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(54) Title: EFFICIENT SYSTEMIC TREATMENT OF DYSTROPHIC PATHOLOGIES

(57) Abstract: A composition comprising a gene therapy product for use in the treatment of a dystrophic disease in a subject, advantageously in humans, wherein: the gene therapy product comprises a nucleic acid sequence encoding a functional microdystrophin; the composition is systemically administered.

EFFICIENT SYSTEMIC TREATMENT OF DYSTROPHIC PATHOLOGIES

The present invention provides an efficient gene therapy product for dystrophic diseases, especially in humans and dogs, defined by the sequence encoding the microdystrophin, the delivery vehicle and the route of administration to be used.

BACKGROUND OF THE INVENTION

Duchenne muscular dystrophy (DMD) is the most frequent progressive muscle degenerative disease, affecting approximately one in 3,500 to 5000 male births. DMD is caused by deletions or mutations in the gene encoding dystrophin, located on the X chromosome. Dystrophin is required for the assembly of the dystrophin-glycoprotein complex, and provides a mechanical and functional link between the cytoskeleton of the muscle fiber and the extracellular matrix. The absence of functional dystrophin causes fiber degeneration, inflammation, necrosis and replacement of muscle with scar and fat tissue, resulting in progressive muscle weakness and premature death due to respiratory and cardiac failure between the second and fourth decade of life (Moser, H., Hum Genet, 1984. **66**(1): p. 17-40).

A milder form of the disease called Becker muscular dystrophy (BMD) is distinguished from DMD by delayed onset, later dependence on wheelchair support, and longer life span. BMD is caused by mutations maintaining the reading frame and the most critical parts of the gene, leading to a truncated but still functional dystrophin protein (Muntoni F *et al*, Lancet Neurol, 2003).

There is no cure nor effective treatment available for DMD (Rodino-Klapac, L.R. et *al.*, Curr Neurol Neurosci Rep, 2013. **13**(3): p. 332) or BMD. Conventional therapies are limited to supportive care, which partially alleviates signs and symptoms, but does not directly target the disease mechanism nor reverse the phenotype.

There currently are several therapeutic strategies being developed for DMD including *in vivo* gene therapy, cell transplantation therapy, pharmacologic rescue of DMD nonsense mutations and exon skipping strategies to repair the DMD gene reading frame. All of these strategies have problems to overcome, including targeting different muscle groups, optimization of delivery, long-term expression of the transgene, and potential immune response (Jamin *et al.*, Expert Opin Biol Ther, 2014).

The dystrophin gene is the largest known gene in the human genome and is too large to fit inside known gene therapy vector systems. Therefore, as of today, there are essentially two gene therapy strategies for DMD with viral vectors: i) constitutive expression of antisense oligonucleotides to promote exon skipping, which is amenable to certain mutations only, and (ii) constitutive expression of a cDNA coding for a functional, reduced-size dystrophin protein ("microdystrophin" also known as "minidystrophin").

Both strategies, use of small antisense sequences or use of microdystrophin, address the major hurdle for the use of AAV vectors in DMD gene therapy, which is their packaging capacity. AAV vectors can accommodate about 4.7 kb while the size of the wild type dystrophin cDNA is about 14 kb. To overcome this issue, a number of studies have developed partially deleted but highly functional dystrophin genes, which can be successfully packaged inside AAV vectors and were shown to improve, though not completely normalize, the dystrophic phenotype in animal models.

The *mdx* mouse model is commonly used to test new constructs encoding microdystrophins. However, this model has drawbacks because the *mdx* mouse displays a less severe form of the disease, without immune reactions. The other animal model is the GRMD dog, which is considered more reliable to predict the therapeutic potential of a gene therapy product in humans (Kornegay *et al.*, Mamm Genome, 2012).

Among all the proposed microdystrophin sequences, Foster H. *et al.* (Mol Ther, 2008. **16**(11): p. 1825-32) compared in mice two different configurations of microdystrophin genes, ΔAB/R3-R18/ΔCT and ΔR4-R23/ΔCT, under the control of a muscle-specific promoter (Spc5-12) in a recombinant AAV vector (rAAV2/8). It was reported that codon human optimization of microdystrophin improved gene transfer and muscle functions in the *mdx* mouse model. Intravenous injection of 3.10^{11} vg total of rAAV/8 allowed efficient cardiac gene transfer and marked dystrophin expression in the skeletal muscle and within the diaphragm.

In relation with CXMDj dogs, Ohshima S. *et al.* (Mol Ther, 2009. **17**(1): p. 73-80) reported the administration into dogs of a rAAV8 encoding a M3 microdystrophin under the control of the CMV promoter by limb perfusion, i.e. intravenous injection under pressure.

Zhang Y. *et al.* (Hum Mol Genet, 2013. **22**(18): p. 3720-29) studied the systemic (5.10^{12} vg total) dual AAV9 gene therapy in DMD mice. By homologous recombination, the dual AAV vectors injected via the tail vain reconstituted a nNOS binding microdystrophin containing dystrophin repeats R16 and R17.

Similarly, Odom G. *et al.* (Mol Ther, 2011. **19**(1): p. 36-45) demonstrated reconstitution of an expression cassette encoding a ΔH2-R19 minidystrophin in mice following intravascular co-delivery of two rAAV6 vectors (2.10^{12} vg total) sharing a central homologous recombinogenic region.

Wang B. *et al.* (J Orthop Res. 2009; 27(4): p 421-6) disclosed the intraperitoneal (i.p.) injection of 3.10^{11}vg total rAAV1 vectors in neonatal mice (dKO and *mdx*). These AAV vectors encode the microdystrophin Δ3990 placed under the control of the MCK or CMV promoter.

Koppanati *et al.* (Gene therapy. 2010; 17(11): p 1355-62) reported *in utero* gene transfer in the *mdx* mouse via the intraperitoneal (i.p.) injection of $6.4.10^{11}$vg total rAAV8 vector encoding a canine microdystrophin placed under the control of the CMV promoter.

Schinkel *et al.* (Human Gene therapy. 2012; 23(6): p 566-75) reported cardiac gene therapy in the *mdx* mouse via the intravenous (IV) injection of 10^{12}vg total rAAV9 vector encoding a microdystrophin placed under the control of the CMV promoter or the cardiac-specific MLC0.26 promoter.

Gregorevic *et al.* (Mol. Therapy 2008; 16(4): p 657-64) reported muscular gene therapy in the *mdx* mouse via the intravenous (IV) injection of 10^{13}vg total rAAV6 vector encoding the ΔR4-R23/ΔCT microdystrophin placed under the control of the CMV promoter.

Shin *et al.* (Gene Therapy 2011; 18(9): p 910-19) reported cardiac gene therapy in the *mdx* mouse via the intravenous (IV) injection of 3.10^{12}vg total rAAV9 vector encoding a microdystrophin (hΔCS2) placed under the control of the CMV promoter.

Shin *et al.* (J. of Gene Medicine 2008; 10(4): p 449) compared the delivery efficiency in mice of rAAV8 encoding the ΔCS2 microdystrophin placed under the control of the CMV promoter, by subcutaneous injection or intravenous injection.

Colgan *et al.* (Mol. Therapy 2014; 22(S1): p S197) reported the microdystrophin and follistatin combinatorial gene delivery by intravenous injection of rAAV6 vectors in dKO mice.

In the context of DMD, a valuable therapeutic solution would be a gene therapy product having the following characteristics:

- A product which can be systemically administered, at a reasonable dose (i.e. a proper gene transfer in the target tissues) and possibly by a unique injection;
- A product which is has acceptable toxicity at that dose, and especially does not induce an adverse immune response against the dystrophin protein;
- A product having a satisfying tropism, i.e. a wide spread gene transfer on large territories of skeletal muscles, but also diaphragm and myocardium;
- A product able to ameliorate the dystrophic disease in humans.

In practice, previous reports have revealed that it is a very challenging task and several attempts have failed:

Studies using AAV2/6 vectors encoding a human-specific, but not codon-optimized, microdystrophin (ΔR4-R23/ΔCT) under a CMV promoter resulted in the limited expression and eventual destruction of injected *CXMDj* dog muscle fibers via the immune system at 6 weeks after discontinuation of immunosuppression, 22 weeks after initial intramuscular injection (Wang, Z. *et al.*, Mol Ther, 2007. **15**: p. 1160-66).

Clinical trials based on the intramuscular injection of AAV2/5 vectors encoding a human-specific, but not codon-optimized, microdystrophin (ΔR3-R21/ΔCT) under a CMV promoter resulted in very limited transgene expression and in an inappropriate immune response (Mendell, JR *et al.*, N Engl J Med, 2010.363(15): p. 1429-37; Bowles, DE. *et al.*, Mol Ther, 2012. **20**(2): p. 443-55).

Therefore, there is a need in the art for an efficient treatment of dystrophic pathologies in humans, including systemic benefits in terms of survival, overall clinical score, digestive, cardiac and/or respiratory function.

BRIEF SUMMARY OF THE INVENTION

The present invention aims at alleviating or curing the devastating Duchenne muscular dystrophy (DMD) by expressing a shorter but functional dystrophin polypeptide called microdystrophin.

For the first time, the present invention offers a promising gene therapy product, a sequence optimized microdystrophin, encapsidated in the AAV8 capsid, for treating dystrophic diseases. After systemic intravenous administration of a single dose, not only is the microdystrophin highly expressed in multiple muscles but it also results in muscle pathology improvement and improved clinical outcome measures.

Indeed, so good results obtained in a dog model, in terms of muscular, digestive, respiratory and cardiac rescue, correlated with a prolonged life in good condition, have never been reported so far in relation with this kind of pathology.

Definitions

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" or "approximately" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that

range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or a RNA or a cDNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

The term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric "nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation,

recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR and the like, and by synthetic means.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

"Identical" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous or identical at that position. The percent of homology/identity between two sequences is a function of the number of matching positions shared by the two sequences divided by the number of positions compared X 100. For example, if 6 of 10 of the positions in two sequences are matched then the two sequences are 60% identical. Generally, a comparison is made when two sequences are aligned to give maximum homology/identity.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the

like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence, which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements, which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one, which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell preferentially if the cell is a cell of the tissue type corresponding to the promoter.

The term "abnormal" when used in the context of organisms, tissues, cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the "normal" (expected) respective characteristic. Characteristics, which are normal or expected for one cell or tissue type, might be abnormal for a different cell or tissue type.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in situ*, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

A disease or disorder is "alleviated" or "ameliorated" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is reduced. This also includes halting progression of the disease or disorder. A disease or disorder is "cured" if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is eliminated.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

As used herein, "treating a disease or disorder" means reducing the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject. Disease and disorder are used interchangeably herein in the context of treatment.

An "effective amount" of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. The phrase "therapeutically effective amount", as used herein, refers to an amount that is sufficient or effective to prevent or treat (delay or prevent the onset of, prevent the progression of, inhibit, decrease or reverse) a disease or condition, including alleviating

symptoms of such diseases. An "effective amount" of a delivery vehicle is that amount sufficient to effectively bind or deliver a compound.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention relates to a composition comprising a gene therapy product for use in the treatment of a dystrophic disease in a subject, wherein:

- the gene therapy product comprises a nucleic acid sequence encoding a functional microdystrophin;
- the composition is systemically administered.

In other words, the present invention provides a method for treating a dystrophic disease in a subject, comprising systemically administrating to the subject a composition comprising a nucleic acid sequence encoding a functional microdystrophin.

In one embodiment, the present invention provides a method for treating a dystrophic disease in a subject, comprising systemically administrating to the subject a gene therapy product comprising a nucleic acid sequence encoding a functional microdystrophin. The invention concerns the use of a gene therapy product comprising a nucleic acid sequence encoding a functional microdystrophin for the preparation of a medicament for the treatment of dystrophic diseases, wherein the medicament is systemically administered.

According to a first aspect, the present invention relates to a gene therapy product for use in the treatment of a dystrophic disease in a subject.

Typically, a gene therapy product is made of 2 components:

- The encapsidated recombinant nucleic acid sequence which defines the expression cassette that provides the therapeutic benefit(s) once expressed in the target cell/tissue; and
- The viral capsid which allows proper gene transfer and to a certain extent, tissue tropism.

According to one aspect, the gene therapy product comprises a nucleic acid sequence encoding a functional microdystrophin.

In the frame of the invention, microdystrophin means a peptide or protein, which is shorter than the native or wild type dystrophin. In the context of the invention, the terms "microdystrophin" and "minidystrophin" have the same meaning. In the rest of the application, the term "microdystrophin" will be used, as well as the abbreviations "MD" or "μDys".

A "functional" microdystrophin means that the corresponding peptide or protein is able to perform at least some of the functions of the wild-type dystrophin protein and is able to alleviate, at least partially, one or more of the symptoms associated with the absence of a native dystrophin, especially fiber degeneration, inflammation, necrosis, replacement of muscle with scar and fat tissue, muscle weakness, digestive, respiratory and cardiac failure, as well as premature death.

The structure of dystrophin is well documented (see Figure 1) and active fragments thereof have been disclosed (Athanasopoulos *et al.*, Gene Ther 2004 Suppl 1:S109-21). As would be understood in the art, an active fragment is a portion or portions of a full length sequence that retain the biological function of the full length sequence.

The full-length dystrophin is characterized by different domains:

- A N-terminal domain which binds to actin;
- 4 hinge domains (H1 to H4);
- 24 spectrin-like repeats or rod domains (1 to 24);
- A cysteine-rich domain;
- A C-terminal domain.

According to one embodiment, the microdystrophin has at least one domain lacking, advantageously at least one spectrin-like-repeat.

According to a particular embodiment, the microdystrophin has the configuration ΔR4-R23/ΔCT, comprising 4 spectrin-like repeats, i.e. spectrin-like repeats 1, 2, 3 and 24 as shown on figure 1. More precisely, this sequence comprises deletions of rod domains 4-23 and exons 71-78 of the CT domain of dystrophin, and contains the last three amino acids of exon 79 of dystrophin followed by three stop codons.

Such a microdystrophin noted ΔR4-R23/ΔCT or MD1 has e.g. the amino acid sequence shown in SEQ ID NO: 3, 4 or 7.

In one embodiment, the nucleic acid sequence encoding the functional microdystrophin, also named ORF for "open reading frame", is a cDNA. However, e.g. single- or double-stranded DNA or RNA can be used.

In a specific embodiment, the present invention provides compositions comprising nucleic acid sequences that are shorter than the wild-type dystrophin cDNA.

When used in the context of AAV vectors, which can accommodate about 4.7 kb, the nucleic acid sequence encoding the functional microdystrophin, as well as all the sequences required for its proper expression, should not exceed this packaging capacity. In one embodiment, the nucleic acid sequence encoding the functional microdystrophin does not exceed 4500, 4000 bp, preferably 3900, 3800, 3700, 3600 or even 3500 bp.

The nucleic acid sequence encoding the functional microdystrophin is advantageously of human origin but can also be a non-human primate, a canine, a rat or a murine sequence. In one embodiment, the nucleic acid sequence originates from the organism it will be administered to (e.g. a human sequence in humans).

According to another embodiment, the nucleic acid sequence encoding said microdystrophin is optimized for use in a given subject, advantageously in humans. Preferably, this optimized sequence is modified as follows:

- The sequence is modified to include a consensus Kozak sequence before AUG start codon within mRNA, to improve initiation of translation.
- The sequence is optimized based on transfer RNA frequencies in human and GC content is increased to promote RNA stability. As a result and in a specific case, codon optimization for humans advantageously leads to 63% of codons being modified and the GC content increased to over 60%. This of course depends on the original (before optimization) microdystrophin sequence and the target host.

According to one embodiment, the nucleic acid sequence encoding a functional microdystrophin corresponds to:

- nucleotides 586 to 4185 of sequence SEQ ID NO: 1 as shown in SEQ ID NO: 5; or
- nucleotides 586 to 4188 of sequence SEQ ID NO: 2 as shown in SEQ ID NO: 6.

According to one embodiment, said sequence can be an isolated nucleic acid encoding a microdystrophin having substantial homology or identity (60%, 70%, 80%, 90% 95% or even 99%) to the peptides disclosed herein, especially of sequence SEQ ID NO: 3, SEQ ID NO: 4, or even SEQ ID NO: 7.

Preferably, the nucleotide sequence of an isolated nucleic acid encoding a peptide of the invention is "substantially homologous/identical", that is, is about 60% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, more preferably about 90% homologous, even more preferably, about 95% homologous, and even more preferably about 97%, 98% or even 99% homologous to a nucleotide sequence of an isolated nucleic acid encoding the functional microdystrophin, especially of sequence SEQ ID NO:5, SEQ ID NO: 6 or even SEQ ID NO: 8.

According to another aspect, the nucleotide sequence harbored by an expression vector according to the invention is "substantially homologous/identical", that is, is about 60% homologous, more preferably about 70% homologous, even more preferably about 80% homologous, even more preferably about 90% homologous, even more preferably about 95% homologous, and even more preferably about 97%, 98% or even 99% homologous to the sequence SEQ ID NO: 1 or SEQ ID NO: 2.

In another embodiment, the composition comprises a plasmid or a vector. According to a specific embodiment, the isolated nucleic acid is inserted into the vector. In brief summary, the expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors to be used are suitable for replication and, optionally, integration in eukaryotic cells. Typical vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

In one embodiment, the composition comprises an expression vector, advantageously a viral vector. Of particular interest are the expression vectors which packaging capacity does not allow accommodation of the (wild type) dystrophin gene, including the (wild type) dystrophin cDNA.

In one embodiment, the viral vector is selected from the group consisting of a baculoviral vector, herpes viral vector, lentiviral vector, retroviral vector, adenoviral vector, and adeno-associated viral (AAV) vector.

According to a specific embodiment of the invention, the viral vector containing the expression construct is an adeno-associated viral (AAV) vector.

Adeno-associated viral (AAV) vectors have become powerful gene delivery tools for the treatment of various disorders. AAV vectors possess a number of features that render them ideally suited for gene therapy, including a lack of pathogenicity, moderate immunogenicity, and the ability to transduce post-mitotic cells and tissues in a stable and efficient manner. Expression of a particular gene contained within an AAV vector can be specifically targeted to one or more types of cells by choosing the appropriate combination of AAV serotype, promoter, and delivery method.

In one embodiment, the encoding sequence is contained within an AAV vector. More than 100 naturally occurring serotypes of AAV are known. Many natural variants in the AAV capsid exist, allowing identification and use of an AAV with properties specifically suited for dystrophic pathologies. AAV viruses may be engineered using conventional molecular biology techniques, making it possible to optimize these particles for cell specific delivery of nucleic acid sequences, for minimizing immunogenicity, for tuning stability and particle lifetime, for efficient degradation, for accurate delivery to the nucleus.

As mentioned above, the use of AAV vectors is a common mode of exogenous delivery of DNA as it is relatively non-toxic, provides efficient gene transfer, and can be easily optimized for specific purposes. Among the serotypes of AAVs isolated from human or non-human primates (NHP) and well characterized, human serotype 2 is the first AAV that was developed as a gene transfer vector. Other currently used AAV serotypes include AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 and AAV12. In addition, non-natural engineered variants and chimeric AAV can also be useful.

Desirable AAV fragments for assembly into vectors include the cap proteins, including the vp1, vp2, vp3 and hypervariable regions, the rep proteins, including rep 78, rep 68, rep 52, and rep 40, and the sequences encoding these proteins. These fragments may be readily utilized in a variety of vector systems and host cells.

Such fragments may be used alone, in combination with other AAV serotype sequences or fragments, or in combination with elements from other AAV or non-AAV viral sequences. As used herein, artificial AAV serotypes include, without limitation, AAV with a non-naturally occurring capsid protein. Such an artificial capsid may be generated by any suitable technique, using a selected AAV sequence (e.g., a fragment of a vp1 capsid protein) in combination with heterologous sequences which may be obtained from a different selected AAV serotype, non-contiguous portions of the same AAV serotype, from a non-AAV viral source, or from a non-viral source. An artificial AAV serotype may be, without limitation, a chimeric AAV capsid, a recombinant AAV capsid, or a "humanized" AAV capsid. Thus exemplary AAVs, or artificial AAVs, include AAV2/8 (US 7,282,199), AAV2/5 (available from the National Institutes of Health), AAV2/9 (WO2005/033321), AAV2/6 (US 6,156,303), and AAVrh8 (WO2003/042397), among others. In one embodiment, the vectors useful in the compositions and methods described herein contain, at a minimum, sequences encoding a selected AAV serotype capsid, e.g., an AAV8 capsid, or a fragment thereof. In another embodiment, useful vectors contain, at a minimum, sequences encoding a selected AAV serotype rep protein, e.g., AAV8 rep protein, or a fragment thereof. Optionally, such vectors may contain both AAV cap and rep proteins. In vectors in which both AAV rep and cap are provided, the AAV rep and AAV cap sequences can both be of one serotype origin, e.g., all AAV8 origin. Alternatively, vectors may be used in which the rep sequences are from an AAV serotype, which differs from that which is providing the cap sequences. In one embodiment, the rep and cap sequences are expressed from separate sources (e.g., separate vectors, or a host cell and a vector). In another embodiment, these rep sequences are fused in frame to cap sequences of a different AAV serotype to form a chimeric AAV vector, such as AAV2/8 (US 7,282,199).

According to one embodiment, the composition comprises an AAV of serotype 2, 5, 8 or 9. Advantageously, the claimed vector is an AAV8 or AAV9 vector, especially an AAV2/8 or AAV2/9 vector. More advantageously, the claimed vector is an AAV8 vector or an AAV2/8 vector.

In the AAV vectors used in the present invention, the AAV genome may be either a single stranded (ss) nucleic acid or a double stranded (ds) / self complementary (sc) nucleic acid molecule.

Advantageously, the nucleic acid sequence encoding the functional microdystrophin is inserted between the ITR (« Inverted Terminal Repeat ») sequences of the AAV vector. Typical ITR sequences correspond to:

- nucleotides 1 to 128 of sequence SEQ ID NO: 1 or of sequence SEQ ID NO: 2 (5'ITR sequences);
- nucleotides 4511 to 4640 of sequence SEQ ID NO: 1 or nucleotides 4514 to 4643 of sequence SEQ ID NO: 2 (3'ITR sequences).

Recombinant viral particles can be obtained by any method known to the one skilled in the art, e.g. by co-transfection of 293 HEK cells, by the herpes simplex virus system and by the baculovirus system. The vector titers are usually expressed as viral genomes per mL (vg/mL).

In one embodiment, the expression vector comprises regulatory sequences, especially a promoter sequence. Such promoters can be natural or synthetic (artificial) promoters, inducible or constitutive.

In one embodiment, the promoter is an ubiquitous promoter or having a low tissue-specificity. As an example, the expression vector can harbor the phosphoglycerate kinase 1 (PGK), EF1, β-actin, CMV promoter.

In a preferred embodiment, the promoter sequence is chosen in order to adequately govern the expression of the nucleic acid sequence placed under its control, in terms of expression level, but also of tissue specificity. In one embodiment, the expression vector comprises a muscle specific promoter. Such a promoter allows a robust expression in the skeletal muscles, and possibly in the cardiac muscle as well as in the diaphragm. Examples of suitable promoters known by the skilled person are e.g. the desmin promoter, the muscle creatine kinase (MCK) promoter, the CK6 promoter, and the Syn promoter. Another promoter is the synthetic promoter C5-12 (spC5-12) as shown in sequences SEQ ID NO: 1 or 2 (nucleotides 215 to 537), which allows a robust expression in skeletal and cardiac muscles.

A non-exhaustive list of other possible regulatory sequences is:

- a polyadenylation signal, e.g. the polyA of the gene of interest, the polyA of SV40 or of beta hemoglobin (HBB2), advantageously in 3' of the sequence encoding the functional microdystrophin ; The poly A of SV40 is disclosed in sequences SEQ ID NO: 1 (nucleotides 4223 to 4353) and SEQ ID NO: 2 (nucleotides 4226 to 4356);
- sequences for transcript stabilization, e.g. intron 1 of hemoglobin (HBB2);
- enhancer sequences ;
- miRNA target sequences, which can inhibit the expression of the sequence encoding the functional dystrophin in non target tissues, in which said expression is not desired, for example where it can be toxic. Preferably, the corresponding miRNA is not present in the skeletal muscles, and possibly not in the diaphragm nor in the heart.

According to one embodiment, the gene therapy product comprises an expression vector, advantageously an AAV vector harboring the sequence SEQ ID NO: 1 or SEQ ID NO: 2, advantageously SEQ ID NO: 1. As mentioned above, the invention also encompasses "substantially homologous" sequences, that is, displaying about 60% homology, more preferably about 70% homology, even more preferably about 80% homology, more preferably about 90% homology, even more preferably about 95% homology, and even more preferably about 97%, 98% or even 99% homology to the sequence SEQ ID NO: 1 or 2.

According to the present invention, the composition comprises at least said gene therapy product, and possibly other active molecules (other gene therapy products, chemical molecules, peptides, proteins…), dedicated to the treatment of the same disease or another disease.

According to a specific embodiment, said composition does not comprise any immunosuppressive agent.

The present invention then provides pharmaceutical compositions comprising a nucleic acid of the invention, or the vector of the invention. Such compositions comprise a therapeutically effective amount of the therapeutic (the nucleic acid or vector of the invention), and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. or European Pharmacopeia or other generally

recognized pharmacopeia for use in animals, and humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, sustained-release formulations and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to release pain at the site of the injection.

In one embodiment, the composition according to the invention is suitable for administration in humans. The composition is preferably in a liquid form, advantageously a saline composition, more advantageously a phosphate buffered saline (PBS) composition or a Ringer-Lactate solution.

The amount of the therapeutic (i.e. a nucleic acid or a vector) of the invention which will be effective in the treatment of dystrophic diseases can be determined by standard clinical techniques. In addition, *in vivo* and/or *in vitro* assays may optionally be employed to help predict optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, the weight and the

seriousness of the disease, and should be decided according to the judgment of the practitioner and each patient's circumstances.

Suitable administration should allow the delivery of a therapeutically effective amount of the gene therapy product to the target tissues, especially skeletal muscles and possibly smooth muscles (e.g. esophagus), diaphragm and heart. In the context of the invention, when the gene therapy product is a viral vector comprising a nucleic acid sequence encoding a functional microdystrophin, the therapeutic dose is defined as the quantity of viral particles (vg for viral genomes) containing the microdystrophin sequence, administered per kilogram (kg) of the subject.

Available routes of administration are topical (local), enteral (system-wide effect, but delivered through the gastrointestinal (GI) tract), or parenteral (systemic action, but delivered by routes other than the GI tract). The preferred route of administration of the compositions disclosed herein is parenteral which includes intramuscular administration (i.e. into the muscle) and systemic administration (i.e. into the circulating system). In this context, the term “injection” (or “perfusion” or “infusion”) encompasses intravascular, in particular intravenous (IV), and intramuscular (IM) administration. Injections are usually performed using syringes or catheters.

In one embodiment, systemic delivery of the composition comprises administering the composition near a local treatment site, i.e. in a vein or artery nearby a weakened muscle. In certain embodiments, the invention comprises the local delivery of the composition, which produces systemic effects. This route of administration, usually called “regional (loco-regional) infusion”, “administration by isolated limb perfusion” or “high-pressure transvenous limb perfusion” has been successfully used as a gene delivery method in muscular dystrophy (Zheng Fan *et al.* (2012, Molecular Therapy 20(2), 456-461).

According to one aspect, the composition is administered to an isolated limb (loco-regional) by infusion or perfusion. In other words, the invention comprises the regional delivery of the composition in a leg and/or arm by an intravascular route of administration, i.e. a vein (transveneous) or an artery, under pressure. This is usually achieved by using a tourniquet to temporarily arrest blood circulation while allowing a regional diffusion of the infused product, as e.g. disclosed by Toromanoff *et al.* (2008, Molecular Therapy 16(7):1291-99), Arruda *et al.* (2010, Blood 115(23):4678-88) and Fan *et al.* (2012, Molecular Therapy 20(2), 456-461).

In one embodiment, the composition is injected in a limb of the subject. In one embodiment, the subject is a mammal, preferably a human, a dog or a nonhuman primate. When the subject is a human, the limb can be the arm or the leg. According to one embodiment, the composition is administered in the lower part of the body of the subject, e.g. below the knee, or in the upper part of the body of the subject, e.g., below the elbow.

In one embodiment, the composition is administered to a peripheral vein, e.g. the cephalic vein. The volume of the composition to be infused can be in a range that varies between about 5 and 40% of the limb volume. The typical dose can vary between 5 and 30 ml/kg of body weight. In one embodiment, the pressure to be applied (tourniquet pressure or maximum line pressure) is below 100 000 Pa, advantageously below 50 000 Pa. In a preferred embodiment, the pressure applied is around 300 torr (40 000 Pa).

In one embodiment, the blood circulation of the limb is stopped using a tourniquet that is tightened for several minutes to more than one hour, typically between about 1 and 80 minutes, for example about 30 minutes. In a preferred embodiment, the tourniquet was applied before, during and after the administration, for example about 10 minutes prior to, about 20 minutes during and about 15 min after the infusion. More generally, the pressure is applied for several minutes, typically between about 1 and 80 minutes, for example about 30 minutes. In a preferred embodiment, the pressure is applied before, during and after the administration, for example about 10 minutes prior to, about 20 minutes during and about 15 minutes after the infusion.

In one embodiment, the average flow rate is comprised between 5 and 150 ml/min, advantageously between 5 and 80 ml/min, for example 10 ml/min. Of course, the flow rate also determines the time period during which the blood circulation is stopped and the pressure applied.

In the context of a loco-regional administration, the dose injected may vary between 10^{12} and 10^{14} vg/kg of the patient body, preferably between 10^{12} and 10^{13} vg/kg.

A preferred method of administration according to the invention is systemic administration. Systemic injection opens the way to an injection of the whole body, in order to reach the entire muscles of the body of the subject including the heart and the diaphragm and then a real treatment of these systemic and still incurable diseases. In certain embodiments, systemic delivery comprises delivery of the composition to the subject such that composition is accessible throughout the body of the subject.

According to a preferred embodiment, systemic administration occurs via injection of the composition in a blood vessel, i.e. intravascular (intravenous or intra-arterial) administration. According to one embodiment, the composition is administered by intravenous injection, through a peripheral vein.

The systemic administration is typically performed in the following conditions:

- a flow rate of between 1 to 10 mL/min, advantageously between 1 to 5 mL/min, e.g. 3 mL/min;
- the total injected volume can vary between 1 and 20 mL, preferably 5 mL of vector preparation per kg of the subject. The injected volume should not represent more than 10% of total blood volume, preferably around 6%.

When systemically delivered, the composition is preferably administered with a dose less than or equal to 10^{15} vg/kg or even 10^{14} vg/kg, advantageously between 10^{12} vg/kg and 10^{14} vg/kg, more advantageously between 5.10^{12} vg/kg and 10^{14} vg/kg, e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9.10^{13} vg/kg. A lower dose of e.g. 1, 2, 3, 4, 5, 6, 7, 8 or 9.10^{12} vg/kg can also be contemplated in order to avoid potential toxicity and /or immune reactions. As known by the skilled person, a dose as low as possible given a satisfying result in term of efficiency is preferred.

In a specific embodiment, the treatment comprises a single administration of the composition.

As it will be illustrated in the examples below, the administration of the gene therapy product according to the invention is not believed to be associated with adverse immune reactions. Therefore, and according to one embodiment, said administration is not combined with any further or extra immunosuppressive treatment (immunosuppression).

In one embodiment, the presence of the gene therapy product and/or the expression of the functional microdystrophin, as well as the associated therapeutic benefits, are observed for up to 1 month, or 3 months or 6 months or even 1 year, 2 years, 5 years, 10 years, or even the whole life of the subject.

According to the invention, the subject is a mammal, preferably a human or a dog, but can also be a mouse, a rat or a nonhuman primate.

"Dystrophic disease" means a disease linked to a defect in the dystrophin gene. This defect can be deletions or mutations leading to low level of expression or absence of expression, introduction of a premature stop codon in the open reading frame, or the production of an inactive protein. Preferred dystrophic diseases are Duchenne and Becker muscular dystrophy (DMD/BMD) caused by mutations of the dystrophin gene. Said mutations can result in the absence or a low level of dystrophin expression, or in the production of a partially or fully inactive, possibly truncated protein.

Subjects that could benefit from the compositions of the invention include all patients diagnosed with a muscular dystrophy or at risk of developing such a muscular dystrophy. A subject to be treated can then be selected based on the identification of mutations or deletions in the dystrophin gene by any method known to the one skilled in the art, including for example sequencing of the dystrophin gene, and/or through the evaluation of the dystrophin level of expression or activity by any method known to the one skilled in the art. Therefore, said subjects include both subjects already exhibiting symptoms of a dystrophic disease and subjects at risk of developing said disease. In one embodiment, said subjects include subjects already exhibiting symptoms of a dystrophic disease. In another embodiment, said subjects are ambulatory patients and early non-ambulant patients.

Such compositions are notably intended for gene therapy, particularly for the treatment of Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD), advantageously DMD.

A first target of the invention is to provide a safe (not toxic) treatment. A further aim is to provide an efficient treatment which allows to postpone, slow down or prevent the development of the disease, and possibly to ameliorate the phenotype of the patient which can be easily monitored at the clinical level.

In a subject, the composition according to the invention can be used:

- for ameliorating muscular function. Of particular interest are the skeletal muscles, but also the cardiac muscle and the diaphragm;
- for ameliorating gait;
- for ameliorating cardiac function;
- for ameliorating respiratory function;
- for ameliorating digestive function; and/or

- for prolonging survival, more generally to ameliorate the quality and the expectancy of life.

According to one aspect, the invention concerns a method for ameliorating muscular function, gait, digestive function, cardiac function and/or respiratory function, and/or for prolonging survival, advantageously without adverse effects (cellular and/or humoral immune response), comprising administering to a subject in need thereof a therapeutic quantity of a gene therapy product as disclosed above.

Advantageously, said ameliorations are observed for up to 1 month after administration, or 3 months or 6 months or 9 months, more advantageously for up to 1 year after administration, 2 years, 5 years, 10 years, or even for the whole life of the subject.

In one embodiment, said ameliorations results in reduced symptom severity and/or frequency and/or delayed appearance, wherein said symptom is chosen within the group consisting of frequent fall, inability to walk, dysphagia, cardiomyopathy, ptyalism, reduced motor skills (running, hopping, jumping), breathing abnormalities, pseudohypertrophy, lumbar hyperlodosis, and muscle stiffness.

An amelioration of said functions can be evaluated based on methods known in the art, e.g.:

- assessment of the percentage of muscle fibers expressing the dystrophin protein;
- walking tests;
- assessment of strength by dynamometer measurements;
- assessment of motor function of a precise limb by motor function measurements;
- assessment of global activity using a movement monitor;
- assessment of gait by accelerometric recording in 3 axes;
- assessment of cardiac function by echocardiographic, Doppler analyses and Speckle tracking analysis;
- assessment of respiratory function by evaluation of diaphragm kinetics;
- assessment of vital functions, especially cardiac, respiratory and digestive functions, by clinical follow-up;
- assessment of quality and expectancy of life by clinical score.

As illustrated in the examples, the claimed treatment allows improving the clinical state and the various parameters disclosed above in comparison with an untreated subject.

According to one embodiment, the present invention concerns a method of treatment of a dystrophic disease comprising administering to a subject the gene therapy product as disclosed above, wherein:

- at least 30% of the muscle fibers, advantageously 40%, more advantageously at least 50% of the muscle fibers express the dystrophin protein; and/or
- a clinical score is maintained at a level corresponding to at least 50% of the score of a healthy subject, advantageously at least 60% or even 70%.

Advantageously, said effects are observed for up to 1 month after administration, or 3 months or 6 months or 9 months, more advantageously for up to 1 year after administration, 2 years, 5 years, 10 years, or even more for the whole life of the subject.

As known in the art, the level of dystrophin expression in muscles is easily determined by the skilled person, advantageously by immunohistochemistry, e.g. by immunostaining of muscular biopsies with an anti-Dystrophin antibody as disclosed above. The calculation of clinical scores is also routine for the skilled person. As detailed above in relation with dogs, this score can be calculated based on dysphagia, breathing, ptyalism and global activity. Concerning patients, Bushby and Connor have e.g. listed clinical outcome measures for trials in Duchenne muscular dystrophy (Clin Investig (Lond). 2011; 1(9): 1217–1235).

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", fourth edition (Sambrook, 2012); "Oligonucleotide Synthesis" (Gait, 1984); "Culture of Animal Cells" (Freshney, 2010); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1997); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Short Protocols in Molecular Biology" (Ausubel, 2002); "Polymerase Chain Reaction: Principles, Applications and Troubleshooting", (Babar, 2011); "Current Protocols in Immunology" (Coligan, 2002). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples and the attached figures. These examples are provided for purposes of illustration only, and are not intended to be limiting.

The results presented below have been obtained in the GRMD (Golden Retriever Muscular Dystrophy) dog model. It is the best animal model for dystrophic pathologies, in order to evaluate the potential of a gene therapy product, in terms of efficiency (therapeutic dose, stability, toxicity, ...) but also of immune response, before clinical trials.

Figure 1: Scheme of the full-length dystrophin (A), of various microdystrophins (B) and of the expression construct (C).

Figure 2: Study plan – General scheme of the systemic treatment in GRMD dogs.

Figure 3:

A/ Muscular biopsies obtained 3 months post rAAV2/8-SPc5.12-cMD vector administered by intravenous systemic delivery into the GRMD2 dog (ICI).

a/ m. biceps femoris before injection

b/ healthy dog

c/ m. extensor carpi radialis right:

82 % of cMD + fibers (cMD detected in 82 % of the fibers)

2.8 vg/dg (vector genome per diploid genome)

d/ m. extensor digitorum communis right:

59 % of cMD + fibers (cMD detected in 59 % of the fibers)

4.1 vg/dg

e/ m. extensor carpi radialis left:

62 % of cMD + fibers (cMD detected in 62 % of the fibers)

6.2 vg/dg

f/ m. extensor digitorum communis left:

66 % of cMD + fibers (cMD detected in 66 % of the fibers)

2.6 vg/dg

B/ Muscular biopsies obtained 8 months post rAAV2/8-SPc5.12-cMD vector administered by intravenous systemic delivery into the GRMD2 dog (ICI):

a/ m. biceps femoris right:

58 % of cMD + fibers (cMD detected in 58 % of the fibers)

1.0 vg/dg

b/ m. biceps femoris left:

56 % of cMD + fibers (cMD detected in 56 % of the fibers)

0.8 vg/dg

Figure 4: Data on clinical score obtained in the GRMD cohort that has received 10^{14} vg/kg of rAAV2/8-SPc5.12-cMD vector systemic at the age of 2 months.

* means that the dog is no longer alive

Figure 5: Data on the Global gait index. Curves were calculated using the model built by Discriminant Analysis and the Data obtained for untreated GRMD and healthy dogs are represented. For the latters, the mean centroid curves and the 95% confidence intervals are shown.

Figure 6: Diaphragm Range of Motion (ROM) monitored on treated and untreated GRMD dogs. Healthy dogs display a ROM value between 90 and 110%.

Figure 7: IFNγ ELISpot using canine µDys peptide pools (kinetics of PBMCs). Data were obtained in the GRMD Dog 2 (ICI) that has received 10^{14} vg/kg of rAAV2/8-SPc5.12-cMD vector systemic at the age of 2 months.

Figure 8: Detection of anti-dystrophin IgG antibodies by Western-Blot in injected dog sera. Data were obtained in the GRMD Dog 2 (ICI) that has received 10^{14} vg/kg of rAAV2/8-SPc5.12-cMD vector systemic at the age of 2 months. The reactivity of each serum was tested on cellular extracts of 293 cells transfected (or not) with a pCMV-canine-MD (cµDys). Sera before injection (Day 0) and after injection (week 3, month 1.5, Month 2.5, month 4 and month 7.5) have been tested. Positive controls consisted in the anti-dystrophin antibody MANEX 1011C, and a positive canine serum (C+) from a GRMD dog immunized against dystrophin.

Materials and Methods:

1/ Animals

The evaluation of a fully systemic injection of the microdystrophin vector (rAAV2/8-SPc5.12-cMD) has been performed in the GRMD dog model (Kornegay *et al.*, Mamm Genome, 2012). Selected male dogs were genotyped for the DMD mutation, which consists of a single base change in the 3' consensus splice site (A>G) of intron 6 of the dystrophin gene that provokes inaccurate mRNA processing.

Dogs were treated as shown in Table 1 below (without immunosuppression):

Dose	Timing of follow-up	Name of the dog		Date of injection	Date of euthanasia
$1^{E}14$vg/kg	Long term	IMAGE	µDys 1	6.11.2013	*Still alive (17 months post-inj°)*
		ICI	µDys 2	6.01.2014	*Still alive (15 months post-inj°)*
	7-8 months post-injection	ICE-T	µDys 3	28.08.2013	16.04.2014
		JAFFAR	µDys 4	16.09.2014	*May 2015*
		JACADI	µDys 5	3.11.2014	*July 2015*

Control dogs correspond to non injected GRMD dogs and healthy dogs.

2/ Microdystrophin vector

The rAAV2/8-SPc5.12-cMD vector encodes an mRNA sequence-optimized canine dystrophin (cMD) under the control of a muscle-specific promoter (SPc5.12).

The construction of canine-specific, mRNA sequence-optimized cMD cDNA, incorporated deletions of rod domains 4-23 and exon 71-78 of the CT domain of dystrophin (ΔR4-R23; Figure 1), containing the last three amino acids of exon 79 of dystrophin followed by three stop codons and incorporating the SV40 poly adenylation site. cDNA sequence was modified to include a consensus Kozak sequence. An mRNA sequence was optimized based on transfer RNA frequencies in human and GC content

was increased to promote RNA stability. mRNA sequence optimization of microdystrophin (GENEART, Regensburg, Germany) resulted in the GC content being increased from 48% to 61% in the canine dystrophin and 23.6% of codons being modified as well. The size of the cMD gene cDNA is 3603 bp and the flanking inverted terminal repeat (ITR)-containing transgene cassette size of this vector is 4643 bp, which corresponds to 99.2% of the 4682 bp of wild-type-AAV2 genome length. 5'- and 3'-untranslated regions of the dystrophin gene were removed to decrease the flanking ITR size of the dystrophin cassette. Expression was under the control of the muscle-specific synthetic promoter (SPc5-12) (Wang, B., *et al.,* Gene Ther, 2008. **15**(22): p. 1489-99).

This expression cassette (SEQ ID NO: 2 including the AAV_ITR, the Spc512 promoter, the canine MD cDNA and the SV40 PolyA) was demonstrated to result in widespread and stable dystrophin expression after intramuscular injections in the Duchenne beagle-based *CXMDJ* model (Koo, T., *et al*., J Gene Med, 2011. **13**(9): p. 497-506). In addition, this construct improved muscle pathology and reduction of inflammatory responses in the target muscle tissue.

3/ Preparation of rAAV2/8-SPc5.12-cMD

The recombinant adeno-associated virus vector containing the canine microdystrophin cDNA regulated by the SPc5- 12 promoter, rAAV2/8-SPc5.12-cMD, was produced in a baculovirus/Sf9 system. Two baculovirus batches were generated, one expressing *rep* (encoding the AAV2 Rep protein) and *cap* (encoding the AAV8 Cap protein) AAV genes and the second being the AAV2 transfer vector. The viruses were produced, banked, and used to co-infect SF9 cells in 200-liter single-use bioreactor (Sartorius). After a three-day culture, cells are harvested, lysed, and the lysate processed by clarification, purification on an immunoaffinity column, concentration through tangential flow filtration, formulation, sterile filtering and filling. Purification is based on a commercial gel (AVB from GE Healthcare) carrying a single-chain antibody binding AAV1, AAV2, AAV3, AAV5, AAV6, and AAV8. The process has an overall yield of >20%, and generates 145 units of 4.5 ml product with a viral titer $>10^{13}$ vg/ml.

4/ Systemic administration

GRMD dogs have been injected by systemic delivery with the therapeutic candidate (rAAV2/8-SPc5.12-cMD) vector. This pilot cohort was administered with 10^{14} vg/kg (total of around $5x10^{14}$ vg/animal). The simple systemic injection was performed through a peripheral vein, in a cannulated cephalic vein, at a flow rate of 3 mL/min. Total injected volume was around 25 mL of vector preparation (5 ml/kg) representing 6 % total blood volume (10% being the recommended upper limit), which turned out to be very well tolerated.

The experimental animals were injected at the age of 2 months and are followed as shown in Table 1. They all were prescreened for the absence of AAV8 neutralizing factors in the serum. Prior the intravenous (IV) injection of the vector, GRMD dogs that exhibited profound weakness and/or swallowing impairment were discarded from the experiment. Immunosuppressive regiments were never used and the only medical care provided was restricted to maintain comfort and wellbeing of the animals. Appropriate regulatory documents (ethics and GMO handling) were obtained in due time. All procedures are carried out accordance with the Guide for the Care and Use of Laboratory Animals and approved by the *ad hoc* Animal Use and Care Committee.

5/ Evaluation of the systemic treatment

Morbidity and mortality are assessed twice daily. Animals found dead would be submitted to necropsy in the presence of the pathologist and tissue samples collected when appropriate in attempt to systematically determine the cause of death.

Clinical and biological tolerance of the protocol

In all dogs, clinical laboratory parameters including electrolytes, kidney and liver function tests and complete blood counts are monitored regularly after injection. The clinical status of each dog, including cardiac, respiratory, digestive, locomotors and neurologic functions, are also carefully and weekly evaluated all along the protocol.

Assessment of vector shedding and vector biodistribution by Q-PCR

Vector shedding and vector biodistribution by Q-PCR are performed regularly until euthanasia on urine, serum, intermediate muscle biopsies, major skeletal muscles from the 4 limbs among flexors and extensors, heart and diaphragm, liver, spleen, kidneys, lymph nodes and testis. Extraction of rAAV DNA from fluids is done using the Qiamp Viral RNA mini-kit (Qiagen). rAAV is extracted from 140 µL of serum. 1/8 of the extraction (10 µL) is used for Q-PCR analysis. Extraction of genomic DNA (gDNA) from tissues is done using the Gentra Puregene kit (Qiagen) and Tissue Lyzer II (Qiagen). The concentration of each gDNA sample is determined using a nano-spectrophotometer (Implen).

Quantitative PCR is conducted on a StepOne Plus (Applied Biosystem) using 50ng of gDNA in duplicates or 10 µL of fluid extracts. Vector copy numbers is determined using primers and probe designed to specifically amplify the SPc5.12-cMD cassette. gDNA copy numbers is determined using primers and probe designed to amplify the canine glucuronidase gene. For each sample, Ct (cycle threshold) values are compared with those obtained with different dilutions of linearized standard plasmids (containing either the SPc5.12-cMD cassette or the canine glucuronidase gene). Results are expressed in vector genome per diploid genome (vg/dg). For fluids, only the transgene specific Q-PCR is performed and results are expressed in vector genome per µl of fluid extracted. The absence of Q-PCR inhibition in the presence of gDNA is previously checked by analyzing 10 µL of fluid extract or 50ng of gDNA extracted from spleen, testis, liver, kidney or skeletal muscle, spiked with different dilutions of standard plasmid.

Assessment of transgene expression in different tissues by Q-RT-PCR

Microdystrophin expression is assessed by Q-RT-PCR in multiple skeletal muscles, heart and diaphragm, liver, spleen, and any other tissue exhibiting high vector copy number. Briefly, total RNA is extracted from muscles, liver and spleen with TRIzol reagent (Invitrogen) and treated with RNAse-free DNAse I from the TURBO DNA-free kit (Ambion) according to the manufacturer's instructions. Reverse transcription is performed using random primers and an M-MLV reverse transcriptase (Invitrogen). A negative control without reverse transcriptase (RT-) is processed for each sample. Quantitative PCR is conducted on a StepOne Plus (Applied Biosystem) diluted cDNA in duplicates. The relative quantification of the cMD messengers is determined using primers designed to specifically amplify this sequence. The results are normalized by a Q-PCR analysis of the canine RPL32 (Ribosomal Protein L32) messenger, known to be similarly expressed in the different tissues of dog (Peters, I.R., *et al.,*. Vet Immunol

Immunopathol, 2007. **117**(1-2): p. 55-66). The absence of Q-PCR inhibition in the presence of cDNA of muscle, liver and spleen is checked by analyzing diluted cDNA spiked with different dilutions of standard plasmid.

For each sample, Ct (cycle threshold) values are compared with those obtained with different dilutions of standard plasmids (containing the cMD expression cassette or the sequence of the canine RPL32 messenger). Results are expressed in relative quantities (RQ):

$RQ = 2^{-\Delta Ct} = 2^{-(Ct\ target\ -\ Ct\ endogenous\ control)}$

Analysis of Dystrophin expression by Western blot

Using a specific Western-Blot analysis, the expression of microdystrophin in different muscles of the injected dogs is evaluated:

- In several skeletal muscles, as well as the heart and the diaphragm, sampled at euthanasia,
- In liver, spleen and any other tissues in which a high level of transgene copy numbers would be found, at euthanasia.

Total proteins are extracted from tissue samples. Protein extracts are separated on SDS-PAGE, transferred on a nitrocellulose membrane. After Red Ponceau staining, membranes are blocked in 5% skim milk in TBS and hybridized with the anti-Dystrophin MANEX1011C antibody and with secondary anti-mouse IgG HRP-conjugated antibody.

Analysis of Dystrophin expression by immunohistochemistry

By immunochemistry, microdystrophin expression is evaluated in the skeletal muscles of the injected dogs:

- In intermediate muscular biopsies,
- In all skeletal muscles, as well as the heart and the diaphragm, sampled at euthanasia,
- In liver, spleen, testes, kidneys and lymph nodes, sampled at euthanasia.

microdystrophin expression and localization are assessed by immunohistochemistry. microdystrophin polypeptide immunostaining is performed on transverse sections of each muscle using the mouse anti-Dystrophin antibody from Novocastra (NCL-DYSB). The restoration of the Dystrophin-associated proteins is evaluated by immunostaining of β-dystroglycan, β-sarcoglycan, gamma-sarcoglycan and Utrophin, including colocalization with laminin at the sarcolemal membrane.

Assessment of the local pathological pattern in the muscles

Pathology assessment is key to address the actual benefit of the gene therapy product at the target tissue level. Using morphometric analyses, the EC Board-certified pathologist evaluates the pathological pattern in the skeletal muscles of the injected dogs. These analyses are done on postural muscles with a majority of type I fibers (proximal limb muscles, paravertebral muscles); locomotor muscles with a majority of type II fibers (flexor and extensors from distal limb muscles); respiratory muscles, diaphragm, intercostal and masticatory muscles. Heart is evaluated extensively as well with the specific difficulty to apprehend fibers diameter due to unparalleled orientation.

Endomysial fibrosis is evaluated after immunohistochemical revelation of Collagen I (immunoperoxydase assay) and automatic measurement of the percentage of the labeled areas.

Total fibrosis is evaluated after immunohistochemical revelation of Collagen I (immunoperoxydase assay), on the same slides than endomysial fibrosis. An automatic measurement of the percentage of the total labeled areas is also performed.

Perimysial fibrosis is calculated by the difference between total fibrosis and endomysial fibrosis in the same fields of muscular tissue.

Anocytosis (variation of fibers diameter) is evaluated by manual morphometry: determination of the minimum fiber diameter on at least 200 myofibers and six fields per analyzed muscle cross-section.

Necrosis is evaluated by measurement of calcium accumulation, by an Alizarin Red staining. The percentage of labeled areas is measured after manual threshold.

Regeneration is evaluated after immunohistochemical revelation myotubes with an antibody specific of a developmental Myosin Heavy Chain isoform (immunoperoxydase assay). The percentage of labeled areas is measured after manual threshold.

Inflammation is evaluated after immunohistochemical revelation of T and B lymphocytes and macrophages on the same slide (immunoperoxydase assay). The percentage of labeled areas is determined after manual threshold.

Assessment of the pathological pattern in the different tissues

Potential adverse side effects due to off target tissues (liver, spleen, kidney, …) is evaluated using HE staining and anatomopathology expertise in the different tissues of the dogs at euthanasia.

NMR imaging and spectroscopy indices of skeletal muscles

Non-invasive muscle imaging and spectroscopy indices are performed a week before euthanasia. The dogs are sent to Institute of Myology, Paris (Pierre Carlier's team) and subjected to a 3T Siemens Trio scanner Nuclear Magnetic Resonance (NMR) to quantitatively and serially describe the dystrophic muscle abnormalities compared to untreated and healthy animals. In addition to that, P31 spectroscopy of the extensor carpi radialis is realized at 4T in a Bruker biospec scanner. Each individual measurement is positioned relative to the reference data during previous NMR studies of disease progression in groups of untreated and healthy dogs Thibaud, J.L., *et al.*, Neuromuscul Disord, 2012. **22 Suppl 2**: p. S85-99; Wary, C., *et al.,* NMR Biomed, 2012. **25**(10): p. 1160-9. Thoracic and pelvic/fore limbs are imaged in a 3T scanner. Standard and fat-saturated *T1*-, *T2*- and proton-density-weighted images are acquired as described in Thibaud, J.L., *et al.* (Neuromuscul Disord, 2007. **17**(7): p. 575-84). A measurement of *T*1 and a two-hour kinetic study of muscle enhancement after gadolinium-chelate injection are also performed. Ten indices that differ between healthy and untreated GRMD dogs have been identified, which allow interpreting the effect of the gene therapy treatment on large muscle territories.

Functional assessment: clinical grading

Clinical examination is also performed twice daily and includes food and water consumption, activity (global comportment, response to external stimuli) and physical appearance (face, fur, limbs). A full examination with body weight is performed on all animals during each anesthesia.

The general *clinical status* of the animals with respect to the muscle disease is evaluated by a clinical grading done weekly after injection, using a previously published protocol (Rouger, K., *et al.*, Am J Pathol, 2011. **179**(5): p. 2501-18). This evaluation includes 11 locomotion criteria and 6 items related to the general health status (including dysphagia, ptyalism, global activity and breathing). Each item is scored from 0 to 2, with 0 corresponding to the absence of symptoms and 2 to maximum severity. The global clinical score is expressed as the percentage of the maximum clinical score (defined as 100% for a healthy dog) and a tendency curve (mobile means order 3) is built to represent the clinical score evolution. The clinical score evolution obtained in the injected dogs is compared to the clinical score evolution of non-injected GRMD dogs.

Functional assessment: Gait analysis (muscular function)

Gait analysis quantified by Locometrix is performed twice a month. Locometrix® is a 3D accelerometric device composed of 3 orthogonally positioned accelerometers. This construction allows the recording of the accelerations along the dorso-ventral, cranio-caudal and medio-lateral axes of the dogs. Speed, stride frequency, stride length, regularity, total power, dorso-ventral power, cranio-caudal power, medio-lateral power and force can be analyzed with this device, and several of these indices are modified during the progression of the disease in GRMD dogs (Barthelemy, I., *et al.*, BMC Musculoskelet Disord, 2011. **12**: p. 75).

Functional assessment: Cardiac function evaluation

Cardiac function of the treated dogs is evaluated monthly using echocardiographic and Doppler analysis, a sensitive approach allowing the detection of contractility defects.

Data acquisition:

Conventional echocardiography and 2D color tissue Doppler imaging (TDI) are performed on conscious dogs in standing position monitored with a continuous ECG, using a Vivid 7 ultrasound unit equipped with 5-7.5 and 2-5MHz phased-array transducers (GE, Waukesha, WI), according to the recommendations from the American College of Veterinary Internal Medicine (Thomas, W.P., *et al.*, J Vet Intern Med, 1993. **7**(4): p. 247-52). All data are transferred for offline analysis using a specific software (Echo Pac 5.4, GE) by two examiners who are unaware of the clinical status of the dogs. Several parameters are measured for the assessment of myocardial contractility as described below.

Conventional parameters: Left ventricular (LV) dimensions, posterior wall and interventricular septal wall thicknesses are measured.

Left ventricular fractional shortening and ejection fraction (Teichholz method) are calculated. Pulsed Doppler of the mitral valve inflow are used for measuring the ratio of early to late diastolic flow velocity (E/A).

Tissue Doppler imaging: Measurement of radial myocardial velocities and strain rate are obtained from a short-axis view at the level of the papillary muscles in the posterior wall and an apical 4-chamber view at the level of the basal portion of the septal and lateral walls.

Speckle tracking imaging: In a short-axis view, segmental strains in each of the 5 predefined segments are measured. Mean circumferential and radial are determined by calculating manually the mean of the measurements obtained. In the 4 chambers view, global longitudinal strains are measured automatically with a program that integrates

the measurements derived from the analysis of 6 automatically detected segments. Pre injection data and mock injected GRMD serve as references.

Functional assessment: respiratory function evaluation

Respiratory function is evaluated monthly and is done by using thoracic radioscopic acquisitions performed on conscious dogs. After extraction of the end-expiratory and end-inspiratory images, 2 indices are calculated:

- the caudal retraction index of the diaphragm (RI) reflects the retraction of the diaphragm;
- the diaphragm range of motion (ROM) reflects the mobility of the diaphragm: It is obtained after (i) superpostion of the 2 images obtained on end-inspiration and on end-expiration, (ii) measurement of the distance between the localization of the ventral point of the caudal vena cava foramen on each image, (iii) normalization of this distance by the length of the 13th thoracic vertebra (T13).

These 2 indices are correlated with the retraction and the mobility of the diaphragm, which are modified during disease progression in GRMD dogs (Barthelemy, I., *et al.,* Myology congress, 2011). The results obtained in the GRMD dogs are positioned relative to the results obtained in non-injected untreated animals.

Clinical follow-up of respiratory function:

Respiratory function is also evaluated via an observation of respiratory movements/cycles with their modification revealing some respiratory abnormalities, that can be the consequences of the diaphragm and other respiratory muscles weakness.

Animal status with respect to the apparition and worsening of respiratory abnormalities is evaluated by clinical examination performed bi-monthly by a specialized veterinarian. In particular, the number and regularity of respiratory movements/cycles are evaluated.

Functional assessment: digestive function evaluation

Clinical follow-up of digestive function:

As seen in DMD patients, dysphagia (i.e. difficulty in swallowing) is a typical symptom of disease evolution in GRMD dogs, as a consequence of pronounced oral and pharyngeal muscular weakness (van den Engel-Hoek, J Neurol, 2013, 260(5): 1295-303).

Animal status with respect to the apparition and worsening of dysphagia is evaluated by a clinical examination performed bi-monthly by a specialized veterinarian. In particular, tongue size, presence of abnormal quantities of saliva within the mouth, and the capacity of the animal to eat solid or soft food are evaluated.

Follow up of the immune responses

During the entire study, blood samples (plasma, serum and peripheral blood mononuclear cells-PBMC) from dogs enrolled in the study are harvested to monitor the:

- humoral immune response against rAAV8
- humoral immune response against microdystrophin
- cellular immune response against rAAV8
- cellular immune response against microdystrophin
- inflammatory immune response in the early times after injection

Blood samples are handled according to the French L2 biosafety requirements and are processed for hematology and clinical biochemistry. Dedicated serum samplings are regularly obtained for the following immunology assessments: (i) anti-AAV antibodies and anti-dystrophin antibodies; (ii) inflammatory cytokines measurement by Luminex; (iii) complement activation. Whole blood was also collected prior and after treatment for isolation of the peripheral blood mononuclear cells (PBMC) and subsequent monitoring a potential cellular immune response against AAV and/or dystrophin polypeptide.

Humoral immune responses to rAAV8 vector:

Dog sera is evaluated at different time points post-vector injection: (i) for the presence of IgG, and IgM specific to rAAV8 detected by customized ELISA; (ii) for the rAAV8 neutralizing capacity revealed by customized neutralizing assay.

Humoral immune responses to Dystrophin:

The detection of IgG anti-Dystrophin antibodies is routinely performed by Western-Blot analysis. Briefly, cellular extracts containing canine dystrophin protein are subjected to SDS-PAGE, and then transferred to a Hybond ECL nitrocellulose membrane. After an overnight saturation, membranes are incubated with experimental canine sera from injected animals. Subsequently, detection is performed by hybridization with peroxydase conjugated rabbit anti-dog IgG antibody, followed by enhanced chemiluminescence detection. Positive control consists in anti-Dystrophin MANEX 1011C antibody (Wolfson Center for Inherited Neuromuscular Diseases).

The *cellular immune responses to AAV8 and dystrophin polypeptide* are evaluated as follows: Briefly, IFN-γ ELISPOT assays are performed with lentiviral vectors (LV) encoding for either VP proteins of AAV8 or canine dystrophin polypeptide. LV vectors are used to transduce PBMC. A complementary approach using an overlapping peptide

library covering the canine sequence of canine dystrophin polypeptide is also used to stimulate lymphocytes.

Inflammatory immune responses (cytokines) are quantified by Luminex technology before and at different time points post-vector administration looking at IL2, IL4, IL6, IL8, IL10, IL15, IFN and TNF.

Results:

As shown on Figure 2, 2-month old GRMD dogs have been injected with $1x10^{14}$vg/kg of the *rAAV2/8-SPc5.12-cMD* vector described above, by simple systemic injection through a peripheral vein. No clinical nor biochemical nor hematology adverse effects were ever detected immediately nor up to several months post vector administration.

Muscular biopsies:

Intermediate biopsies from several different muscles were obtained for the GRMD dogs, 3 and 8 months post systemic injection.

Following the methodology described above, the percentage of muscle fibers expressing the dystrophin polypeptide, 3 months post systemic delivery of the vector, was investigated. The results for GRMD Dog 2 are shown on Figure 3A.

Along with the percentage of fibers expressing the therapeutic transgene, the number of vector genomes per diploid cell (vg/dg) is indicated after following the methodology also described above. For an average of 2-4 vg/dg, the average percentage of fibers expressing dystrophin ranked from 59 to 82% on the biopsies (Figure 3A c/ to f/), which was interpreted as very encouraging. One can notice the absence of major cell infiltration and a pretty remarkable preserved tissue architecture.

8 months post systemic delivery of the vector, for 1 vg/dg, the percentage of fibers expressing dystrophin was about 50% on the biopsies (Figure 3B).

All the data available are compiled in Table 2 below:

Timing	Muscle	µDys 1 (IMAGE)		µDys 2 (ICI)		µDys 3 (ICE-T)		µDys 4 (JAFFAR)		µDys 5 (JACADI)	
		% Dys	vg/dg	% Dys	vg/dg	% Dys	vg/dg	% Dys	vg/dg	% Dys	vg/dg
Before injection	Biceps femoris	<0.5%	<0.003	<0,5%	<0.003	<0,5%	<0.003	<0,5%	<0.003	<0,5%	<0.003
3 months p.i.	Ext. carpi radialis R	62%	1.3	82%	2.8	43%	2.2	71%	3.4	81%	2.9
	Ext. digit. communis R	68%	1.2	59%	4.1	31%	2.0	73%	4.7	23%	1.5
	Ext. carpi radialis L	40%	1.1	62%	6.2	61%	2.6	20%	1.5	12%	2.0
	Ext. digit. communis L	40%	0.9	66%	2.6	42%	1.8	21%	1.0	11%	1.9
8 months p.i.	Biceps femoris R	N/A	0.5	58%	1.0	9%	1.0				
	Biceps femoris L	N/A	0.9	56%	0.8	38%	1.3				
14 months p.i.	Biceps femoris R	44%	1.0	44%	1.7						
	Biceps femoris L	36%	0.7	40%	1.4						

N/A: not applicable because of the low quality of the biopsy

Moreover, a further quantification of the vector genome copies found in the tissues of GRMD dog 3, 7,5 months post injection, is shown in the table below:

	Tissu	vg/dg
Skeletal muscles of the right forelimb	m. flexor carpi ulnaris	**0,16**
	m. extensor digitorum communis	**0,25**
	m. flexor digitorum superficialis	**0,12**
	m. flexor carpi radialis	**0,19**
	m. extensor carpi radialis	**0,97**
	m. pectoralis	**1,01**
	m. deltoideus	**1,86**
Skeletal muscles of the left forelimb	m. flexor carpi ulnaris	**0,24**
	m. extensor digitorum communis	**0,56**
	m. flexor digitorum superficialis	**0,11**
	m. flexor carpi radialis	**0,13**
	m. extensor carpi radialis	**0,29**
	m. pectoralis	**2,47**
	m. deltoideus	**1,88**
Skeletal muscles of the body	m. paravertebral lumbar	**0,82**
	m. intercostales externi	**0,35**
	m. rhomboideus cervicis	**1,42**
	m. rectus abdominis	**0,68**
Skeletal muscles of the right hindlimb	m. biceps femoris	**1,03**
	m.tibialis cranialis	**1,70**
	m. semi---membranous	**2,06**
	m. semi---tendinous	**1,33**
	m. gluteus superficialis	**0,56**
	m. vastus lateralis	**0,46**
	m. sartorius	**0,80**
	m. gastrocnemius lateralis	**0,81**
	m. extensor digitorum longus	**0,53**
	m. gracilis	**0,22**
Skeletal muscles of the left hindlimb	m. biceps femoris	**1,26**
	m. tibialis cranialis	**0,96**
	m. semi---membranous	**0,39**
	m. semi---tendinous	**0,15**
	m. gluteus superficialis	**1,11**
	m. vastus lateralis	**2,19**
	m. sartorius	**0,30**
	m. gastrocnemius lateralis	**0,83**
	m. extensor digitorum longus	**0,25**
	m. gracilis	**0,37**
Diaphragm	diaphragm	**1,26**
Heart	heart (right + left ventricles)	**1,78**
	heart (septum + part of the atrioventricular node)	**0,97**

Table 3: Vector genome copies found in the muscles of GRMD3 at sacrifice (7,5 months post-injection).

In a very interesting manner, it is observed that even at this late time point, a significant amount of transgenic particles is detected in all the skeletal muscles of the body (even at distance of the injection site, i.e. the right cephalic vein), but also in the heart and in the diaphragm. This is in favor of an excellent biodistribution of the transgene within the whole organism.

Clinical evaluation:

Preliminary data on clinical evaluation of the 5 treated GRMD dogs was performed as described above against 8 other untreated age-matched GRMD dogs. Figure 4 shows, at different post vector injection time points, an improvement of the clinical score based essentially on dysphagia, breathing, ptyalism, global activity. 100% scoring corresponds to healthy individuals. Even if clinical outcomes may vary between treated individuals within the same group (as it is often the case between untreated GRMD), these results suggest that the treated GRMD animals exhibit so far a rather stable phenotype, better than the majority of the untreated animals. The clinical score evaluated in the treated dogs is maintained at a level corresponding to at least 50% of the maximal score obtained in healthy dogs (100%), with some animals being above 70%, whereas the clinical score of the large majority of the untreated animals rapidly dropped under 40% even less (Figure 4).

Remarkably, the clinical follow up shows that even at one year old, the treated GRMD dogs are able to run, to jump obstacles, to stand up on their hind limbs. This was never observed for the untreated GRMD dogs, for which life expectancy is rarely more than one year.

The data shown on Figure 4 also support an amelioration of the cardiac and respiratory functions in treated dogs and a prolonged survival in comparison with untreated dogs, together with an improved quality of life.

Gait characterization:

As mentioned above, a bi-monthly gait evaluation was performed using the Locometrix® device. Accelerometric was recorded in 3 axes: dorso-ventral (DV), medio-lateral (ML) and cranio-caudal (CC). The gait characterization by a statistical discrimant factor analysis of 7 gait variables (stride frequency, regularity, total power, cranio-caudal power, dorso-ventral power, medio-lateral power and stride length) is shown on Figure 5.

The results obtained in the injected dogs are positioned relative to the reference data collected during a previous 3D-accelerometers study of disease progression in a group of 25 untreated GRMD and 9 normal dogs (Barthelemy, I., et al., BMC Musculoskelet Disord, 2011. 12: p. 75).

Data show that µdys-treated GRMD dogs developed a global gait index that was very different and much improved to that observed for age-matched untreated GRMD dogs. They rapidly improved their gait performances to exhibited gait very close to that of healthy dogs, after only 3 to 4 months post-injection. From these data, it appears that the µdys-treated GRMD dogs present a gait that is close to healthy dogs of the same breed.

Cardiac and respiratory functions:

The clinical scores shown on Figure 4 support an amelioration of the cardiac and respiratory function.

More specifically, improved respiratory function is supported by Figure 6 which, despite a significant variability among dogs, reveals superior values for the ROM indice in the treated GRMD dogs, in connection with an improved mobility of the diaphragm.

These data also corroborate the clinical follow up of the dogs reported in Table 4 below:

	Age of respiratory abnormalities appearance
µDys 1 (IMAGE)	19 months
µDys 2 (ICI)	None
µDys 3 (ICE-T)	None
µDys 4 (JAFFAR)	None
µDys 5 (JACADI)	None
GRMD Control 1 (JAMES)	7 months
GRMD Control 2 (JESSY)	7 months
GRMD Control 3 (JOSS)	6 months
GRMD Control 4 (EBOUGE)	6 months
GRMD Control 5 (FELIX)	4 months
GRMD Control 6 (FIASKO)	7 months

Respiratory abnormalities in GRMD dogs are characterized by a modification of the respiratory movements/cycles. Like in DMD patients, these abnormalities are the consequence of diaphragm and other respiratory muscle weakness. Table 4 reveals a delay or even suppression in the apparition of respiratory abnormalities in the treated GRMD (µDys) dogs.

Digestive functions:

Dysphagia (difficulty in swallowing) is a typical symptom of disease evolution in GRMD dogs. Like in DMD patients, dysphagia is the consequence of pronounced oral and pharyngeal muscular weakness.

A possible advantage of the treatment on digestive functions has been investigated and the results are reported in Table 5 below:

	Age of dysphagia appearance		Severity
µDys 1 (IMAGE)	12 months	10.2 months +/- 2.0 months	Weak
µDys 2 (ICI)	12 months		Very weak
µDys 3 (ICE-T)	10 months		Weak
µDys 4 (JAFFAR)	10 months		Weak
µDys 5 (JACADI)	7 months		Weak
GRMD Control 1 (JAMES)	8 months	6.2 months +/-1.3 months	Marked
GRMD Control 2 (JESSY)	4 months		Severe
GRMD Control 3 (JOSS)	6 months		Marked
GRMD Control 4 (EBOUGE)	6 months		Weak
GRMD Control 5 (FELIX)	6 months		Very weak
GRMD Control 6 (FIASKO)	7 months		weak

This clinical follow up reveals a delay in the apparition of dysphagia in the treated GRMD (µDys) dogs, with less severe symptoms.

Immune response / Toxicity:

The detection of the protein, 3 and 8 months post injection (Figure 3), as well as the good clinical scores shown on Figure 4, indicate the absence of adverse and deleterious immune responses to the recombinant AAV vector and to the microdystrophin.

The muscle biopsies (Figure 3), as well as the good clinical scores shown on Figure 4, support the absence of toxicity of the gene therapy product.

In terms of biosafety, the cellular immune response against cMD was evaluated, by interferon gamma Elispot using cMDYF peptides pools incubated on a kinetic of PBMCs (Figure 7). Whatever the injected dose, none of the injected animals exhibited a detectable secretion of Interferon gamma, suggesting an absence of cellular immune response against cMDYF.

The humoral immune response against cMD was also evaluated by an immuno-western-blot (Figure 8). All the available results are compiled in Table 6 below:

		Before injection	Month +0.5	Month +1.5	Month +2	Month +4	Month +7.5
$1^{E}14$ vg/kg	µDys 1	Nd	Nd	++	Nd	Nd	Nd
	µDys 2	Nd	+	++	++	+	Nd
	µDys 3	Nd	Nd	Nd	Nd	Nd	Nd
	µDys 4	*Pending*	*Pending*	*Pending*	*Pending*	*Pending*	*Pending*
	µDys 5	*Pending*	*Pending*	*Pending*	*Pending*	*Pending*	*Pending*

Here, the presence of anti-μdystrophin antibodies was detected in 2 out of 5 dogs injected with 10^{14}vg/kg of the AAV-cMD vector. Of importance, this humoral immune response against the cMD is only transient (maximal range of detection = between 2 weeks and 4 months post-injection) and doesn't seem to be associated to any clinical deleterious effect, suggesting that an immune tolerance could occur in these animals.

Survival:

Prolonged survival clearly appears from Figure 4:

- at age 8-9 months, only 2 over 8 untreated GRMD dogs are still alive. On the contrary all the treated GRMD dogs are still alive and healthy;
- in a general manner, the life expectancy of untreated GRMD dogs is around 12 months with a very bad clinical state at this age. On the contrary, the 2 treated GRMD dogs tested for long-term follow up (μDys 1 and 2) remain alive after this deadline (with an age of 19 and 17 months, respectively) and are in a good clinical state.

Therapeutic dose:

This study reveals that 10^{14} vg/kg, a relatively low dose for systemic administration, is an appropriate dose in terms of efficiency and toxicity in dogs.

CONCLUSIONS:

Altogether, these functional data correlated well with a substantial expression of dystrophin polypeptide (>50 % microdystrophin-expressing fibers) on intermediate muscle biopsies. They show the therapeutic effect of the MD microdystrophin construct and support that the systemic delivery may be beneficial to halt/reduce the progression of the disease. The results obtained from this systemic pilot cohort of GRMD indicate that several outcome measures from molecular, pathology and functional aspects support the systemic gene therapy in humans.

This study brings the proof of concept that the *SPc5.12-cMD* therapeutic cassette encoding for a sequence optimized microdystrophin and encapsidated in the AAV8 capsid provides clinical benefit to the dog model of the Duchenne myopathy after systemic intravenous administration of a single dose. Not only was the microdystrophin polypeptide highly expressed in multiple muscles but it also resulted in gait

improvement and improved clinical outcome measures, prolonged survival, without adverse immune response. To the knowledge of the inventors, this is the first report of so encouraging and surprising results, especially in the context of a systemic administration.

5

CLAIMS

1. A composition comprising a gene therapy product for use in the treatment of a dystrophic disease in humans or dogs, wherein:
   - the gene therapy product comprises an adeno-associated viral (AAV) vector which harbors a nucleic acid sequence encoding a ΔR4-R23/ΔCT microdystrophin;
   - the composition is systemically administered.

2. A composition for its use according to claim 1, wherein the composition is administered by intravascular injection, advantageously by intravenous injection.

3. A composition for its use according to any of claims 1 to 2, wherein the nucleic acid sequence encoding the microdystrophin is of human or canine origin, advantageously a sequence optimized for use in humans or dogs.

4. A composition for its use according to any of claims 1 to 3, wherein the expression of the nucleic acid sequence encoding the microdystrophin is under the control of a muscle specific promoter, advantageously Spc5-12.

5. A composition for its use according to any of claims 1 to 4, wherein the gene therapy product comprises a sequence 90% homologous to SEQ ID NO: 1 or SEQ ID NO: 2.

6. A composition for its use according to claim 5, wherein the gene therapy product comprises a sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

7. A composition for its use according to any of claims 1 to 6, wherein the AAV vector is an AAV of serotype 2, 8 or 9.

8. A composition for its use according to claim 7, wherein the AAV vector is an AAV8 vector, advantageously an AAV2/8 vector.

9. A composition for its use according to any of the preceding claims, wherein the AAV8 vector, advantageously the AAV2/8 vector, harbors a nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

10. A composition for its use according to any of claims 1 to 9, wherein the composition is administered with a dose less or equal to 10^{15} vg/kg, advantageously between 10^{12} vg/kg and 10^{14} vg/kg.

11. A composition for its use according to any of claims 1 to 10, comprising a single administration of the composition.

12. A composition for its use according to any of claims 1 to 11, wherein the disease is Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD), advantageously DMD.

13. A composition for its use according to any of claims 1 to 12, for ameliorating the muscular function.

14. A composition for its use according to any of claims 1 to 13, for ameliorating the cardiac function.

15. A composition for its use according to any of claims 1 to 14, for ameliorating the respiratory function.

16. A composition for its use according to any of claims 1 to 15, for ameliorating the digestive function.

17. A composition for its use according to any of claims 1 to 16, for ameliorating gait.

18. A composition for its use according to any of claims 1 to 17, for ameliorating the quality and/or expectancy of life.

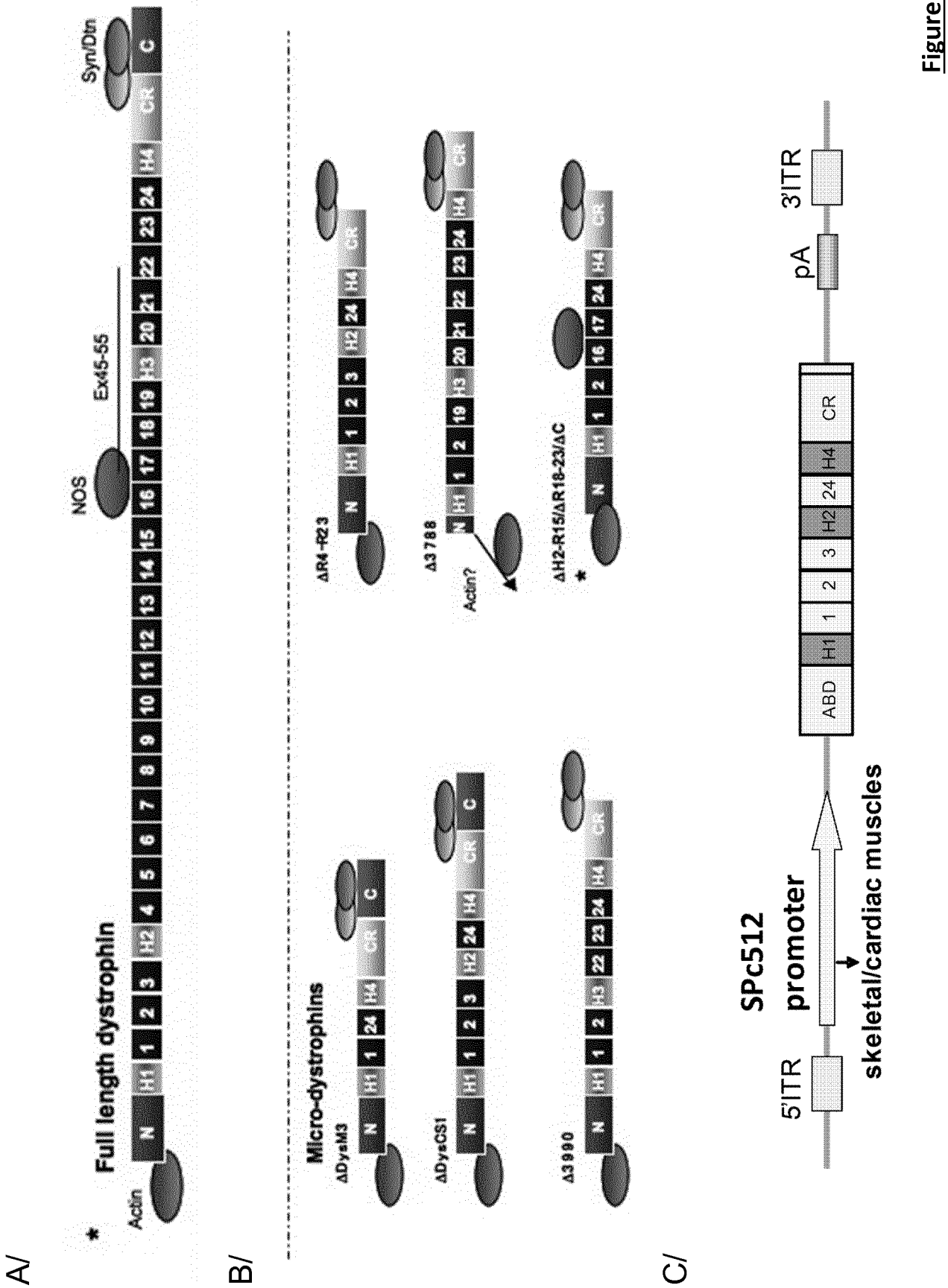

A/
B/
C/
Full length dystrophin
Actin
NOS
Ex45-55
Syn/Dtn
Micro-dystrophins
ΔDysM3
ΔDysCS1
Δ3990
ΔR4-R23
Δ3788
Actin?
ΔH2-R15/ΔR18-23/ΔC
5'ITR
SPc512
promoter
skeletal/cardiac muscles
ABD
H1
H2
CR
pA
3'ITR

Figure 1

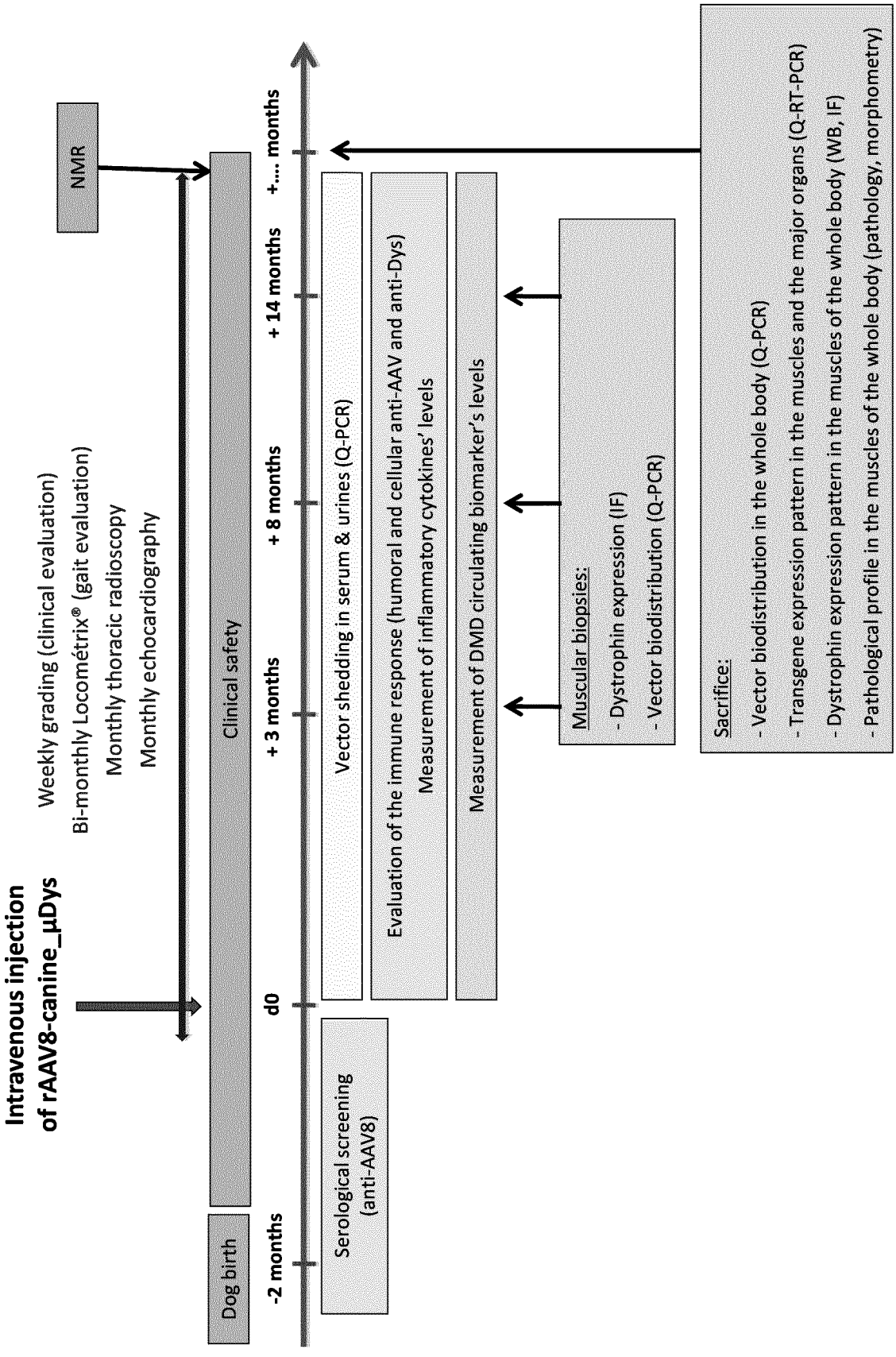
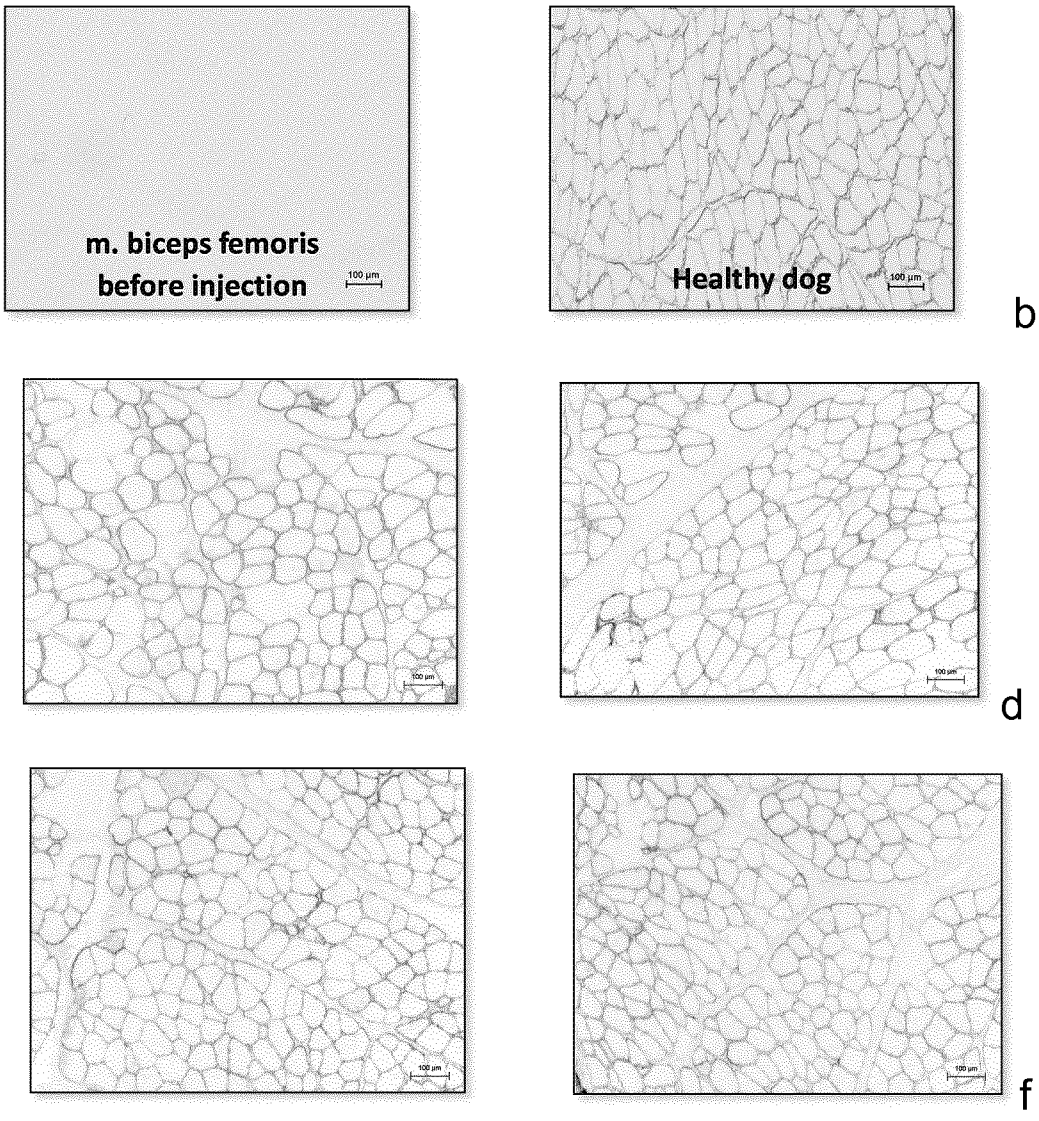

Intravenous injection of rAAV8-canine_µDys
Weekly grading (clinical evaluation)
Bi-monthly Locométrix® (gait evaluation)
Monthly thoracic radioscopy
Monthly echocardiography
NMR
Clinical safety
Dog birth
-2 months
d0
+ 3 months
+ 8 months
+ 14 months
+.... months
Serological screening (anti-AAV8)
Vector shedding in serum & urines (Q-PCR)
Evaluation of the immune response (humoral and cellular anti-AAV and anti-Dys)
Measurement of inflammatory cytokines' levels
Measurement of DMD circulating biomarker's levels
Muscular biopsies:
- Dystrophin expression (IF)
- Vector biodistribution (Q-PCR)
Sacrifice:
- Vector biodistribution in the whole body (Q-PCR)
- Transgene expression pattern in the muscles and the major organs (Q-RT-PCR)
- Dystrophin expression pattern in the muscles of the whole body (WB, IF)
- Pathological profile in the muscles of the whole body (pathology, morphometry)

Figure 2

A/


m. biceps femoris
before injection
100 µm
Healthy dog
100 µm
a
b
c
d
e
f

B/

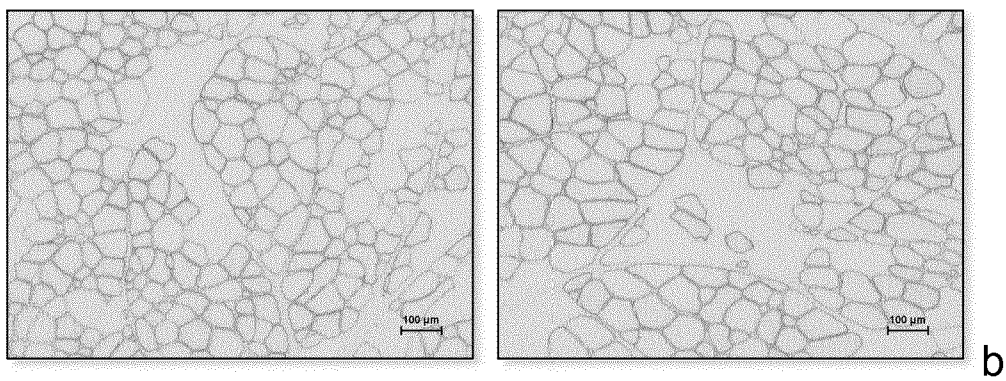

100 µm
100 µm
a
b

Figure 3

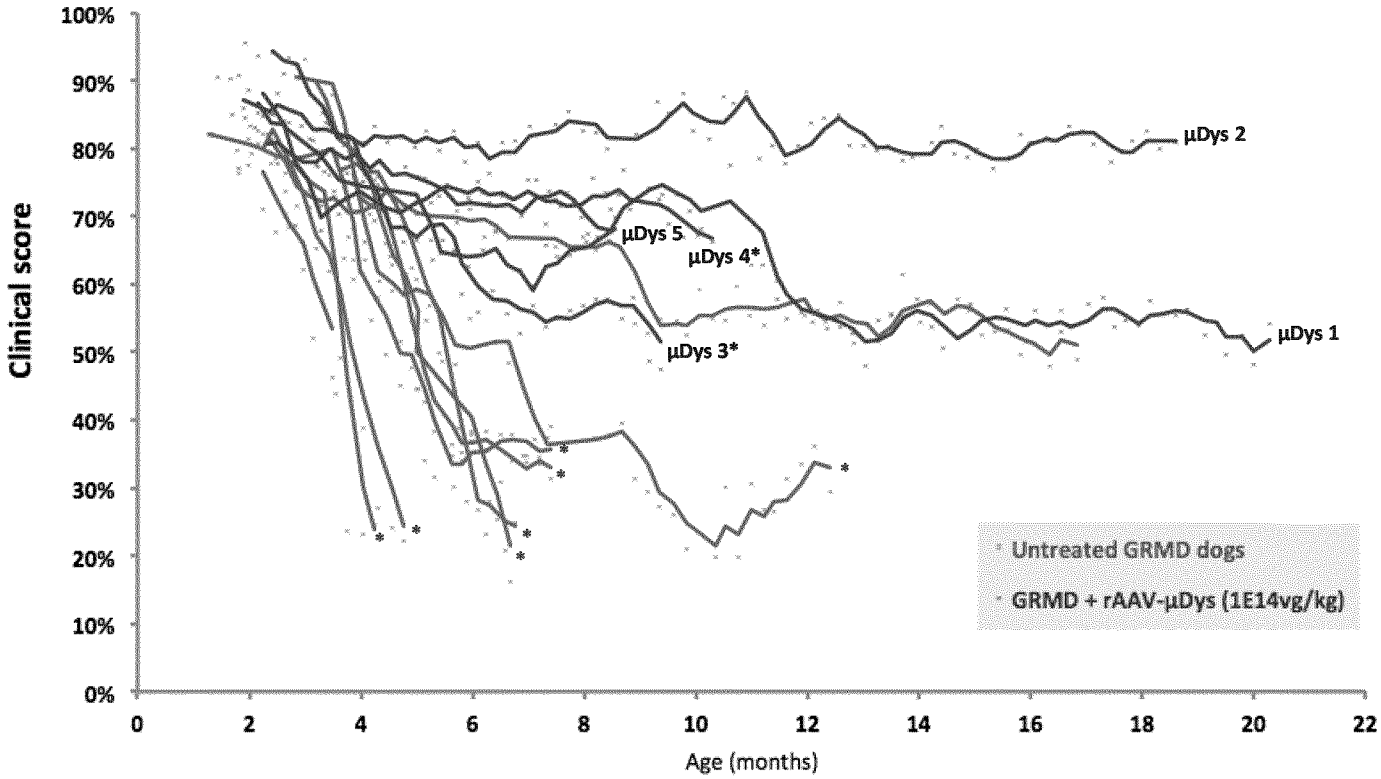


Figure 4

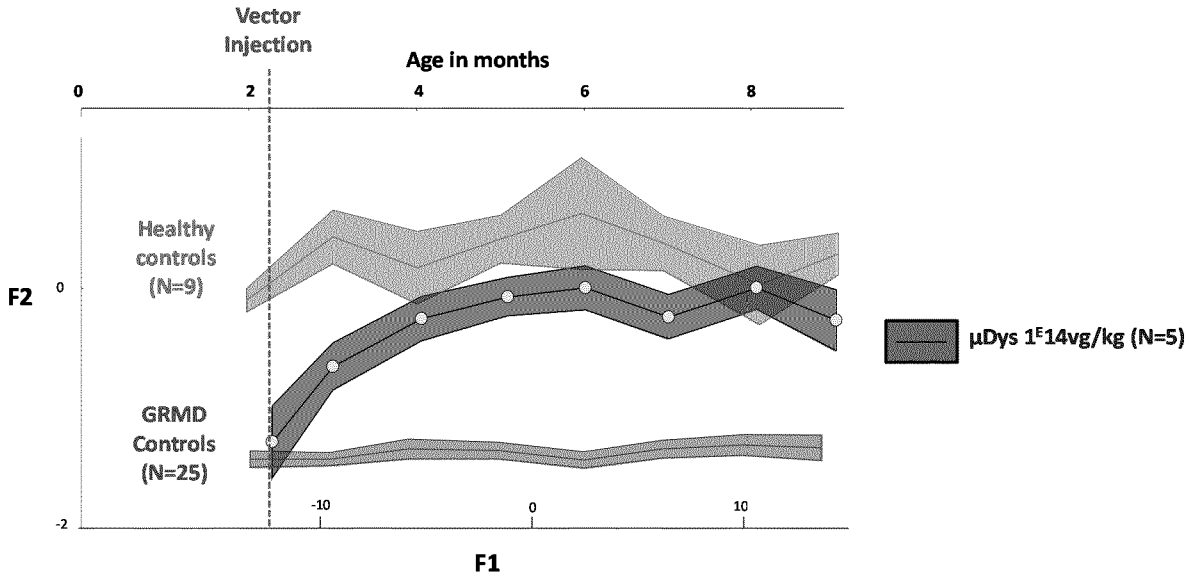


Figure 5

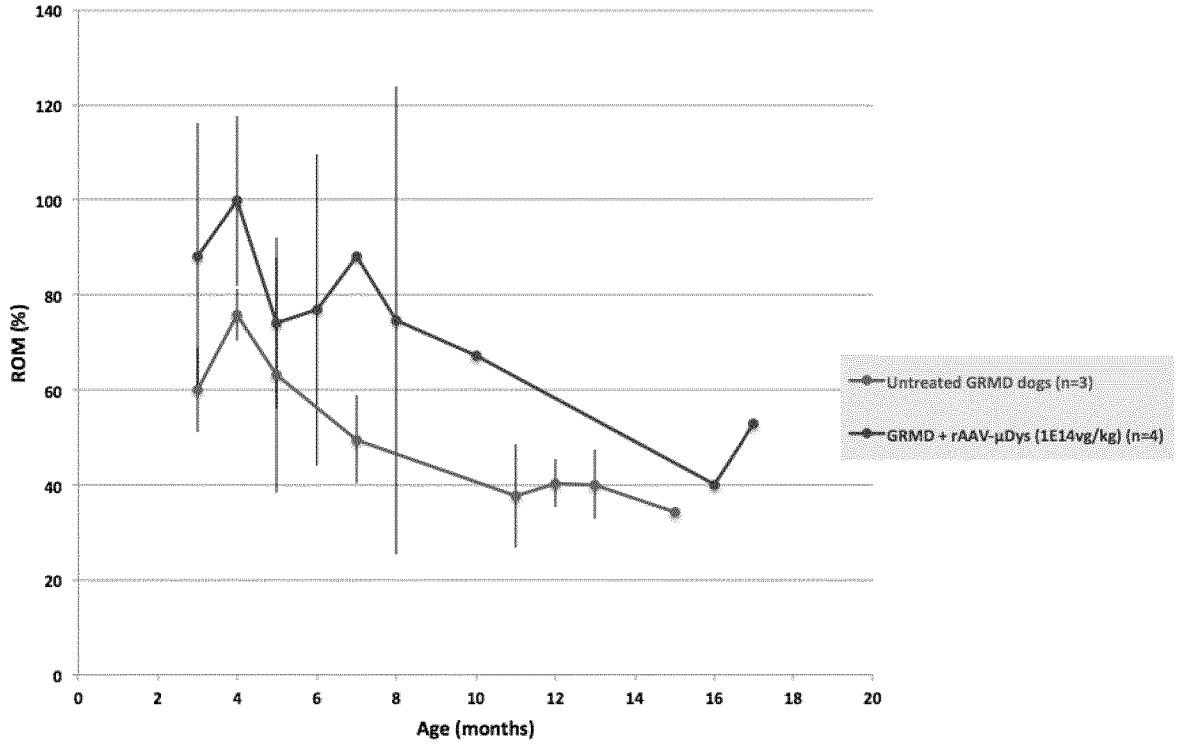

140
120
100
80
60
40
20
0
ROM (%)
0
2
4
6
8
10
12
14
16
18
20
Age (months)
Untreated GRMD dogs (n=3)
GRMD + rAAV-µDys (1E14vg/kg) (n=4)

Figure 6

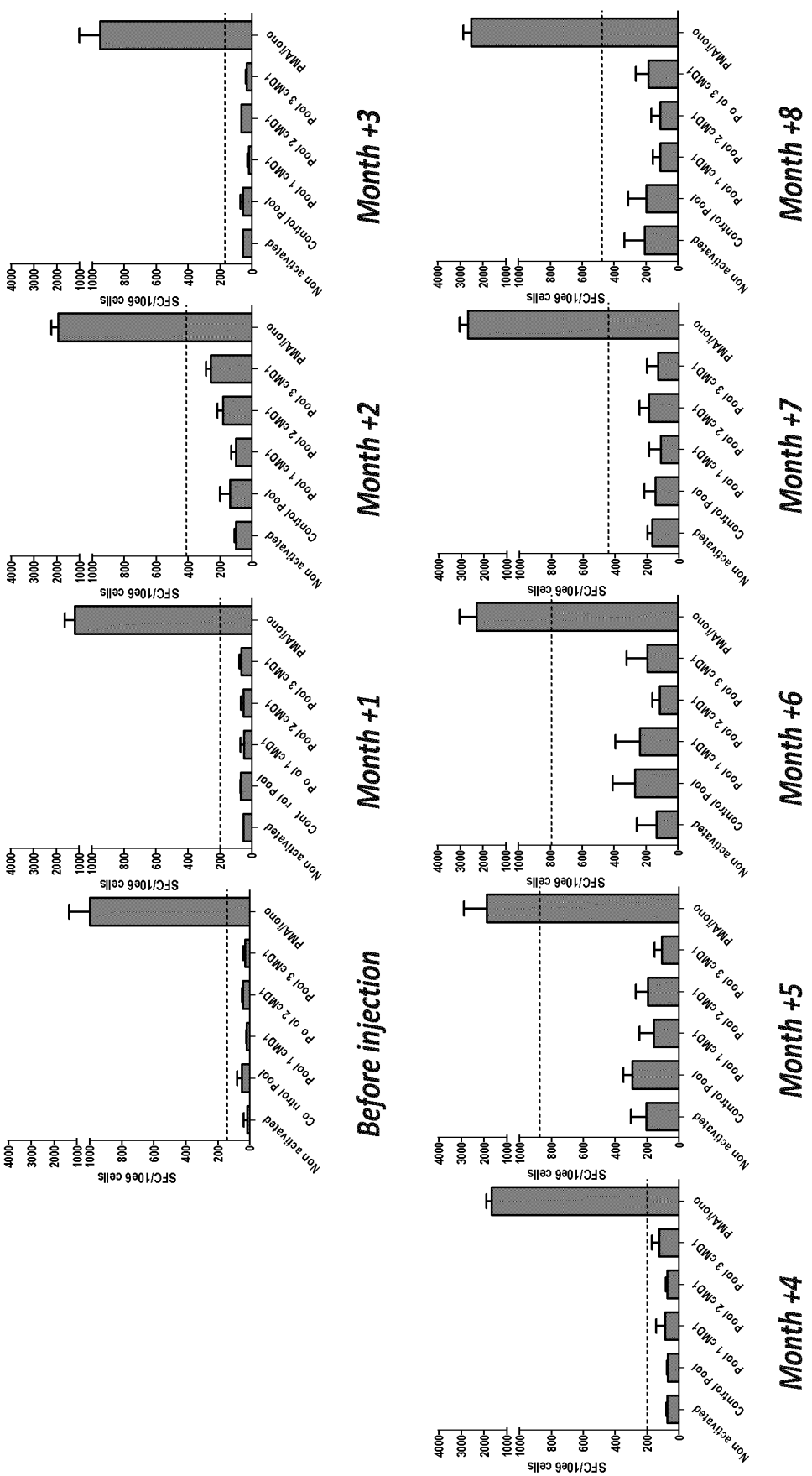

Before injection
Month +1
Month +2
Month +3
Month +4
Month +5
Month +6
Month +7
Month +8
SFC/10e6 cells
Non activated
Control Pool
Pool 1 cMDT
Pool 2 cMDT
Pool 3 cMDT
PMA/Iono

Figure 7

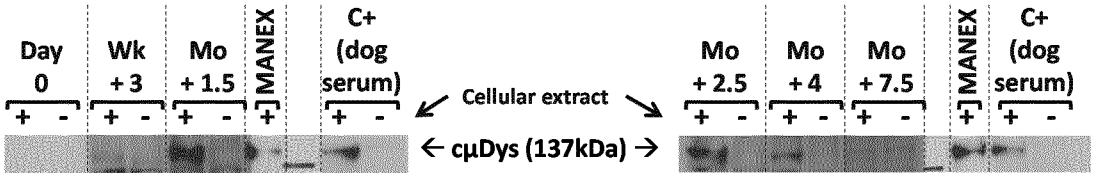

Day 0
Wk +3
Mo +1.5
MANEX
C+ (dog serum)
Cellular extract
Mo +2.5
Mo +4
Mo +7.5
MANEX
C+ (dog serum)
+ -
← cμDys (137kDa) →

Figure 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/064703

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/861 A61K48/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAEYOUNG KOO ET AL: "Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog", THE JOURNAL OF GENE MEDICINE, vol. 13, no. 9, 1 September 2011 (2011-09-01), pages 497-506, XP055152320, ISSN: 1099-498X, DOI: 10.1002/jgm.1602 cited in the application page 497 - page 505	1-18

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[X] 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

"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	Date of mailing of the international search report
24 August 2015	04/09/2015

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FOSTER HELEN ET AL: "Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer", MOLECULAR THERAPY, NATURE PUBLISHING GROUP, GB, vol. 16, no. 11, 1 November 2008 (2008-11-01), pages 1825-1832, XP002601851, ISSN: 1525-0024, DOI: 10.1038/MT.2008.186 [retrieved on 2008-09-02] cited in the application page 1826 - page 1830	1-18
A	BRIAN BOSTICK ET AL: "AAV micro-dystrophin gene therapy alleviates stress-induced cardiac death but not myocardial fibrosis in >21-m-old mdx mice, an end-stage model of Duchenne muscular dystrophy cardiomyopathy", JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, ACADEMIC PRESS, GB, vol. 53, no. 2, 5 May 2012 (2012-05-05), pages 217-222, XP028501316, ISSN: 0022-2828, DOI: 10.1016/J.YJMCC.2012.05.002 [retrieved on 2012-05-12] abstract page 218 - page 221; table 1	1-18
A	SACHIKO OHSHIMA ET AL: "Transduction Efficiency and Immune Response Associated With the Administration of AAV8 Vector Into Dog Skeletal Muscle", MOLECULAR THERAPY, vol. 17, no. 1, 21 October 2008 (2008-10-21), pages 73-80, XP055152686, ISSN: 1525-0016, DOI: 10.1038/mt.2008.225 page 73 - page 78	1-18
A	Y. ZHANG ET AL: "Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy", HUMAN MOLECULAR GENETICS, vol. 22, no. 18, 15 May 2013 (2013-05-15), pages 3720-3729, XP055152228, ISSN: 0964-6906, DOI: 10.1093/hmg/ddt224 page 3720 - page 3772; figures 1-4	1-18

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUY L ODOM ET AL: "Gene Therapy of mdx Mice With Large Truncated Dystrophins Generated by Recombination Using rAAV6", MOLECULAR THERAPY, vol. 19, no. 1, 1 January 2011 (2011-01-01), pages 36-45, XP055074634, ISSN: 1525-0016, DOI: 10.1038/mt.2010.205 page 36 - page 44	1-18
A	BING WANG ET AL: "Systemic human minidystrophin gene transfer improves functions and life span of dystrophin and dystrophin/utrophin-deficient mice", JOURNAL OF ORTHOPAEDIC RESEARCH, vol. 27, no. 4, 1 April 2009 (2009-04-01), pages 421-426, XP055152407, ISSN: 0736-0266, DOI: 10.1002/jor.20781 the whole document	1-18
A	B M KOPPANATI ET AL: "Improvement of the mdx mouse dystrophic phenotype by systemic in utero AAV8 delivery of a minidystrophin gene", GENE THERAPY, vol. 17, no. 11, 10 June 2010 (2010-06-10) , pages 1355-1362, XP055152677, ISSN: 0969-7128, DOI: 10.1038/gt.2010.84 page 1355 - page 1360	1-18
A	STEFANIE SCHINKEL ET AL: "Long-Term Preservation of Cardiac Structure and Function After Adeno-Associated Virus Serotype 9-Mediated Microdystrophin Gene Transfer in mdx Mice", HUMAN GENE THERAPY, vol. 23, no. 6, 1 June 2012 (2012-06-01), pages 566-575, XP055152623, ISSN: 1043-0342, DOI: 10.1089/hum.2011.017 page 566 - page 573	1-18
A	PAUL GREGOREVIC ET AL: "Systemic Microdystrophin Gene Delivery Improves Skeletal Muscle Structure and Function in Old Dystrophic mdx Mice", MOLECULAR THERAPY, vol. 16, no. 4, 4 March 2008 (2008-03-04), pages 657-664, XP055152553, ISSN: 1525-0016, DOI: 10.1038/mt.2008.28 page 657 - page 663; figures 1-4	1-18

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J-H SHIN ET AL: "Improvement of cardiac fibrosis in dystrophic mice by rAAV9-mediated microdystrophin transduction", GENE THERAPY, vol. 18, no. 9, 31 March 2011 (2011-03-31), pages 910-919, XP055152487, ISSN: 0969-7128, DOI: 10.1038/gt.2011.36 page 910 - page 918; figures 1-8; table 1	1-18
A	JIN-HONG SHIN ET AL: "Efficient systemic delivery of RAAV8 into dystrophic animals by subcutaneous injection", JOURNAL OF GENE MEDICINE, vol. 10, no. 4, 26 March 2008 (2008-03-26), - 30 June 2007 (2007-06-30), page 449, XP055152502, US ISSN: 1099-498X, DOI: 10.1002/jgm the whole document	1-18
A	TIMOTHY D COLGAN ET AL: "511: Microdystrophin and Follistatin Combinatorial Gene Delivery To Treat a Severe Mouse Model of Duchenne Muscular Dystrophy", MOLECULAR THERAPY, vol. 22, no. Suppl. 1, 1 May 2014 (2014-05-01), - 24 May 2014 (2014-05-24), page S197, XP055152509, GB ISSN: 1525-0016, DOI: 10.1038/mt.2014.64 the whole document	1-18
A	ZEJING WANG ET AL: "Sustained AAV-mediated Dystrophin Expression in a Canine Model of Duchenne Muscular Dystrophy with a Brief Course of Immunosuppression", MOLECULAR THERAPY, 10 April 2007 (2007-04-10), XP055152230, ISSN: 1525-0016, DOI: 10.1038/sj.mt.6300161 cited in the application page 1160 - page 1165	1-18

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAWN E BOWLES ET AL: "Phase 1 Gene Therapy for Duchenne Muscular Dystrophy Using a Translational Optimized AAV Vector", MOLECULAR THERAPY, vol. 20, no. 2, 8 November 2011 (2011-11-08), pages 443-455, XP055152225, ISSN: 1525-0016, DOI: 10.1038/mt.2011.237 page 443 - page 454	1-18
A	JERRY R. MENDELL ET AL: "Dystrophin Immunity in Duchenne's Muscular Dystrophy", NEW ENGLAND JOURNAL OF MEDICINE, vol. 363, no. 15, 7 October 2010 (2010-10-07), pages 1429-1437, XP055152462, ISSN: 0028-4793, DOI: 10.1056/NEJMoa1000228 cited in the application page 1429 - page 1436	1-18
T	Taeyoung Koo ET AL: "22 Genetic Therapy for Duchenne Muscular Dystrophy: Principles and Progress", , 9 May 2012 (2012-05-09), XP055152256, Retrieved from the Internet: URL:http://cdn.intechopen.com/pdfs-wm/36746.pdf [retrieved on 2014-11-11] page 446, line 1 - page 448, line 2	