate (ATP) production; or (iv) any combination thereof, relative to the stem cells in the first composition. Each possibility represents a separate embodiment of the invention.

The term “increased mitochondrial DNA content” as used herein refers to the content
15 of mitochondrial DNA which is detectably higher than the mitochondrial DNA content in the first composition, prior to mitochondria enrichment. Mitochondrial DNA content may be measured by performing quantitative PCR of a mitochondrial gene prior and post mitochondrial enrichment, normalized to a nuclear gene.

The term “increased content of at least one mitochondrial protein” as used herein
20 refers to the content of either nuclear-encoded or mitochondrial-encoded mitochondrial proteins, e.g., CS, COX1 and SDHA, which is detectably higher than content of said mitochondrial protein in the first composition, prior to mitochondrial enrichment.

The term “increased rate of oxygen (O₂) consumption” as used herein refers to a rate
25 of oxygen (O₂) consumption which is detectably higher than the rate of oxygen (O₂) consumption in the first composition, prior to mitochondrial enrichment.

The term “increased content or activity level of citrate synthase” as used herein refers to a content or activity level of citrate synthase which is detectably higher than the content value or activity level of citrate synthase in the first composition, prior to mitochondrial
enrichment.

30 The term “increased rate of adenosine triphosphate (ATP) production” as used herein refers to a rate of adenosine triphosphate (ATP) production which is detectably higher than the rate of adenosine triphosphate (ATP) production in the first composition, prior to mitochondrial enrichment.

In certain embodiments, the term “detectably higher” as used herein refers to a statistically-significant increase between the normal and increased values. In certain embodiments, the term “detectably higher” as used herein refers to a non-pathological increase, *i.e.* to a level in which no pathological symptom associated with the substantially higher value becomes apparent. In certain embodiments, the term “increased” as used herein refers to a value which is 1.05 fold, 1.1 fold, 1.25 fold, 1.5 fold, 2 fold, 3 fold, 4 fold, 5 fold, 6 fold, 7 fold or higher than the corresponding value found in corresponding cells or corresponding mitochondria of a plurality of healthy subjects or in the stem cells of the first composition prior to mitochondrial enrichment. Each possibility represents a separate embodiment of the invention.

In specific situations the same cells, prior to mitochondrial enrichment, serve as controls to measure CS and ATP activity and determine enrichment level.

The phrase “conditions allowing the human functional exogenous mitochondria to enter the human stem cells” as used herein generally refers to parameters such as time, temperature, and proximity between the exogenous mitochondria and the human stem cells. While identifying those conditions are within the capabilities of any man of ordinary skill in the field, such conditions are provided by the present invention. For example, human cells and human cell lines are routinely incubated with mitochondria in liquid medium, in a sterile environment, at 37 °C and 5% CO₂ atmosphere. In certain embodiments, the stem cells were incubated with the mitochondria for 24 hours at R.T. in saline containing 4.5% HSA (human serum albumin).

In certain embodiments, the human stem cells are incubated with the healthy functional exogenous mitochondria for a time ranging from 0.5 to 30 hours, at a temperature ranging from 16 to 37°C. In certain embodiments, the human stem cells are incubated with the healthy functional exogenous mitochondria for a time ranging from 1 to 30 or from 5 to 25 hours. Each possibility represents a separate embodiment of the present invention. In specific embodiments, incubation is for 20 to 30 hours. In some embodiments, incubation is at room temperature (18 °C to 30 °C) for at least 1, 5, 10, 15 or 20 hours. Each possibility represents a separate embodiment of the present invention. In other embodiments, incubation is up to 1, 5, 10, 15, 20 or 30 hours. Each possibility represents a separate embodiment of the present invention. In specific embodiments, incubation is for 24 hours. In some embodiments, incubation is at room temperature (20 °C to 30 °C). In certain embodiments, incubation is at 37 °C. In some embodiments, incubation is in a 5% CO₂ atmosphere for at least 1, 5, 10, 15

or 20 hours, and/or up to 1, 5, 10, 15 or 20 hours. In other embodiments, incubation does not include added CO₂ above the level found in air.

In some embodiments, incubation is in a medium supporting cell survival. In some embodiments, the medium is Dulbecco's Modified Eagle Medium (DMEM). In other
5 embodiment, the medium is saline containing HSA (human serum albumin). In some embodiments, the saline contains between 2% to 10% HSA. In further embodiments, the saline contains between 3 to 6 % HSA. In yet further embodiments, the saline contains 4.5% HSA. In specific embodiments, incubation of the stem cells with the healthy functional mitochondria is at a temperature ranging between 16 to 30°C, for a time ranging between 15
10 to 30 hours, in a saline containing between 3 to 6 % HSA, without added CO₂ above the level found in air.

In certain embodiments, the methods described above in various embodiments thereof, further include centrifugation before, during or after incubation of the stem cells with the exogenous mitochondria. Each possibility represents a separate embodiment of the
15 present invention. In some embodiments, the methods described above in various embodiments thereof, include a single centrifugation step before, during or after incubation of the stem cells with the exogenous mitochondria. In some embodiments, the centrifugation force ranges from 1000g to 8500g. In some embodiments, the centrifugation force ranges from 2000g to 4000g. In some embodiments, the centrifugation force is above 2500g. In
20 some embodiments, the centrifugation force ranges from 2500g to 8500g. In some embodiments, the centrifugation force ranges from 2500g to 8000g. In some embodiments, the centrifugation force ranges from 3000g to 8000g. In other embodiments, the centrifugation force ranges from 4000g to 8000g. In specific embodiments, the centrifugation force is 7000g. In other embodiments, the centrifugation force is 8000g. In some
25 embodiments, centrifugation is performed for a time ranging from 2 minutes to 30 minutes. In some embodiments, centrifugation is performed for a time ranging from 3 minutes to 25 minutes. In some embodiments, centrifugation is performed for a time ranging from 5 minutes to 20 minutes. In some embodiments, centrifugation is performed for a time ranging from 8 minutes to 15 minutes.

In some embodiments, centrifugation is performed in a temperature ranging from 4 to
30 37°C. In certain embodiments, centrifugation is performed in a temperature ranging from 4 to 10°C or 16-30°C. Each possibility represents a separate embodiment of the present invention. In specific embodiments, centrifugation is performed at 2-6°C. In specific embodiments, centrifugation is performed at 4°C. In some embodiments, the methods described above in

various embodiments thereof include a single centrifugation before, during or after incubation of the stem cells with the exogenous mitochondria, followed by resting the cells at a temperature lower than 30°C. In some embodiments, the conditions allowing the human functional mitochondria to enter the human stem cells include a single centrifugation before, 5 during or after incubation of the stem cells with the exogenous mitochondria, followed by resting the cells at a temperature ranging between 16 to 28°C.

By manipulating the conditions of the incubation, one can manipulate the features of the product. In certain embodiments, the incubation is performed at 37 °C. In certain 10 embodiments, the incubation is performed for at least 6 hours. In certain embodiments, the incubation is performed for at least 12 hours. In certain embodiments, the incubation is performed for 12 to 24 hours. In certain embodiments, the incubation is performed at a ratio of $1 \cdot 10^5$ to $1 \cdot 10^7$ naïve stem cells per amount of exogenous mitochondria having or exhibiting 4.4 units of CS. In certain embodiments, the incubation is performed at a ratio of $1 \cdot 10^6$ naïve stem cells per amount of exogenous mitochondria having or exhibiting 4.4 units 15 of CS. In certain embodiments, the conditions are sufficient to increase the mitochondrial content of the naïve stem cells by about 5% to about 100% as determined by CS activity. In certain embodiments, the conditions are sufficient to increase the mitochondrial content of the naïve stem cells by at least about 5% as determined by CS activity.

Heteroplasmy is the presence of more than one type of mitochondrial DNA within a 20 cell or individual. The heteroplasmy level is the proportion of mutant mtDNA molecules vs. wild type/functional mtDNA molecules and is an important factor in considering the severity of mitochondrial diseases. While lower levels of heteroplasmy (sufficient amount of mitochondria are functional) are associated with a healthy phenotype, higher levels of heteroplasmy (insufficient amount of mitochondria are functional) are associated with 25 pathologies. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 1% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 3% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth 30 composition is at least 5% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 10% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 15% lower than the heteroplasmy level of the stem cells in the first

composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 20% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 25% lower than the heteroplasmy level of the stem cells in the first composition. In certain embodiments, the heteroplasmy level of the stem cells in the fourth composition is at least 30% lower than the heteroplasmy level of the stem cells in the first composition.

The term “mitochondrial content” as used herein refers to the amount of mitochondria within a cell. The term “mitochondrial content” as used herein further refers to the amount of functional mitochondria within a cell, or to the average amount of functional mitochondria within a plurality of cells.

The term “enriched human stem cells” refers to human stem cells, and to populations of human stem cells, which their mitochondrial content was increased, on average, by an active step of a method, compared to their naïve counterparts. The term “increased mitochondrial content” as used herein further refers to a mitochondrial content of the cells in after incubation with mitochondria which is detectably higher than the mitochondrial content of the cells prior to mitochondria enrichment.

The phrase stem cells “obtained” from a subject/patient as used herein refers to cells that were stem cells in the subject/patient at the time of their isolation from said subject/patient.

The phrase stem cells “derived” from a subject/patient as used herein refers to cells that were not stem cells in the patient, and have been manipulated to become stem cells. The phrase further includes stem cells of a certain type which have been manipulated to become stem cells of a different type. The term “manipulated” as used herein refers to the use of any one of the methods known in the field (Yu J. et al., *Science*, 2007, Vol. 318(5858), pages 1917-1920) for reprogramming somatic cells to an undifferentiated state and becoming induced pluripotent stem cells (iPSc), and, optionally, further reprogramming the iPSc to become cells of a desired lineage or population (Chen M. et al., *IOVS*, 2010, Vol. 51(11), pages 5970–5978), such as bone-marrow cells (Xu Y. et al., 2012, *PLoS ONE*, Vol. 7(4), page e34321).

The term “peripheral blood” as used herein refers to blood circulating in the blood system. The term “isolating from the peripheral blood” as used herein refers to the isolation of the stem cells from the other constituents found in the blood.

Citrate synthase (CS) is localized in the mitochondrial matrix, but is encoded by nuclear DNA. Citrate synthase is involved in the first step of the Krebs cycle, and is commonly used as a quantitative enzyme marker for the presence of intact mitochondria (Larsen S. et al., 2012, *J. Physiol.*, Vol. 590(14), pages 3349–3360; Cook G.A. et al., 5 *Biochim. Biophys. Acta.*, 1983, Vol. 763(4), pages 356-367). In certain embodiments, the mitochondrial content of the stem cells in the first composition or in the fourth composition is determined by determining the content of citrate synthase. In certain embodiments, the mitochondrial content of the stem cells in the first composition or in the fourth composition is determined by determining the activity level of citrate synthase. In certain embodiments, the 10 mitochondrial content of the stem cells in the first composition or in the fourth composition correlates with the content of citrate synthase. In certain embodiments, the mitochondrial content of the stem cells in the first composition or in the fourth composition correlates with the activity level of citrate synthase.

CS activity can be measured by commercially available kits, e.g., using the CS 15 activity kit CS0720 (Sigma).

Mitochondrial DNA content may be measured by performing quantitative PCR of a mitochondrial gene prior and post mitochondrial enrichment, normalized to a nuclear gene.

In certain embodiments, the donor of the HLA-matched stem cells is the patient. In certain embodiments, the donor of the HLA-matched stem cells is a family relative of the 20 patient. The term “HLA-matched” as used herein refers to the desire that the patient and the donor of the stem cells be as closely HLA-matched as possible, at least to the degree in which the patient does not develop an acute immune response against the stem cells of the donor. The prevention and/or therapy of such an immune response may be achieved with or without acute or chronic use of immune-suppressors. In certain embodiments, the stem cells from the 25 donor are HLA-matched to the patient to a degree wherein the patient does not reject the stem cells. In certain embodiment, the patient is further treated by an immunosuppressive therapy to prevent immune rejection of the stem cells graft.

As used herein, the term “autologous cells” or “cells that are autologous”, refers to being the patient’s own cells. The term “autologous mitochondria”, refers to mitochondria 30 obtained from the patient’s own cells or from maternally related cells. The terms “allogeneic cells” or “allogeneic mitochondria” refer to cells or mitochondria being from a different donor individual.

The term “syngenic” as used herein and in the claims refers to genetic identity or genetic near-identity sufficient to allow grafting among individuals without rejection. The term syngenic in the context of mitochondria is used herein interchangeably with the term autologous mitochondria meaning of the same maternal bloodline.

5 The term “exogenous mitochondria” refers to a mitochondria that are introduced to a target cell (i.e., stem cell), from a source which is external to the cell. For example, in some embodiments, exogenous mitochondria may be derived or isolated from a cell which is different than the target cell. For example, exogenous mitochondria may be produced/made in a donor cell, purified/isolated obtained from the donor cell and thereafter introduced into
10 the target cell.

 The term “endogenous mitochondria” refers to mitochondria that is being made/expressed/produced by cell and is not introduced from an external source into the cell. In some embodiments, endogenous mitochondria contains proteins and/or other molecules which are encoded by the genome of the cell. In some embodiments, the term “endogenous
15 mitochondria” is equivalent to the term “host mitochondria”.

 In some embodiments, the identification/discrimination of endogenous mitochondria from exogenous mitochondria, after the latter have been introduced into the target cell, can be performed by various means, including, for example, but not limited to: identifying differences in mitochondrial DNA (mtDNA) sequences, for example different haplotypes,
20 between the endogenous mitochondria and endogenous mitochondria, identify specific mitochondrial proteins originating from the tissue of the exogenous mitochondria, such as, for example, cytochrome p450 Cholesterol side chain cleavage (P450SCC) from placenta, UCP1 from brown adipose tissue, and the like, or any combination thereof.

 In certain embodiments, the method described above further comprises a step of
25 administering to the patient an agent which promotes mitochondrial biogenesis. The term “mitochondrial biogenesis” as used herein refers to the growth and division of mitochondria. In certain embodiments, the agent which promotes mitochondrial biogenesis is erythropoietin (EPO) or a salt thereof. In certain embodiments, the agent is selected from the group consisting of recombinant human erythropoietin and isolated human erythropoietin.

30 The term “pre-transplant conditioning agent” as used herein refers to any agent capable of killing bone-marrow cells within the bone-marrow of a human subject.

 As used herein, the term “flash-freezing” refers to rapidly freezing the mitochondria by subjecting them to cryogenic temperatures.

The term “about” as used herein means a range of 10% below to 10% above the indicated integer, number or amount. For example, the phrase “about 1×10^5 ” means “ 1.1×10^5 to 9×10^4 ”. Typically, the numerical values as used herein refer to $\pm 10\%$ of the indicated numerical value.

5 While the present invention has been described with reference to certain embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the present invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the present invention without departing from its scope. Therefore,
10 it is intended that the present invention not be limited to the particular embodiment disclosed, but that the present invention will include all embodiments falling within the scope of the appended claims.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as limiting the
15 broad scope of the invention.

EXAMPLES

Example 1. Mitochondria augmentation therapy in murine cells.

Different murine cells were incubated in DMEM (24 hours, 37°C, 5% CO₂) with isolated
20 murine or human mitochondria in order to increase their mitochondrial content and activity. Table 1 describes representative results of the mitochondria augmentation therapy process, determined by the relative increase in CS activity of the cells after the process compared to the CS activity of the cells before the process.

25 **Table 1.**

Origin of cells	Origin of mitochondria	CS activity of mitochondria / number of cells	Relative increase in CS activity of cells	Dose of cells injected
ICR Mouse - Isolated from whole bone marrow	Human mitochondria	4.4 U CS/1X10 ⁶ Cells	+ 41%	1x10 ⁶ cells
FVB/N Mouse - Isolated from whole bone marrow	C57/BL placental mitochondria	4.4 U CS/1X10 ⁶ Cells	+ 70%	1x10 ⁶ cells
FVB/N Mouse - Isolated from whole bone marrow	C57/BL liver mitochondria	4.4 U CS/1X10 ⁶ Cells	+ 25%	2x10 ⁶ cells
Human - CD34 ⁺ - Isolated by pheresis - Frozen	Human blood mitochondria	4.4 U CS/1X10 ⁶ Cells	+ 33%	

Example 2. Pre-clinical bio-distribution studies.

Mitochondria were isolated from placenta of C57/BL mice (donor of wild type mtDNA).

- 5 Bone marrow cells were isolated from FVB/N mice (carry a mtDNA mutation in ATP8). FVB/N cells were loaded with C57/BL exogenous mitochondria (4.4 U of mitochondria per 1x10⁶ cells), and then I.V. injected back to FVB/N mice. FVB/N mice were then sacrificed in different time points, and organ bio-distribution was tested.

10 In order to test the possible effects of conditioning of the recipient before reintroduction of the FVB/N cells, mice were treated with either Busulfan (a cell cycle non-specific alkylating antineoplastic agent, a form of chemotherapy) or total body irradiation (TBI, a form of radiotherapy).

15 **Figure 1** presents the level of FVB/N mtDNA found in the brain as a function of conditioning treatment before the I.V. injection (* P<0.05; ** P<0.005). The data teach that the level of mutated mtDNA in the brain was significantly reduced after treatment, independent of pre-conditioning.

20 Bone marrow harvested from FVB/N females was enriched with C57BL/6 placenta mitochondria (4.4 mU CS activity per 1X10⁶ cells). Recipient mice underwent IV administration of 1 million augmented cells per animal. Digital PCR was used to detect a C57BL/6-specific SNP.

Figure 2 demonstrates the presence of C57BL/6-derived mtDNA in the brains of mice 1 day or 3 months post MAT. Grey histograms are untreated controls, white indicate treated animals.

5 **Example 3. Compassionate treatment using autologous CD34⁺ cells enriched with MNV-BLD (blood derived mitochondria) for a young patient with Pearson Syndrome (PS) and PS-related Fanconi Syndrome (FS).**

Patient 1 was a 6.5-years old male patient diagnosed with Pearson Syndrome, having a deletion of nucleotides 5835-9753 in his mtDNA. Prior to mitochondrial augmentation therapy (MAT), his weight was 14.5 KG. His growth was significantly delayed for 3 years prior to treatment with no improvement despite being fed by a gastrostomy tube (G-tube) for more than a year. The patient had renal failure (GFR 22ml/min), proximal tubulopathy requiring electrolyte supplementation and hypoparathyroidism requiring calcium supplementation.

15 Mobilization of hematopoietic stem and progenitor cells (HSPC) from the patient was performed by subcutaneous administration of GCSF, given alone for 5 days. Leukapheresis was performed using a Spectra Optia system (TerumoBCT), via peripheral vein access, according to institutional guidelines. CD34 positive selection was performed on mobilized peripheral blood derived cells by using the CliniMACS CD34 reagent according to the
20 manufacturer's instructions. Mitochondria were isolated from maternal peripheral blood mononuclear cells (PBMCs) using 250 mM sucrose buffer pH 7.4 by differential centrifugation.

For MAT, the autologous CD34⁺ cells were incubated with the healthy mitochondria from the patient's mother (1×10^6 cells per amount of mitochondria having 4.4 units of citrate synthase (CS)), which resulted in a 1.56 fold increase in the cells mitochondrial content (56% increase in mitochondrial content as demonstrated by CS activity). Incubation with
25 mitochondria was performed for 24 hours at R.T. in saline containing 4.5% HSA. Enriched cells were suspended in 4.5% HSA in saline solution.

The patient received a single round of treatment, by IV infusion, of 1.1×10^6 autologous
30 CD34⁺ cells enriched with healthy mitochondria per kilogram body weight, according to the timeline presented in **Figure 3A**.

Table 2 presents the Pediatric Mitochondrial Disease Scale (IPMDS) – Quality of Life (QoL) Questionnaire results of the patient as a function of time post cellular therapy. In both the

“Complaints & Symptoms” and the “Physical Examination” categories, 0 represents “normal” to the relevant attribute, while aggravated conditions are scored as 1-5, dependent on severity.

Table 2.

	Pre-treatment	+6 months
Complaints & Symptoms	24	11
Physical Examination	13.4	4.6

5

It should be noted that the patient has not gained weight in the 3 years before treatment, i.e. did not gain any weight since being 3.5 years old. **Figure 3B** shows the growth measured by standard deviation score of the weight and height of the patient, with data starting 4 years prior to MAT and during the follow-up period. The data indicates that approximately 9 months or 15 months following a single treatment, there was an increase in the weight and height of the patient, respectively.

Another evidence for the patient’s growth comes from his Alkaline Phosphatase levels. An alkaline phosphatase level test (ALP test) measures the amount of alkaline phosphatase enzyme in the bloodstream. Having lower than normal ALP levels in the blood can indicate malnutrition, which could be caused by a deficiency in certain vitamins and minerals. The data presented in **Figure 3C** indicates that a single treatment was sufficient to elevate the Alkaline Phosphatase levels of the patient from 159 to 486 IU/L in only 12 months.

Blood lactate is lactic acid that appears in the blood as a result of anaerobic metabolism when mitochondria are damaged or when oxygen delivery to the tissues is insufficient to support normal metabolic demands, one of the hallmarks of mitochondria dysfunction. **Figure 3D** presents the level of lactate found in the blood of the patient as a function of time post the I.V. injection. As can be seen in Figure 3D after MAT, blood lactate level of patient 1 has decreased to normal values.

A genetic indication to the success of the therapy used, is the prevalence of normal mtDNA compared to total mtDNA. As illustrated in **Figure 4 (Pt.1)**, the prevalence of normal mtDNA in the patient was increased from a baseline of about 1 to as high as 1.6 (+ 60%) in just 4 months, and to 1.9 (+90%) after 20 months from treatment. Notably, normal mtDNA levels were above the baseline level on most of the time points.

25

According to a Hospital's neurologist report, neurological improvement has been demonstrated in the patient after transplantation of autologous cells with healthy mitochondria not carrying the deletion mutation; the patient improved his walking skills, climbing steps, using scissors and drawing. Substantial improvements were noted in
5 executing commands and response time as well as in motor and language skills. Also, the mother reported an improvement in memory and stuttering. These findings are particularly relevant and important in the cognitive aspect.

WPSSI showed improved processing speed – visual attention (score improved from 0 to 13), visual perception (score improved from 15 to 22) and verbal skills (score improved from 1 to
10 10).

Example 4. Compassionate treatment using autologous CD34⁺ cells enriched with MNV-BLD (blood derived mitochondria) for a young patient with Pearson Syndrome (PS) and PS-related Fanconi Syndrome (FS).

Patient 2 was a 7-years female patient diagnosed with Pearson Syndrome, having a deletion
15 of 4977 nucleotides in her mtDNA. The patient also suffered from anemia, endocrine pancreatic insufficiency, and is diabetic (hemoglobin A1C 7.1%). Patient has high lactate levels (>25 mg/dL), low body weight, and problems with eating and gaining weight. The patient further suffers from hypermagnesuria (high levels of magnesium in urine, low levels in blood). Patient has memory and learning problems, astigmatism, and low mitochondrial
20 activity in peripheral lymphocytes as determined by TMRE, ATP content and O₂ consumption rate (relative to the healthy mother).

Mobilization of autologous hematopoietic stem and progenitor cells (HSPC), leukapheresis and CD34 positive selection were performed similar to patient 1 (Example 4) with the addition of plerixafor (n=2) on day -1 prior to leukapheresis. Mitochondria were isolated from
25 maternal peripheral blood mononuclear cells (PBMCs) using 250 mM sucrose buffer pH 7.4 by differential centrifugation.

For MAT, the autologous CD34⁺ cells were incubated with the healthy mitochondria from the patient's mother (1*10⁶ cells per amount of mitochondria having 4.4 units of citrate synthase (CS)), resulting in a 1.62 fold increase in the cells mitochondrial content (62%
30 increase in mitochondrial DNA content as demonstrated by CS activity. Incubation with mitochondria was performed for 24 hours at R.T. in saline containing 4.5% HSA. It should be noted that after mitochondrial enrichment, the CD34⁺ cells from the patient increased the rate of colony formation by 26%.

Patient 2 (15 KG at day of treatment) was treated by IV infusion with 1.8×10^6 autologous CD34⁺ cells enriched with healthy mitochondria per kilogram body weight, according to the timeline presented in **Figure 5A**.

Figure 5B presents the level of lactate found in the blood of the patient as a function of time post the I.V. injection. The data teach that the level of blood lactate was significantly reduced over time.

Figure 5C presents the ATP content in lymphocytes of the patient as a function of time post the I.V. injection. The control is the ATP content in lymphocytes of the patient's mother, which is the donor of the mitochondria. The data teach that the patient's ATP content was improved over time.

Figure 4 (Pt.2) presents the prevalence of normal mtDNA as a function of time post the I.V. injection. As can be seen in Figure 4 (Pt.2), the prevalence of normal mtDNA was increased from a baseline of about 1 to as high as 2 (+ 100%) in just 1 month, remaining relatively high until 10 months post treatment. Notably, normal mtDNA levels were above the baseline level at all time points.

Example 5. Compassionate treatment using autologous CD34⁺ cells enriched with MNV-BLD (blood derived mitochondria) for a young patient with Pearson Syndrome (PS).

Patient 3 was a 10.5-years old female patient, diagnosed with Pearson Syndrome, having a deletion of nucleotides 12113-14421 in her mtDNA. The patient also suffered from anemia, and from Fanconi's Syndrome that developed into kidney insufficiency stage 4. Patient was treated with dialysis three times a week. In the last two months, patient also suffered from a severe vision disorder, narrowing of the vision field and loss of near vision. Patient was incapable of any physical activity at all (no walking, sits in a stroller). Patient had high lactate levels (>50 mg/dL), and a pancreatic disorder which was treated with insulin. Brain MRI showed many lesions and atrophic regions. Patient was fed only through a gastrostomy. Patient had memory and learning problems. Patient had low mitochondrial activity in peripheral lymphocytes as determined by Tetramethylrhodamine, Ethyl Ester (TMRE), ATP content and O₂ consumption rate (relative to the healthy mother) tests.

Mobilization of autologous hematopoietic stem and progenitor cells (HSPC) as well as leukapheresis and CD34 positive selection were performed similar to patient 1 (Example 4) with the addition of plerixafor (n=1) on day -1 prior to leukapheresis. Leukapheresis was performed via a permanent dialysis catheter. Mitochondria were isolated from maternal

peripheral blood mononuclear cells (PBMCs) using 250 mM sucrose buffer pH 7.4 by differential centrifugation.

For MAT, the autologous CD34⁺ cells were incubated with healthy mitochondria from the patient's mother (1×10^6 cells per amount of mitochondria having 4.4 units of citrate synthase (CS)), resulting in a 1.14 fold increase in the cells mitochondrial content (14% increase in mitochondrial content as demonstrated by CS activity). Cells were incubated with mitochondria for 24 hours at R.T. in saline containing 4.5% HSA. It should be noted that after mitochondrial enrichment, the CD34⁺ cells from the patient increased the rate of colony formation by 52%.

10 Patient 3 (21 KG) was treated by IV infusion with 2.8×10^6 autologous CD34⁺ cells enriched with healthy mitochondria from her mother per kilogram body weight, according to the timeline presented in **Figure 6A**.

Figure 6B presents the level of lactate found in the blood of the patient as a function of time before and after therapy. The data teach that the level of blood lactate was significantly
15 reduced over time.

Figure 4 (Pt.3) presents the change in heteroplasmy level as a function of time after MAT. It can be seen that there was a decrease in heteroplasmy (less deleted mtDNA) following MAT in patient 3 who had relatively low levels of heteroplasmy at baseline. This was ongoing throughout the follow-up period. Notably, normal mtDNA levels were above the baseline
20 level at most time points.

Example 6. Compassionate treatment using autologous CD34⁺ cells enriched with MNV-BLD (blood derived mitochondria) for a young patient with Kearns–Sayre syndrome (KSS).

25 Patient 4 was a 14-years old, 19.5 kg female patient, diagnosed with Kearns–Sayre syndrome, experiencing tunnel vision, ptosis, ophthalmoplegia and retinal atrophy. The patient had vision problems, CPEO, epileptic seizures, pathologic EEG, severe myopathy with disability to sit or walk, cardiac arrhythmia. The patient had a 7.4 Kb deletion in her mitochondrial DNA, including the following genes: TK, NC8, ATP8, ATP6, CO3, TG, ND3, TR, ND4L,
30 TH, TS2, TL2, ND5, ND6, TE, NC9 and CYB.

Mobilization of hematopoietic stem and progenitor cells (HSPC) as well as leukapheresis and CD34 positive selection were performed similar to patient 3 (Example 5). For MAT, the

autologous CD34+ cells were incubated for 24 hours at R.T. with healthy mitochondria from the patient's mother (1×10^6 cells per amount of mitochondria having 4.4 units of citrate synthase (CS)), in saline containing 4.5% HSA. The enrichment resulted in a 1.03 fold increase in the cells mitochondrial content (3% increase in mitochondrial content as demonstrated by CS activity).

Patient 4 was treated with 2.2×10^6 autologous CD34+ cells enriched with healthy mitochondria per kilogram body weight, according to the timeline presented in **Figure 6A**.

Unexpectedly, 4 months after a single treatment with CD34+ that were enriched by only 3% with healthy mitochondria, the patient showed marked improvement in EEG with no epileptic seizures. Five months after treatment the patient suffered disease-related atrioventricular (AV) block and a pacemaker was installed. The patient recovered and improvement continued. The ATP content in the peripheral blood was measured 6 months post-treatment, showing an increase of about 100% in ATP content compared to that before treatment. Seven months after treatment, the patient could sit by herself, walk with assistance, talk, has better appetite and has gained 3.6 KG.

Example 7: Mitochondrial enrichment therapy increases exploratory behavior in middle-aged mice

Middle aged (12-month old) C57BL/6J mice were administered with either mitochondria-enriched bone marrow (BM) cells (MNV-BM-PLC, 1×10^6 cells, n=24), bone marrow cells (BM, 1×10^6 cells, n=23) or a control vehicle solution (VEHICLE, 4.5% Albumin in 0.9% w/v NaCl, n=23), with repeated administration every approximately 3 months. 1 week prior to initial administration and 9 months post initial administration, the mice were subjected to an open field behavioral test

The results presented in **Figures 7A-7B** demonstrate that MNV-BM-PLC treated mice exhibited an extensive exploratory behavior - increase in the time spent in the arena's center, which was statistically significant compared with the center duration of the BM control group.

As shown in **Figure 7C**, unlike BM and VEHICLE control groups, which exhibited an increased thigmotactic behavior reflected by an increased time spent by the walls of the arena, 9 months post administration, MNV-BM-PLC treated mice decreased their wall duration in a statistically significant manner compared with BM control group.

The increase in time spent in the central zone of the arena indicates an extensive exploratory behavior of mice that underwent mitochondrial augmentation therapy. Along with the

reduction in thigmotaxis, which is associated with anxiety-like behaviors, it attests to an anxiolytic effect of mitochondrial augmentation.

Example 8. Mitochondrial enrichment attenuates age-related motor function decline in middle-aged mice

- 5 Gross motor performance and coordination were assessed by using a Rotarod device in middle-aged (12-month old) C57BL/6J mice administered with either mitochondria-enhanced bone marrow (BM) cells (MNV-BM-PLC, 1×10^6 cells, n=24), bone marrow cells (BM, 1×10^6 cells, n=23) or a control vehicle solution (VEHICLE, 4.5% Albumin in 0.9% w/v NaCl, n=23).
- 10 As shown in **Figures 8A-8B**, 1 month post administration, VEHICLE and BM control groups showed a decrease in latency to fall off the rotating rod (-2.82% and -2.18% from baseline, ns) which further declined by 14.15% and 21.79% (**p=0.0008) relative to baseline 3 months post administration. MNV-BM-PLC mice exhibited a 16.17% reduction in latency to fall off the rod 1 month post mitochondria enrichment therapy (*p=0.0464), halted 3 months
- 15 post enrichment (-8.72% from baseline, ns).

The results demonstrate a moderation of motor function impairment in mitochondria-enriched middle-aged mice relative to age-matched controls, implying that mitochondrial enrichment therapy can attenuate age-related motor function deterioration.

Example 9. Treatment using stem cells enriched with mitochondria for brain diseases and disorders.

20 Title: A phase I/II, open label, single dose clinical study to evaluate the safety, engraftment and therapeutic effects of transplantation of stem cells enriched with mitochondria in patients with brain diseases and disorders.

Design: All patients enrolled have a confirmed diagnosis of a brain disease or disorder. The donor of the mitochondria is confirmed as carrying no mtDNA abnormalities. Eligible patients are enrolled into the study and admitted to hospital for a short period and undergo the treatment procedure. Treatment safety, adverse events (AEs) and disease assessments are recorded throughout the duration of treatment and the post-treatment follow up period.

25

Treatment Doses: Therapeutic cell dose of up to 4×10^6 cells/kg body weight, are transplanted by IV infusion according to the routine standard procedure at the clinical department.

30

Primary Safety Endpoints Assessments: Subjects are assessed for adverse events following treatment with the cells, according to CTCAE v4.03, starting enrollment.

Primary Efficacy Endpoints Assessments: Change in annual rate of metabolic crisis; Change in relative abundance of wild-type mtDNA.

5 Secondary Efficacy Endpoints Assessment: Systemic benefit of MNV-BM-BLD and effect on distal organs (Hospitalization avoidance, Transfusion avoidance, Weight gain on standard growth curves, as compared to rate of gain in the year prior to the therapy). Additional patient-specific individualized outcomes should include change in disease target organs (Renal glomerular function measured by creatinine clearance, compared to the year prior to the study; Renal tubular function, measured by serum and urine levels of potassium and magnesium; Endocrine pancreatic function as assessed by insulin requirement, C-peptide and hemoglobin A1c; Exocrine pancreatic function and rate of diarrhea; Change in Brain MRI findings compared to baseline; Change in Quality of Life (QoL) questionnaire/PEDI-CAT (Pediatric Evaluation of Disability Inventory – Computer Adaptive Test) scores).

15 Exploratory Efficacy Assessments: Functional Assessment (Neuromuscular function as assessed by Gross Motor Function Measure; 6-min walk test; Stress test; Developmental test; WIPSSI (Wechsler Preschool and Primary Scale of Intelligence); Memory test; Reaction time; Box and Blocks test; 30-Second Chair Stand; Standing without Support); Pathologic Assessment (Echocardiography; Bone marrow aspiration + Biopsy; Lymphocyte O₂ consumption, ATP content, TMRE/MTG).

20 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

CLAIMS

1. A pharmaceutical composition for use in treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the composition comprising at least 10^5 to 2×10^7 human stem cells per kilogram bodyweight of the patient in a
5 pharmaceutically acceptable liquid medium capable of supporting the viability of the cells, wherein the human stem cells are enriched with frozen-thawed healthy functional human exogenous mitochondria, wherein the brain disease or disorder is not a mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial protein.
- 10 2. The pharmaceutical composition of claim 1, wherein the enrichment comprises introducing into the stem cells a dose of mitochondria of at least 0.088 up to 176 milliunits of CS activity per million cells
3. The pharmaceutical composition of claim 2 wherein the enrichment comprises contacting the stem cells with a dose of mitochondria of 0.88 up to 17.6 milliunits of CS
15 activity per million cells.
4. The pharmaceutical composition according to any one of claims 1 to 3, wherein the healthy human exogenous mitochondria are syngenic or allogeneic.
5. The pharmaceutical composition of any one of claims 1 to 4, wherein the disease or disorder is associated with acquired mitochondrial dysfunction.
- 20 6. The pharmaceutical composition of any one of claims 1 to 4, wherein the disease or disorder is not associated with acquired mitochondrial dysfunction.
7. The pharmaceutical composition of any one of claims 1 to 6, wherein the disease or disorder is selected from the group consisting of Parkinson Disease (PD), Alzheimer Disease (AD), stroke, epilepsy, multiple sclerosis (MS), dementia, adrenoleukodystrophy (ALD),
25 Huntington Disease (HD), Guillain Barre Syndrome (GBS), autism and Friedreich's ataxia.
8. The pharmaceutical composition of any one of claims 1 to 6, wherein the symptom is selected from the group consisting of impaired ability to execute commands, impaired ability to speak, impaired response time, impaired motor skills, impaired learning, impaired ability to walk, impaired ability to climb stairs, impaired EEG, seizures, impaired ability to draw,
30 impaired language skills, and impaired memory.

9. The pharmaceutical composition of any one of claims 1 to 8, wherein said pharmaceutical composition is administered directly to the central nervous system (CNS).
10. The pharmaceutical composition of any one of claims 1 to 8, wherein the pharmaceutical composition is administered by systemic administration.
- 5 11. The pharmaceutical composition of claim 10, comprising about 10^6 mitochondrially-enriched human stem cells per kilogram body weight of the patient.
12. The pharmaceutical composition of any one of claims 1 to 9, comprising about total of 5×10^5 to 5×10^9 human stem cells enriched with human mitochondria.
13. The pharmaceutical composition of any one of claims 1 to 12, wherein the
10 mitochondrially-enriched human stem cells have at least one of:
- (i) an increased mitochondrial DNA content;
 - (ii) an increased level of CS activity;
 - (iii) an increased content of at least one mitochondrial protein selected from SDHA and COX1;
 - 15 (iv) an increased rate of O_2 consumption;
 - (v) an increased rate of ATP production; or
 - (vi) any combination thereof,
- relative to the corresponding level in the stem cells prior to mitochondrial enrichment
14. The pharmaceutical composition of any one of claims 1 to 13, wherein the human
20 stem cells are obtained or derived from the patient before enrichment with the exogenous mitochondria.
15. The pharmaceutical composition of any one of claims 1 to 13, wherein the human stem cells are obtained or derived from a donor different than the patient before enrichment with the exogenous mitochondria.
- 25 16. The pharmaceutical composition of claim 15, wherein the donor is at least partly HLA-matched with the patient.
17. The pharmaceutical composition of any one of claims 1 to 16, wherein the human stem cells are hematopoietic stem cells.

18. The pharmaceutical composition of any one of claims 1 to 16, wherein the human stem cells are mesenchymal stem cells.
19. The pharmaceutical composition of any one of claims 1 to 16, wherein the human stem cells are pluripotent stem cells (PSCs) or induced pluripotent stem cells (iPSCs).
- 5 20. The pharmaceutical composition of any one of claims 1 to 19, wherein the human stem cells are CD34⁺.
21. The pharmaceutical composition of any one of claims 2 to 20, wherein human stem cells have undergone at least one freeze-thaw cycle prior to introducing healthy functional human exogenous mitochondria into said human stem cells.
- 10 22. The pharmaceutical composition of any one of claims 1 to 21, wherein the human stem cells are isolated, derived or obtained from cells of the bone marrow, adipose tissue, oral mucosa, skin fibroblasts, blood or umbilical cord blood.
23. The pharmaceutical composition of any one of claims 1 to 22, wherein the healthy functional human exogenous mitochondria are isolated or obtained from placenta, placental cells grown in culture or blood cells.
- 15 24. The pharmaceutical composition of any one of claims 1 to 23, wherein the human stem cells have undergone at least one freeze-thaw cycle after enrichment with the healthy functional human exogenous mitochondria.
25. The pharmaceutical composition of any one of claims 1 to 24, wherein the healthy functional human exogenous mitochondria constitute at least 3% of the total mitochondria in the mitochondrially-enriched human stem cells.
- 20 26. The pharmaceutical composition of claim 25, wherein the healthy functional human exogenous mitochondria constitute at least 10% of the total mitochondria in the mitochondrially-enriched human stem cells.
- 25 27. The pharmaceutical composition of any one of claim 1 to 26, further comprising non-enriched stem cells, megakaryocytes, erythrocytes, mast cells, myeloblasts, basophils, neutrophils, eosinophils, monocytes, macrophages, natural killer (NK) cells, small lymphocytes, T lymphocytes, B lymphocytes, plasma cells, reticular cells, or any combination thereof.

28. A method of treating a brain disease, disorder or a symptom thereof in a human patient in need of such treatment, the method comprising the step of administering parenterally a pharmaceutical composition to the patient, the pharmaceutical composition comprising at least about 5×10^5 to 5×10^9 human stem cells, wherein the human stem cells are enriched with frozen-thawed healthy functional human exogenous mitochondria, wherein the brain disease or disorder is not a primary mitochondrial disease or disorder caused by a pathogenic mutation in mitochondrial DNA or by a pathogenic mutation in nuclear DNA encoding a mitochondrial protein.
29. The method of claim 28, wherein said administering the pharmaceutical composition is directly to the central nervous system (CNS).
30. The method of claim 29 wherein said administering is intrathecal.
31. The method of claim 28, wherein said administering the pharmaceutical composition is by systemic administration.
32. The method of any one of claims 28 to 31, wherein the human stem cells are obtained or derived from the patient before enrichment with the exogenous mitochondria.
33. The method of any one of claims 28 to 31, wherein the human stem cells are obtained or derived from a donor different than the patient before enrichment with the exogenous mitochondria.
34. The method of claim 33, wherein the donor is at least partly HLA-matched with the patient.
35. The method of claim 33 or claim 34, further comprising a step of administering to the patient an agent which prevents, delays, minimizes or abolishes an adverse immunogenic reaction between the patient and the mitochondrially-enriched human stem cells.
36. The method of claim 35, wherein the adverse immunogenic reaction is a graft-versus-host disease (GvHD).

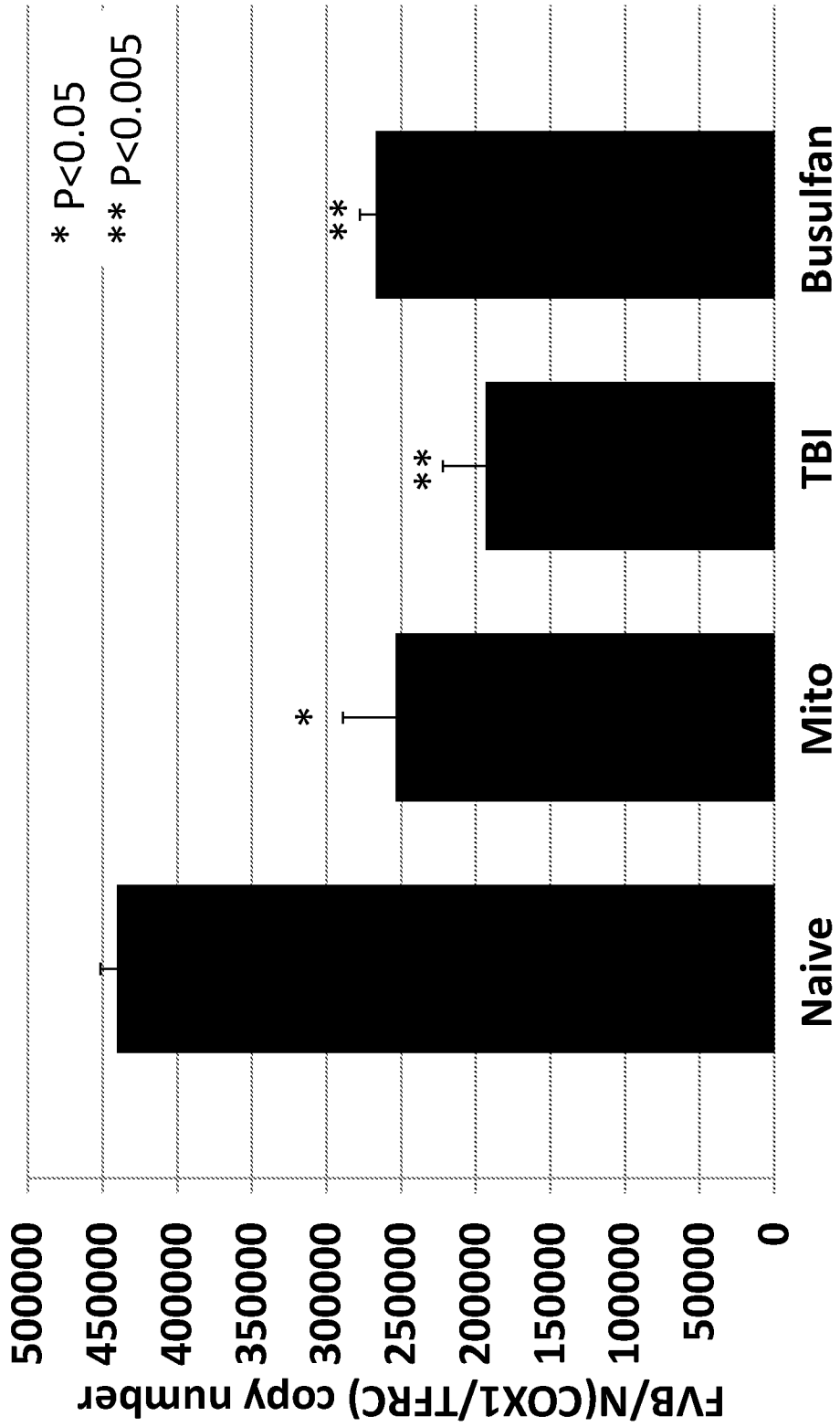


Figure 1

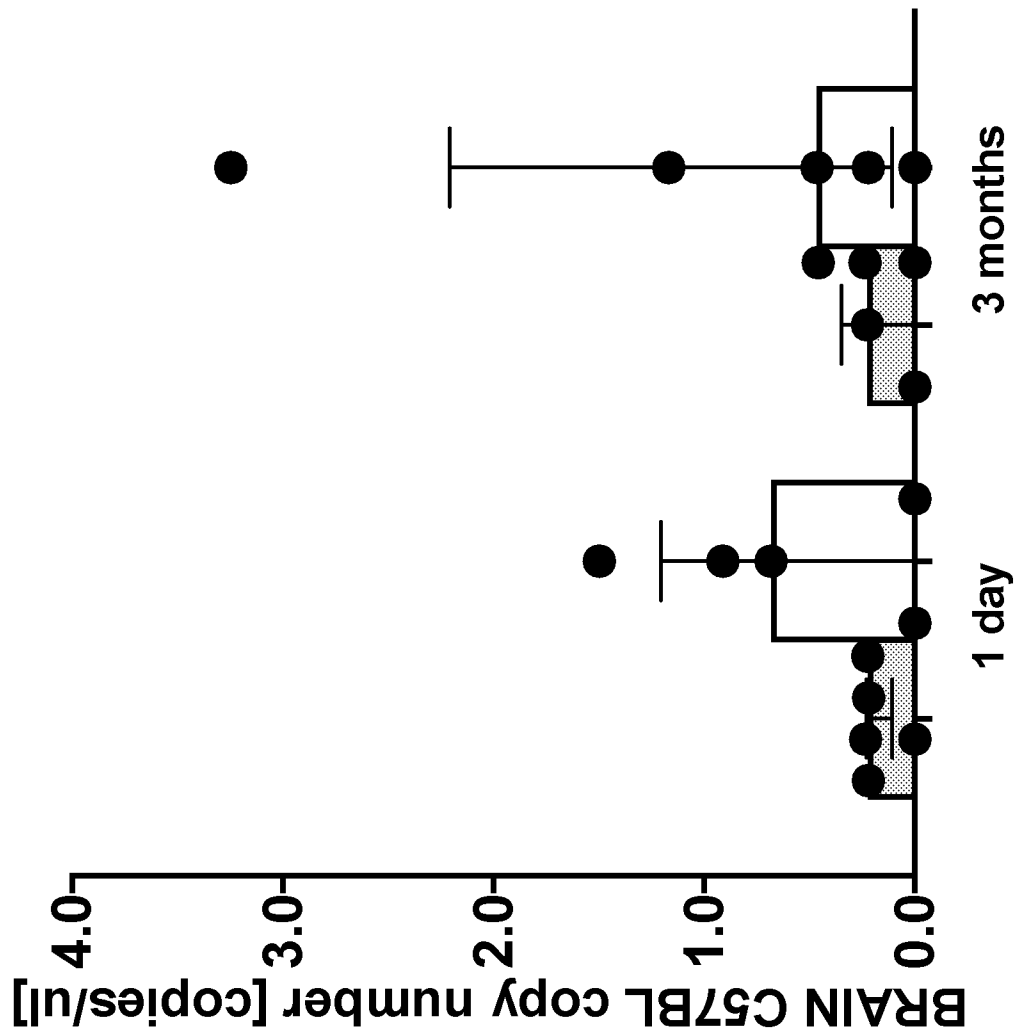


Figure 2

3/17

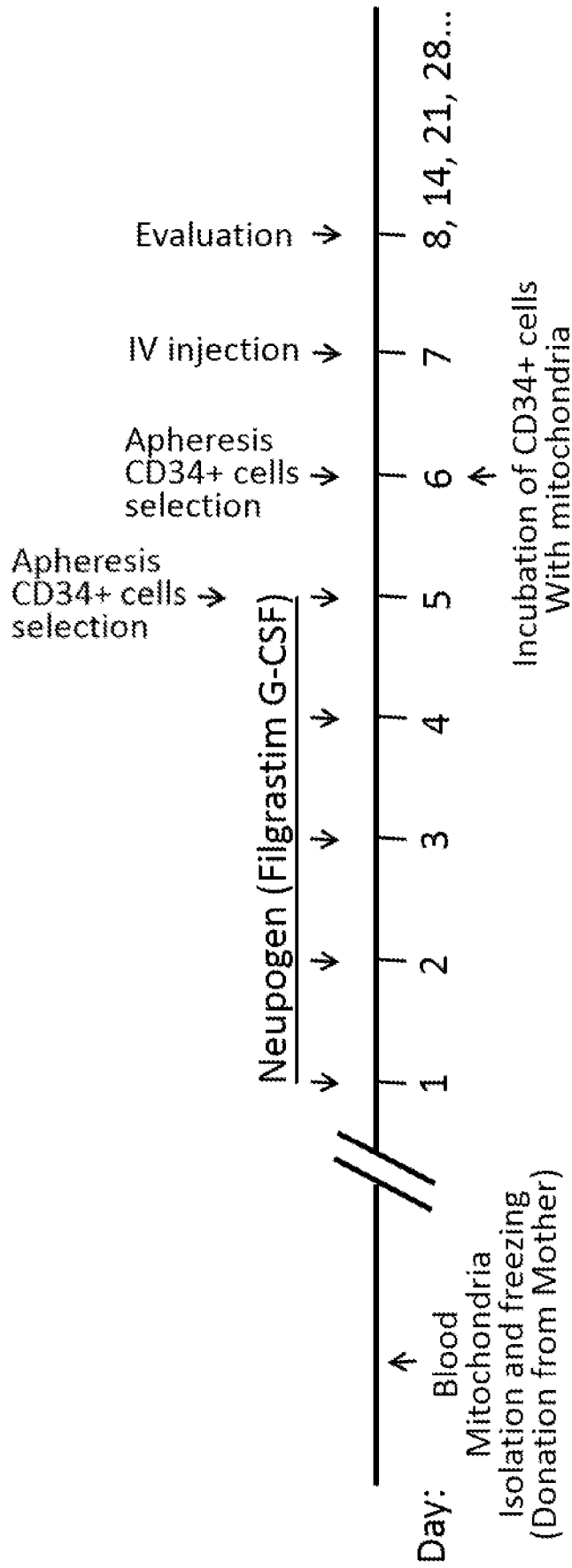


Figure 3A

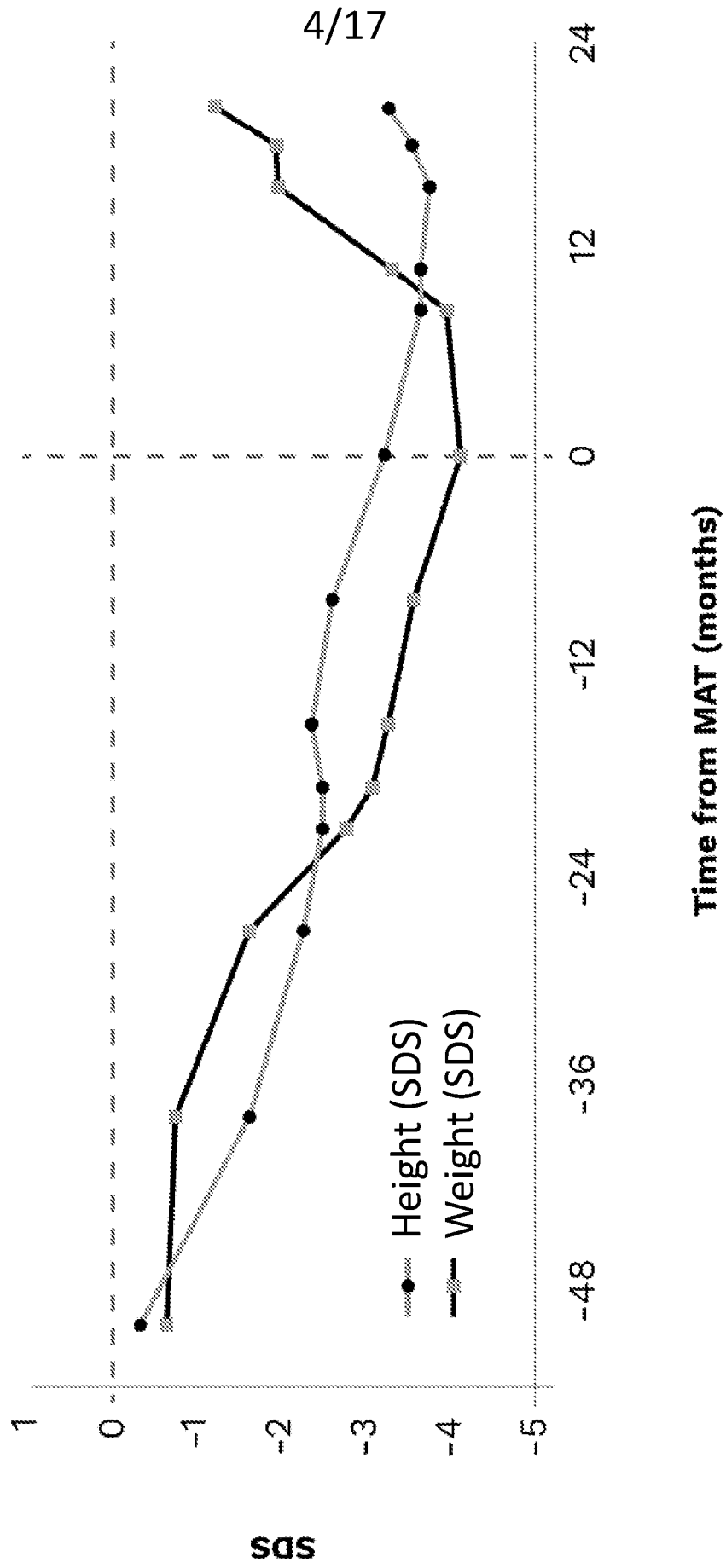


Figure 3B

5/17

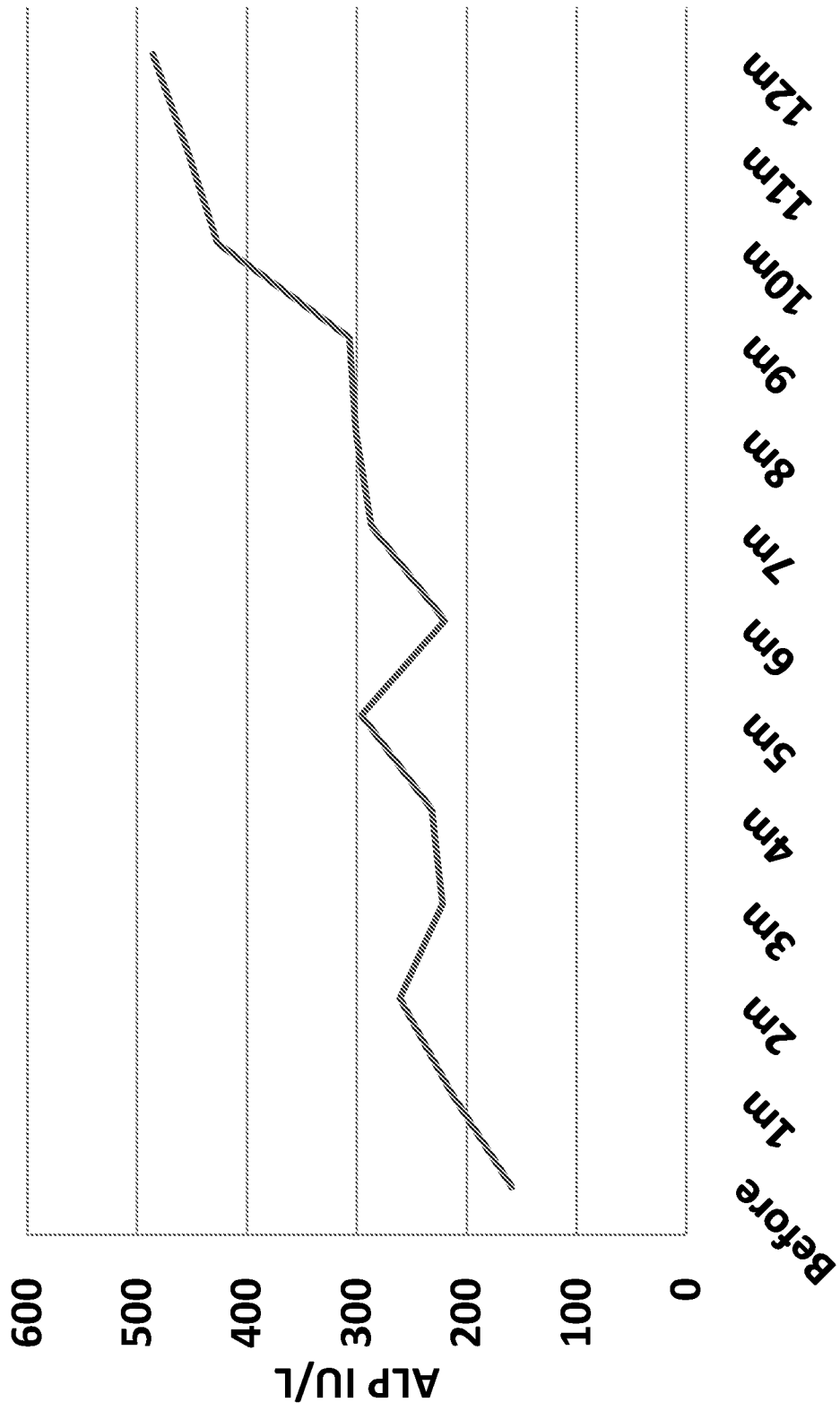


Figure 3C

6/17

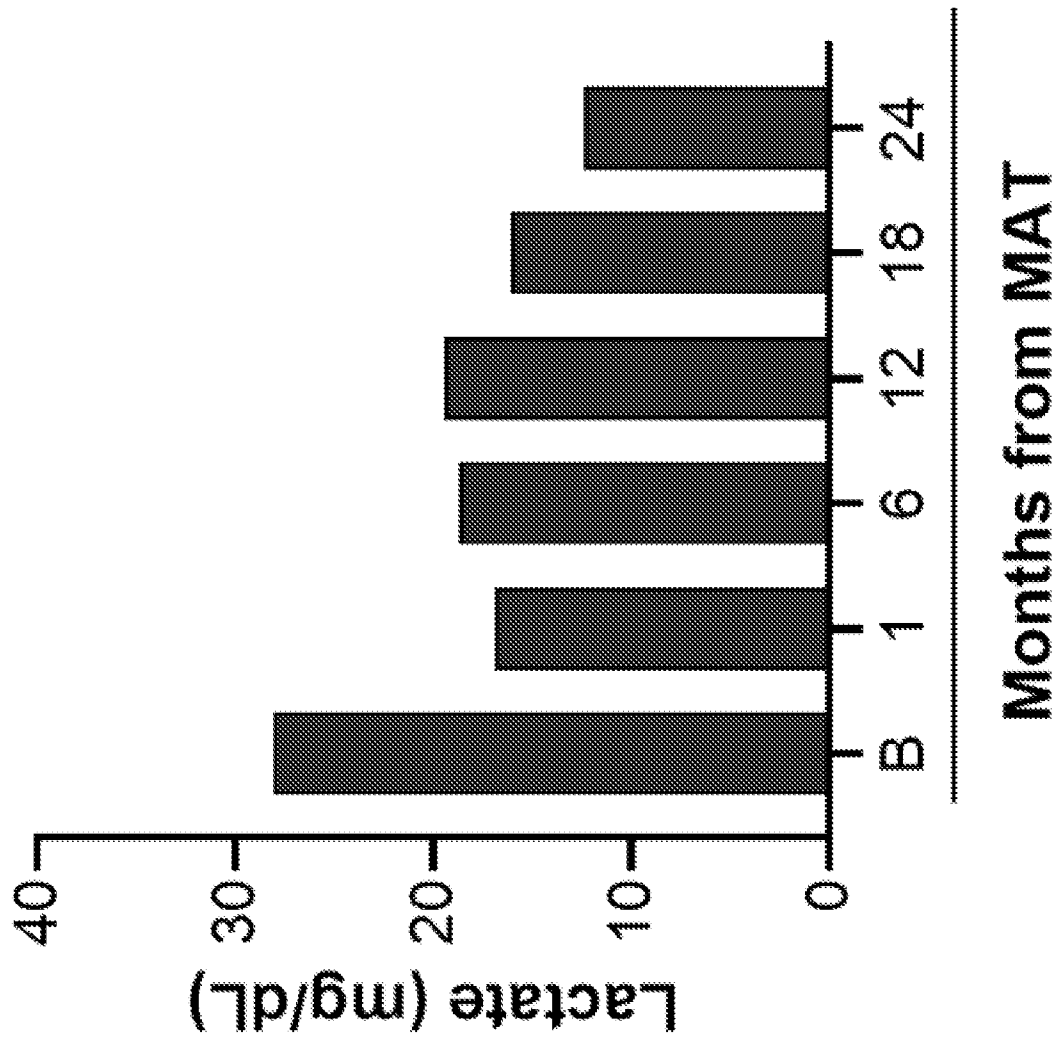


Figure 3D

7/17

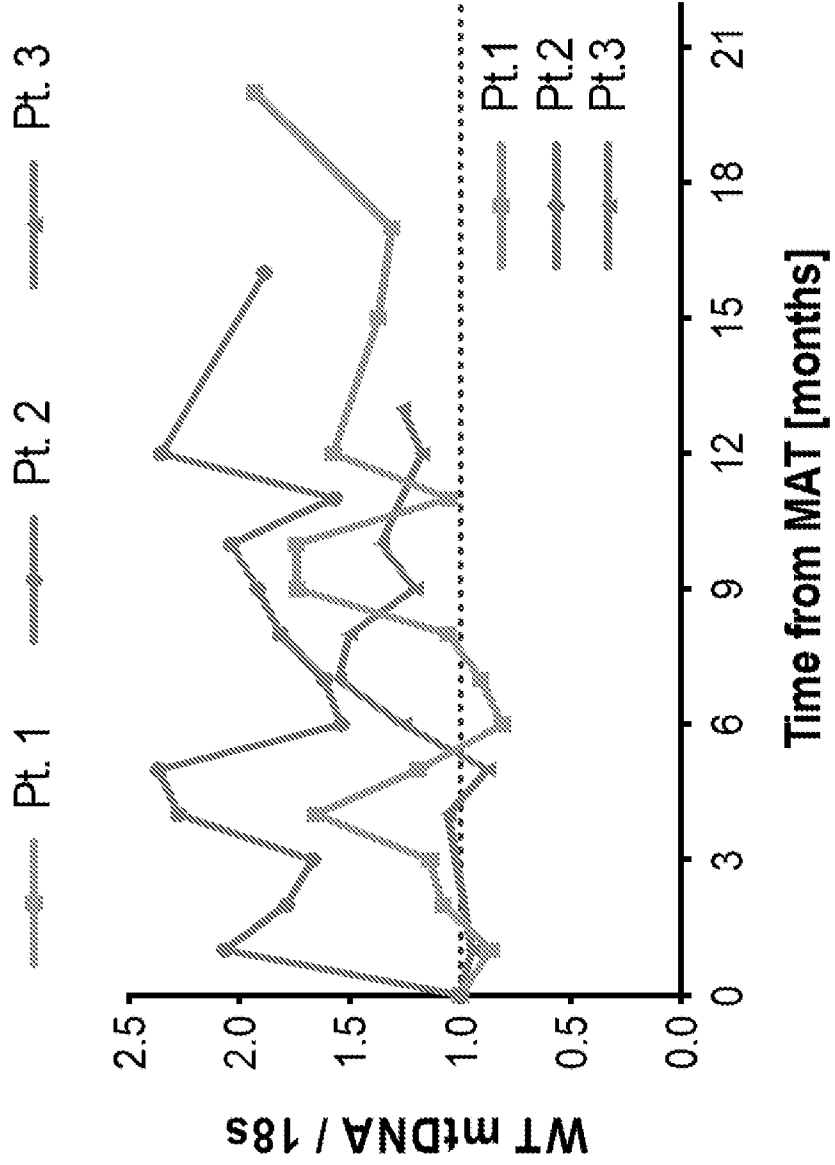


Figure 4

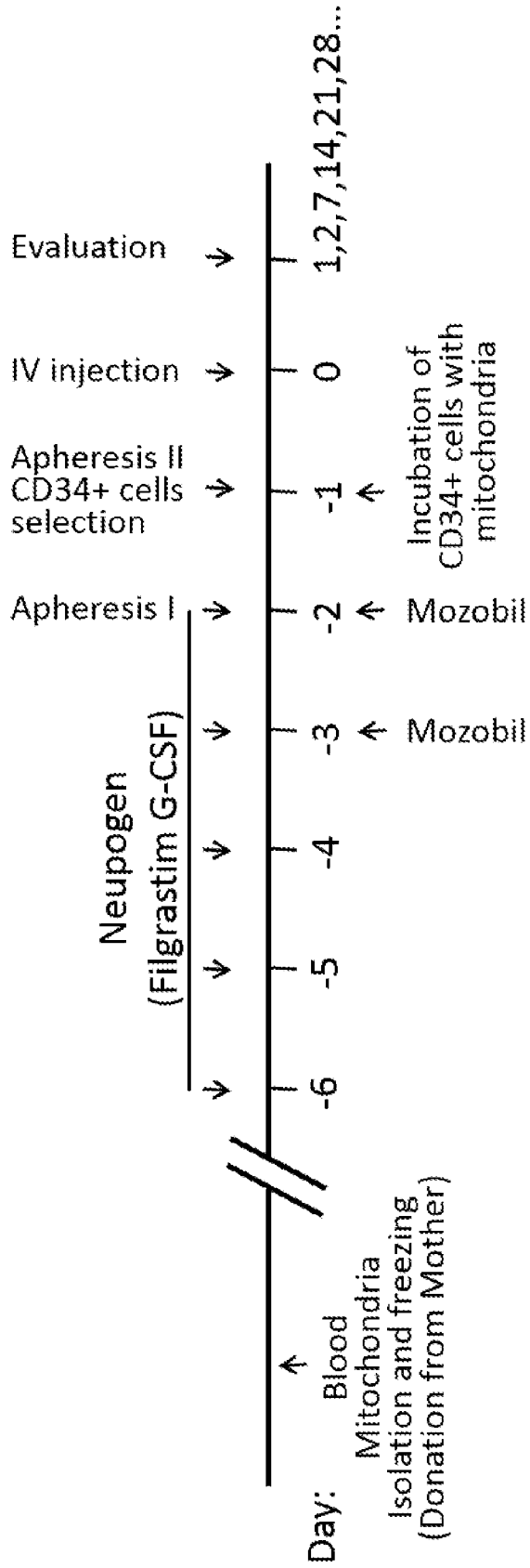


Figure 5A

9/17

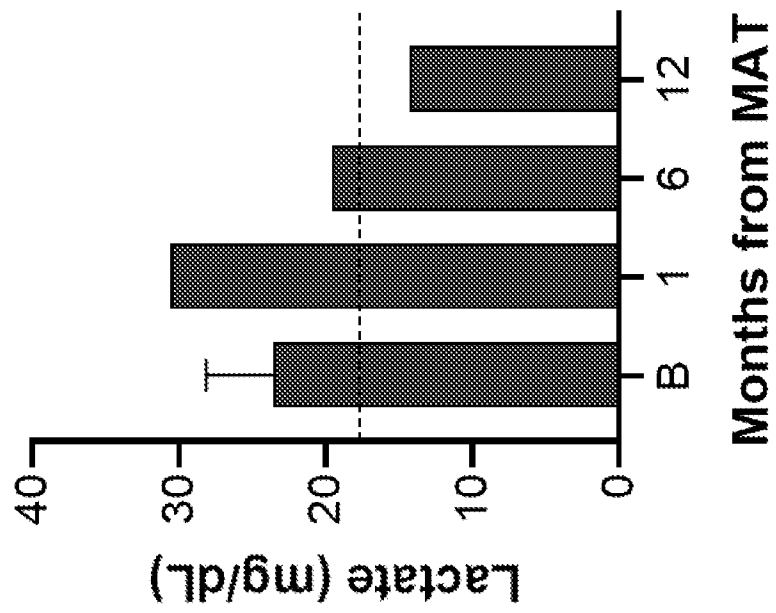


Figure 5B

10/17

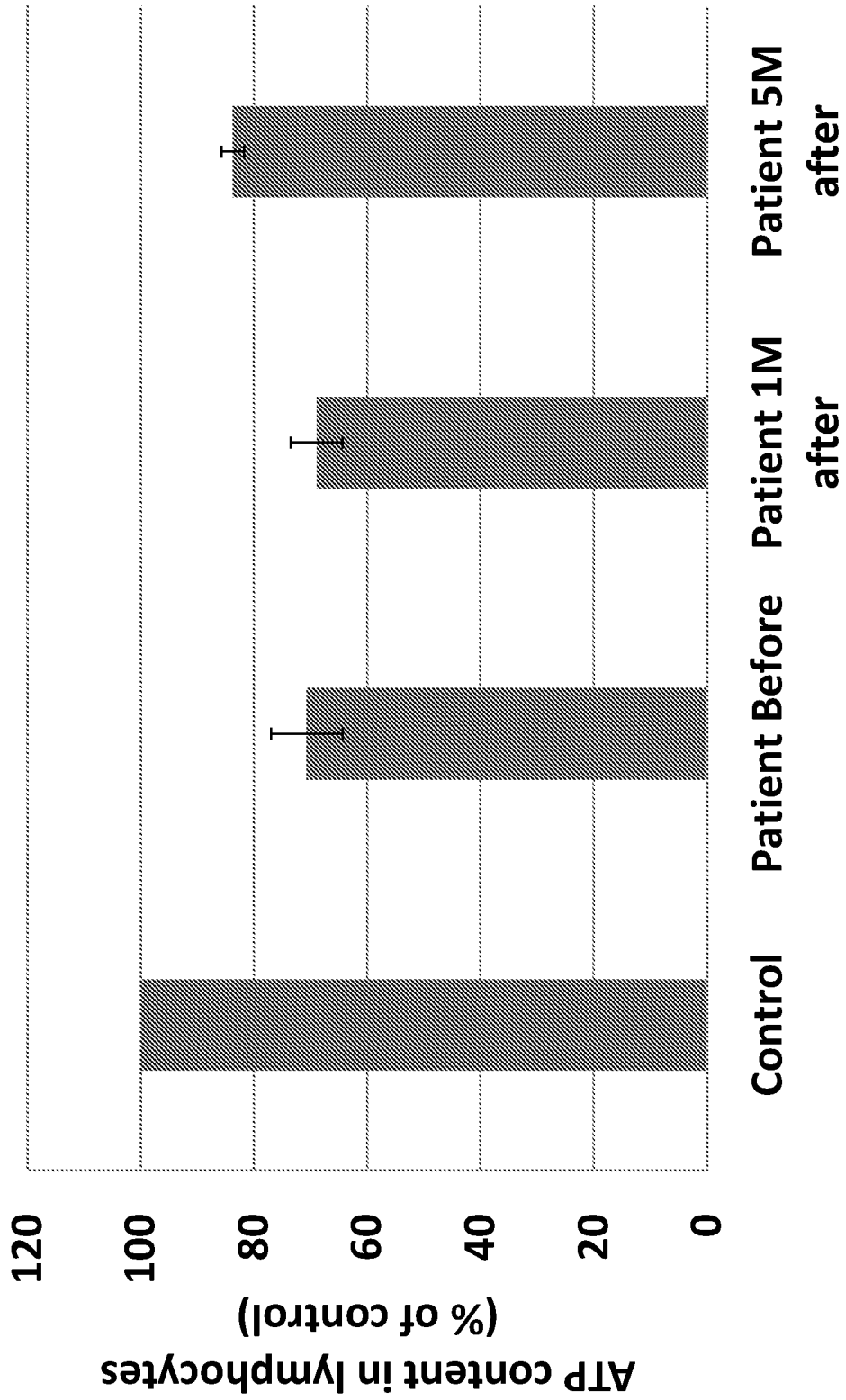


Figure 5C

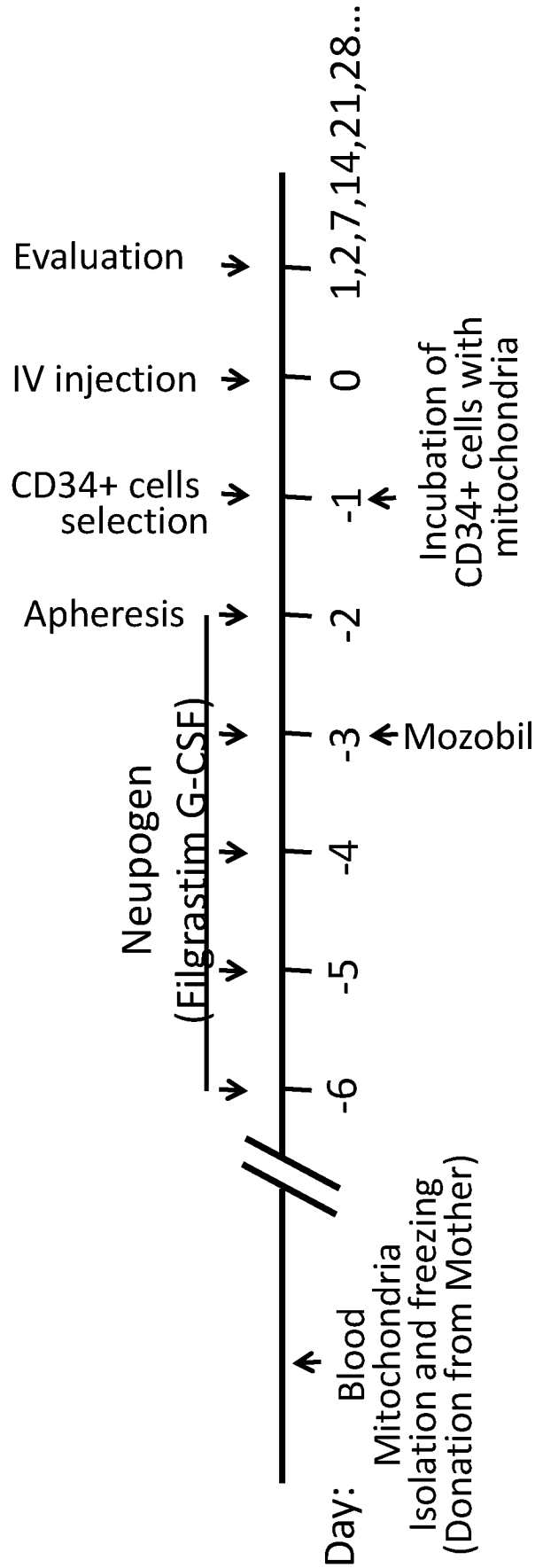


Figure 6A

12/17

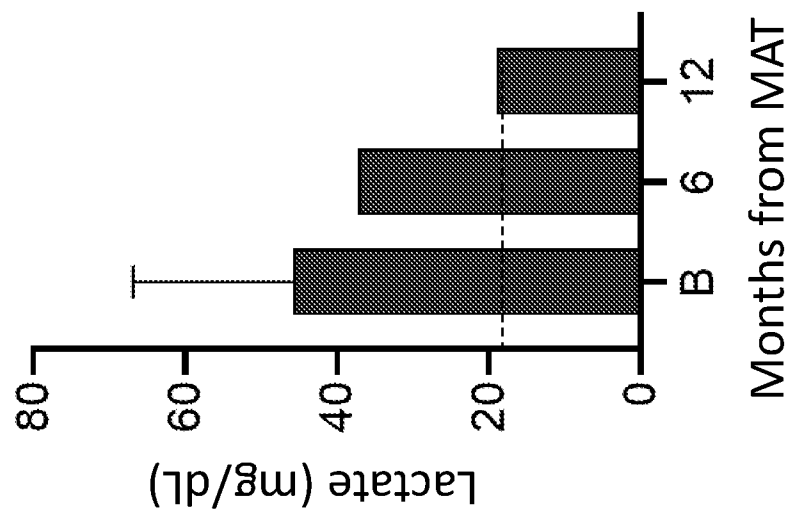


Figure 6B

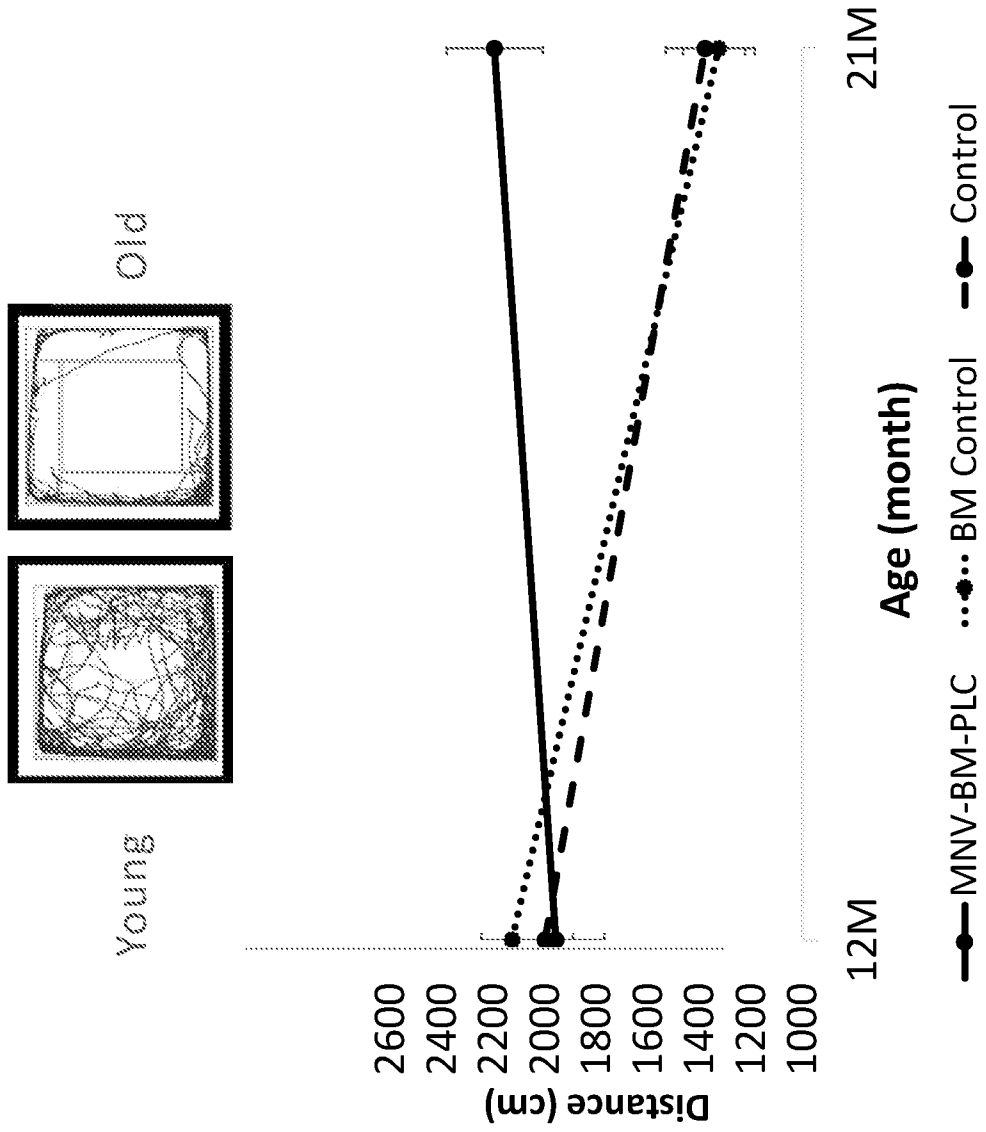


Figure 7A

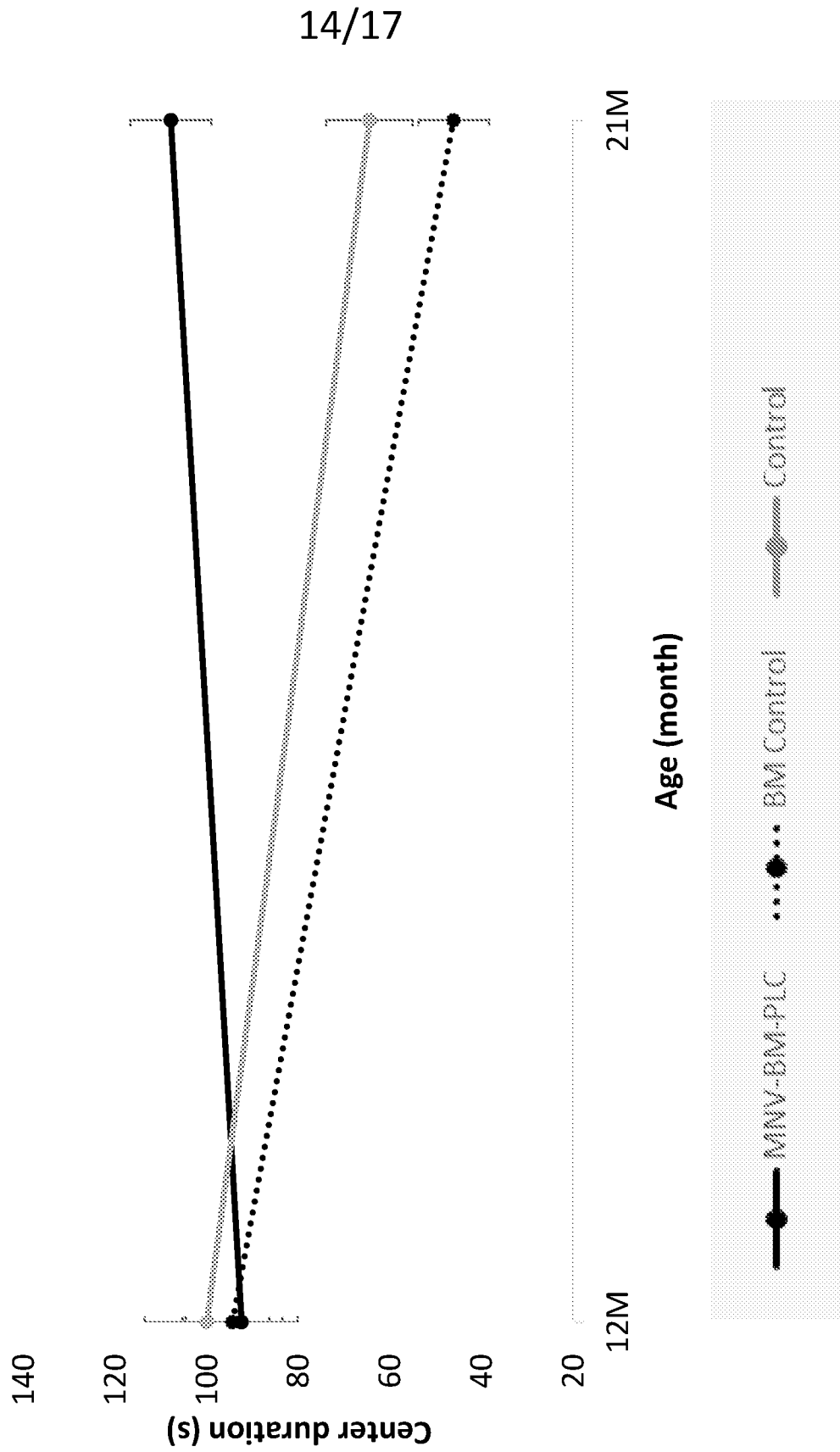


Figure 7B

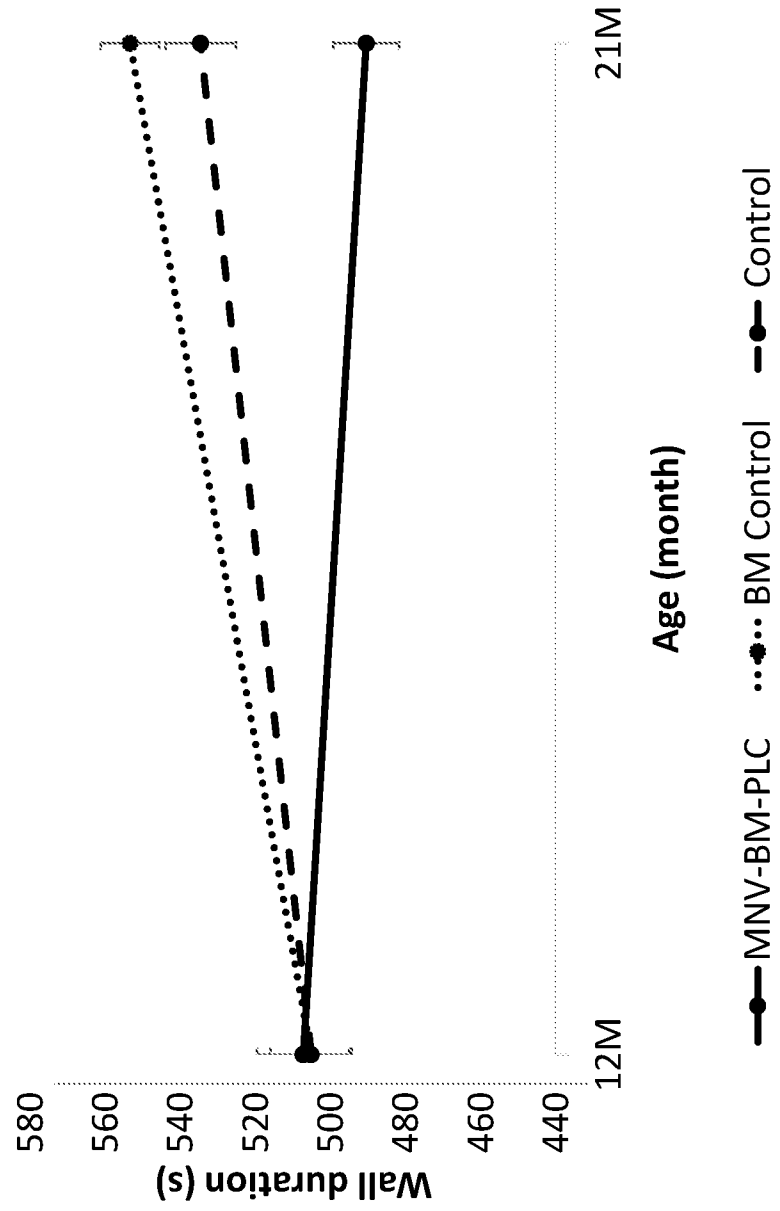


Figure 7C

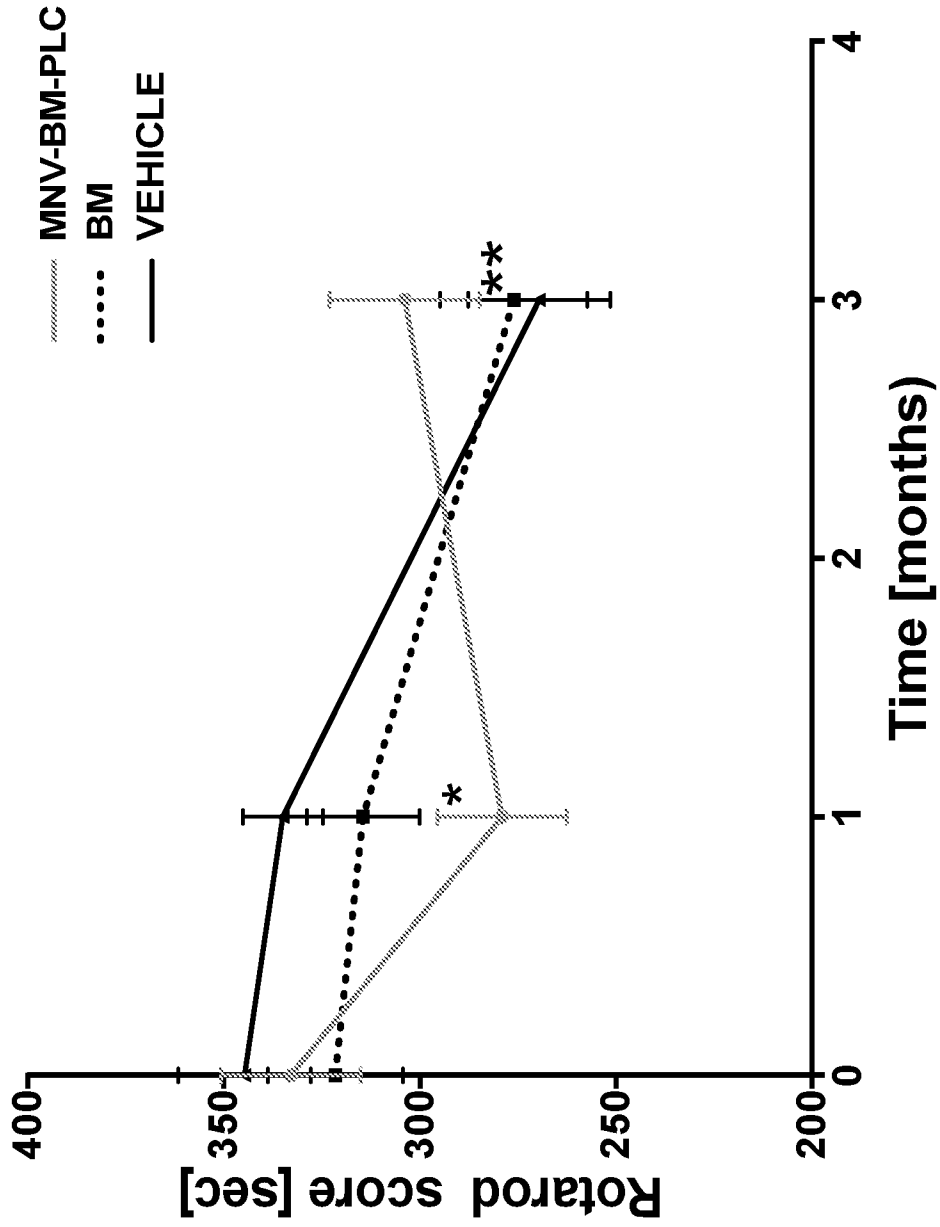


Figure 8A

*p < 0.05, ** < 0.01, *** < 0.001

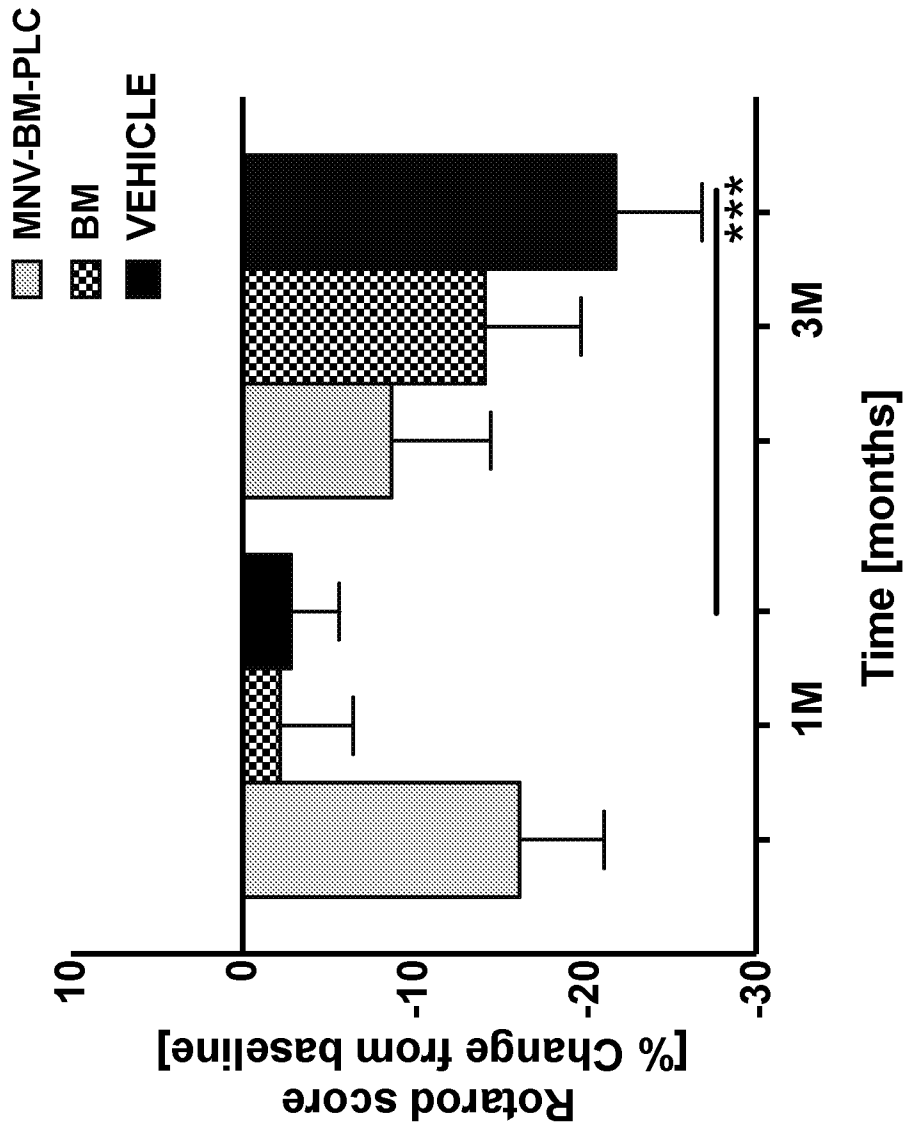


Figure 8B

*p < 0.05, ** < 0.01, *** < 0.001

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2019/050825

A. CLASSIFICATION OF SUBJECT MATTER
 IPC (20190101) A61K 35/28, A61P 25/28, A61P 25/16
 CPC (20150115) A61K 35/28, A61P 25/28, A61P 25/16
 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)
 IPC (20190101) A61K 35/28, A61P 25/28, A61P 25/16
 CPC (20150115) A61K 35/28, A61P 25/28, A61P 25/16

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 Databases consulted: PATENTSCOPE, THOMSON INNOVATION, Esp@cenet, Google Patents, Google Scholar
 Search terms used: mitochondria therapy treatment "brain disease" "brain disorder" "neurological disease" "neurological disorder"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Mitochondrial transplantation strategies as potential therapeutics for central nervous system trauma. Neural regeneration research, 2018, 13.2: 194. GOLLIHUE, Jenna L.; PATEL, Samir P.; RABCHEVSKY, Alexander G 01 Feb 2018 (2018/02/01) abstract, paragraph 2	1-3,8,10,28,31,35, 36
Y	abstract	9,29
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Y	WO 2018101708 A1 CHOI et al. 07 Jun 2018 (2018/06/07) paragraph 63	11,12
Y	WO 2016135723 A1 YIVGI-OHANA et al. 01 Sep 2016 (2016/09/01) page 6 lines 8-9,15-18 page 13 lines 20-23, page 14 line 6 page 15 lines 1-4 page 17 lines 26-31, page 18 lines 20-30 page 27 lines 6-8 page 28 lines 26-30	13-26,33,34

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
 "A" document defining the general state of the art which is not considered to be of particular relevance
 "D" document cited by the applicant in the international application
 "E" earlier application or patent but published on or after the international filing date
 "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 28 Nov 2019	Date of mailing of the international search report 28 Nov 2019
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Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Email address: pctoffice@justice.gov.il	Authorized officer POUNY Yehonathan Telephone No. 972-73-3927124
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No. PCT/IL2019/050825
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