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WO-A-2006/000057
WO-A-2007/135105
WO-A-2009/054725
WILTON STEVE D ET AL: "Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript.", MOLECULAR THERAPY : THE JOURNAL OF THE AMERICAN SOCIETY OF GENE THERAPY JUL 2007, vol. 15, no. 7, July 2007 (2007-07), pages 1288-1296, XP002544728, ISSN: 1525-0024
AARTSMA-RUS A ET AL: "Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy", NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB, vol. 12, no. SUPPL. 1, 1 October 2002 (2002-10-01), pages S71-S77, XP002250906, ISSN: 0960-8966
AARTSMA-RUS ANNEMIEKE ET AL: "Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites", OLIGONUCLEOTIDES, MARY ANN LIEBERT, NEW YORK, NY, US, vol. 15, no. 4, 1 December 2005 (2005-12-01), pages 284-297, XP002406791, ISSN: 1545-4576
AARTSMA-RUS A ET AL: "therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients", HUMAN MOLECULAR GENETICS, OXFORD UNIVERSITY PRESS, SURREY, vol. 12, no. 8, 1 January 2003 (2003-01-01), pages 907-914, XP008084159, ISSN: 0964-6906

Fortsættes ...

VAN DEUTEKOM J C T ET AL: "Advances in Duchenne Muscular Dystrophy Gene Therapy", NATURE REVIEWS GENETICS, MACMILLAN MAGAZINES, GB, vol. 4, 1 October 2003 (2003-10-01), pages 774-783, XP002993824,

VAN OMMEN GERT-JAN ET AL: "The therapeutic potential of antisense-mediated exon skipping.", CURRENT OPINION IN MOLECULAR THERAPEUTICS APR 2008, vol. 10, no. 2, April 2008 (2008-04), pages 140-149, XP009107079, ISSN: 1464-8431

SQUIRES K E: "AN INTRODUCTION TO NUCLEOSIDE AND NUCLEOTIDE ANALOGUES", ANTIVIRAL THERAPY- AN OFFICIAL PUBLICATION OF THE INTERNATIONAL SOCIETY FOR ANTIVIRAL RESEARCH, MTM PUBLICATIONS, LONDON, GB, vol. 6, no. SUPPL. 03, 1 January 2001 (2001-01-01), pages 1-14, XP009042950, ISSN: 1359-6535

DESCRIPTION

Field of the invention

[0001] The invention relates to the field of genetics, more specifically human genetics. The invention in particular relates to the modulation of splicing of the human Duchenne Muscular Dystrophy gene.

Background

[0002] Myopathies are disorders that result in functional impairment of muscles. Muscular dystrophy (MD) refers to genetic diseases that are characterized by progressive weakness and degeneration of skeletal muscles. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are the most common childhood forms of muscular dystrophy. They are recessive disorders and because the gene responsible for DMD and BMD resides on the X-chromosome, mutations mainly affect males with an incidence of about 1 in 3500 boys.

[0003] DMD and BMD are caused by genetic defects in the DMD gene encoding dystrophin, a muscle protein that is required for interactions between the cytoskeleton and the extracellular matrix to maintain muscle fiber stability during contraction. DMD is a severe, lethal neuromuscular disorder resulting in a dependency on wheelchair support before the age of 12 and DMD patients often die before the age of thirty due to respiratory- or heart failure. In contrast, BMD patients often remain ambulatory until later in life, and have near normal life expectancies. DMD mutations in the dystrophin gene are characterized by frame shifting insertions or deletions or nonsense point mutations, resulting in the absence of functional dystrophin. BMD mutations in general keep the reading frame intact, allowing synthesis of a partly functional dystrophin.

[0004] Several possible treatments have been investigated over the last 20 years, including myoblast-transplantation, DNA-targeted gene therapy, and antisense-mediated exon skipping (van Deutekom and van Ommen, (2003), Nat. Rev. Genet., 4(10):774-83). Antisense-mediated exon skipping aims at transforming out-of-frame mutations present in DMD patients into in-frame BMD-like mutations that result in synthesis of an at least partially functional dystrophin, which will prolong the viability of the muscles (Aartsma-Rus and van Ommen, (2007), RNA, 13(10): 1609-24).

[0005] Exon skipping can be induced by antisense oligonucleotides (AON) directed against the splice donor or splice acceptor site of a splice junction that are involved in the enzymatic process of exon joining, or against exon-internal sequences. In general, splice donor and splice acceptor sites comprise conserved sequences and targeting these sequences has the inevitable risk of co-targeting splice sites of additional exons from DMD or other gene

transcripts.

[0006] Exon 44 of the DMD gene consists of 148 base pairs. Therapeutic skipping of exon 44 would restore the correct reading frame in DMD patients having deletions including but not limited to exons 03-43, 05-43, 06-43, 10-43, 13-43, 14-43, 17-43, 19-43, 28-43, 30-43, 31-43, 33-43, 34-43, 35-43, 36-43, 37-43, 38-43, 40-43, 41-43, 42-43, 43, 45, 45-54, and 45-68, or having a duplication of exon 44. Furthermore, for some DMD patients the mutations are such that the simultaneous skipping of one or more exons is required in addition to exon 44 skipping to restore the reading frame. Non-limiting examples of such mutations are nonsense point mutations in the flanking exons 43 or 45, requiring exon 43+44 skipping or exon 44+45 skipping respectively. The aforementioned mutations in total occur in about 6-8 % of all DMD patients. The majority of resulting dystrophin proteins will be truncated in the central rod domain of the protein, leaving the essential N-terminal actin-binding domain and the C-terminal domain binding to dystrobrevin and syntrophin, and the β -dystroglycan-binding C-terminal cysteine-rich domain, intact.

Description

[0007] The present invention identifies four different regions in exon 44 that are particularly suited for inducing skipping of exon 44. The invention thus provides a method for modulating splicing of exon 44 of the DMD gene in a cell, the method comprising providing said cell with a molecule that binds to a nucleotide sequence comprising SEQ ID NO. 1: 5'-GUGGCUAACAGAAGCU; SEQ ID NO. 2: 5'-GGGAACAUGCUGAAAAC, SEQ ID NO. 3: 5'-AGACACAAAUCCUGAGA, or SEQ ID NO. 4: 5'-CUGUUGAGAAA. This molecule preferably binds or is complementary to any of SEQ ID NO: 1, 2, 3, or 4 when SEQ ID NO:1, 2, 3, or 4 is present within exon 44 of the DMD pre-mRNA.

[0008] Throughout the application, the expression "inducing skipping" is synonymous of "modulating splicing".

[0009] It was found that a molecule that binds to a nucleotide sequence comprising SEQ ID NO. 1: 5'-GUGGCUAACAGAAGCU; SEQ ID NO. 2: 5'-GGGAACAUGCUGAAAAC, SEQ ID NO. 3: 5'-AGACACAAAUCCUGAGA, or SEQ ID NO. 4: 5'-CUGUUGAGAAA results in highly efficient skipping of exon 44 in cells provided with this molecule. Furthermore, none of the indicated sequences is derived from conserved parts of splice-junction sites. Therefore, said molecule is not likely to mediate differential splicing of other exons from the DMD pre-mRNA or exons from other genes. In addition, other (immuno)toxicity is preferably avoided by avoiding CpG pairs in the molecule that binds to a nucleotide sequence as defined herein above.

[0010] Exon skipping refers to the induction in a cell of a mature mRNA that does not contain a particular exon that is normally present therein. Exon skipping is achieved by providing a cell expressing the pre-mRNA of said mRNA, with a molecule capable of interfering with sequences such as, for example, the splice donor or splice acceptor sequence required for allowing the

enzymatic process of splicing, or that is capable of interfering with an exon inclusion signal required for recognition of a stretch of nucleotides as an exon to be included into the mRNA. The term pre-mRNA refers to a non-processed or partly processed precursor mRNA that is synthesized from a DNA template in the cell nucleus by transcription.

[0011] Certain methods of the disclosure will alleviate one or more characteristics of a myogenic cell or muscle cell of a DMD patient having deletions including, but not limited to, exons 03-43, 05-43, 06-43, 10-43, 13-43, 14-43, 17-43, 19-43, 28-43, 30-43, 31-43, 33-43, 34-43, 35-43, 36-43, 37-43, 38-43, 40-43, 41-43, 42-43, 43, 45, 45-54, and 45-68, or having a duplication of exon 44. Furthermore, the removal of a flanking exon, such as, for example, exon 43 or exon 45, because of a nonsense point mutation in the flanking exon, will result in an out of frame transcript. The additional skipping of exon 44, in combination with skipping of the flanking exon, will restore the reading frame of the DMD gene in myogenic cells or muscle cells of DMD patients. Non-limiting examples of such mutations are nonsense point mutations in the flanking exons 43 or 45, requiring exon 43+44 skipping or exon 44+45 skipping respectively.

[0012] In an embodiment, a method of the disclosure may also alleviate one or more characteristics of a myogenic cell or muscle cell of a strong BMD patient, to the characteristics of a mild BMD patient. The characteristics of a cell of a DMD or BMD patient include increased calcium uptake by muscle cells, increased collagen synthesis, altered morphology, altered lipid biosynthesis, increased oxidative stress, and/or damaged sarcolemma. Preferred embodiments of a method of the invention are later defined herein.

[0013] In one embodiment, a molecule as defined herein can be a compound molecule that binds and/or is complementary to the specified sequence, or a protein such as an RNA-binding protein or a non-natural zinc-finger protein that has been modified to be able to bind to the indicated nucleotide sequence on a RNA molecule. Methods for screening compound molecules that bind specific nucleotide sequences are, for example, disclosed in PCT/NL01/00697 and US Patent 6,875,736. Methods for designing RNA-binding Zinc-finger proteins that bind specific nucleotide sequences are disclosed by Friesen and Darby, Nature Structural Biology 5: 543- 546 (1998). Binding to one of the specified SEQ ID NO: 1, 2, 3, or 4 sequence, preferably in the context of exon 44 of DMD may be assessed via techniques known to the skilled person. A preferred technique is gel mobility shift assay as described in EP 1 619 249. In a preferred embodiment, a molecule is said to bind to one of the specified sequences as soon as a binding of said molecule to a labelled sequence SEQ ID NO: 1, 2, 3 or 4 is detectable in a gel mobility shift assay. Alternatively or in combination with previous embodiment, a molecule is an oligonucleotide which is complementary or substantially complementary to SEQ ID NO:1, 2, 3, or 4 or part thereof as later defined herein. The term "substantially" complementary used in this context indicates that one or two or more mismatches may be allowed as long as the functionality, i.e. inducing skipping of exon 44, is still acceptable.

[0014] The disclosure provides a method for designing a molecule, preferably an oligonucleotide able to induce the skipping of exon 44 of the DMD gene. First said

oligonucleotide is selected to bind to one of SEQ ID NO: 1, 2, 3, or 4 or parts thereof as earlier defined herein. Subsequently, in a preferred method at least one of the following aspects has to be taken into account for designing, improving said molecule any further:

- The molecule does not contain a CpG,
- The molecule does not contain a G-quartet motif,
- The molecule has acceptable RNA binding kinetics and/or thermodynamic properties.

[0015] The presence of a CpG in an oligonucleotide is usually associated with an increased immunogenicity of said oligonucleotide (Dorn and Kippenberger, Curr Opin Mol Ther 2008 10(1) 10-20). This increased immunogenicity is undesired since it may induce the breakdown of muscle fibers. Immunogenicity may be assessed in an animal model by assessing the presence of CD4⁺ and/or CD8⁺ cells and/or inflammatory mononucleocyte infiltration in muscle biopsy of said animal. Immunogenicity may also be assessed in blood of an animal or of a human being treated with an oligonucleotide of the invention by detecting the presence of a neutralizing antibody and/or an antibody recognizing said oligonucleotide using a standard immunoassay known to the skilled person. An increase in immunogenicity may be assessed by detecting the presence or an increasing amount of a neutralizing antibody or an antibody recognizing said oligonucleotide using a standard immunoassay.

[0016] An oligonucleotide comprising a G-quartet motif has the tendency to form a quadruplex, a multimer or aggregate formed by the Hoogsteen base-pairing of four single-stranded oligonucleotides (Cheng and Van Dyke, Gene. 1997 Sep 15;197(1-2):253-60), which is of course not desired: as a result the efficiency of the oligonucleotide is expected to be decreased. Multimerisation or aggregation is preferably assessed by standard polyacrylamide non-denaturing gel electrophoresis techniques known to the skilled person. In a preferred embodiment, less than 20% or 15%, 10%, 7%, 5% or less of a total amount of an oligonucleotide of the invention has the capacity to multimerise or aggregate assessed using the assay mentioned above.

[0017] The disclosure allows designing an oligonucleotide with acceptable RNA binding kinetics and/or thermodynamic properties. The RNA binding kinetics and/or thermodynamic properties are at least in part determined by the melting temperature of an oligonucleotide (T_m; calculated with the oligonucleotide properties calculator (www.unc.edu/~cail/biotool/oligo/index.html) for single stranded RNA using the basic T_m and the nearest neighbour model), and/or the free energy of the AON-target exon complex (using RNA structure version 4.5). If a T_m is too high, the oligonucleotide is expected to be less specific. An acceptable T_m and free energy depend on the sequence of the oligonucleotide. Therefore, it is difficult to give preferred ranges for each of these parameters. An acceptable T_m may be ranged between 35 and 65°C and an acceptable free energy may be ranged between 15 and 45 kcal/mol.

[0018] The skilled person may therefore first choose an oligonucleotide as a potential therapeutic compound as binding and/or being complementary to SEQ ID NO:1, 2, 3, or 4 of exon 44 or parts thereof as defined herein. The skilled person may check that said oligonucleotide is able to bind to said sequences as earlier defined herein. Optionally in a second step, he may use the disclosure to further optimise said oligonucleotide by checking for the absence of CpG, the absence of a G-quartet motif, and/or by optimizing its T_m and/or free energy of the AON-target complex. He may try to design an oligonucleotide wherein no CpG and/or no G-quartet motif are present and/or wherein a more acceptable T_m and/or free energy are obtained by choosing a distinct sequence of exon 44 (for example SEQ ID NO:1, 2, 3, or 4) to which the oligonucleotide is complementary. Alternatively, if an oligonucleotide complementary to a given stretch within SEQ ID NO:1, 2, 3 or 4 of exon 44, comprises a CpG, a G-quartet motif and/or does not have an acceptable T_m and/or free energy, the skilled person may improve any of these parameters by decreasing the length of the oligonucleotide, and/or by choosing a distinct stretch within any of SEQ ID NO: 1, 2, 3, or 4 to which the oligonucleotide is complementary and/or by altering the chemistry of the oligonucleotide.

As an example, if one chooses SEQ ID NO:1, several oligonucleotides were designed which were found to bind this sequence: SEQ ID NO: 5, 49, and 54. The oligonucleotide comprising SEQ ID NO:5 was found to have the most optimal RNA binding kinetics and/or thermodynamic properties, such as the most optimal T_m. When we tested the functionality of these oligonucleotides to induce the skipping of exon 44, it was confirmed that an oligonucleotide comprising SEQ ID NO:5 is the most efficient of these four oligonucleotides. Each of these oligonucleotides is functional in the sense of the invention. However, an oligonucleotide comprising SEQ ID NO:5 is the most preferred oligonucleotide identified that binds and/or is complementary to SEQ ID NO:1.

[0019] At any step of the method, an oligonucleotide of the disclosure is preferably an oligonucleotide, which is still able to exhibit an acceptable level of a functional activity. A functional activity of said oligonucleotide is preferably to induce the skipping of exon 44 of the DMD gene to a certain extent, to provide an individual with a functional dystrophin protein and/or mRNA and/or at least in part decreasing the production of an aberrant dystrophin protein and/or mRNA. Each of these features is later defined herein. Such functional activity may be measured in a muscular tissue or in a muscular cell of an individual or *in vitro* in a cell. The assessment of the functionality may be carried out at the mRNA level, preferably using RT-PCR. The assessment of the functionality may be carried out at the protein level, preferably using western blot analysis or immunofluorescence analysis of cross-sections. In a preferred embodiment, an oligonucleotide is said to induce skipping of exon 44 of a DMD gene, when tested in a muscle cell of a DMD patient, by RT-PCR, the exon 44 skipping percentage is of at least 30% , or at least 35%, or at least 40%, or at least 45%, or at least 50%, or at least 55%, or at least 60%, or at least 65%, or at least 70%, or at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 95%, or 100%.

[0020] In a preferred embodiment, such oligonucleotide is preferably a medicament. More preferably, said medicament is for preventing or treating Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual or a patient. As defined herein a DMD pre-mRNA

preferably means the pre-mRNA of a DMD gene of a DMD or BMD patient. A patient is preferably intended to mean a patient having DMD or BMD or a patient susceptible to develop DMD or BMD due to his or her genetic background.

In the case of a DMD patient, an oligonucleotide used will preferably correct at least one of the DMD mutations as present in the DMD gene of said patient and therefore will preferably create a dystrophin that will look like a BMD dystrophin: said dystrophin will preferably be a functional dystrophin as later defined herein.

[0021] In the case of a BMD patient, an oligonucleotide as used will preferably correct at least one of the BMD mutations as present in the DMD gene of said patient and therefore will preferably create a, or more of a, dystrophin, which will be more functional than the dystrophin which was originally present in said BMD patient. Even more preferably, said medicament increases the production of a functional or more functional dystrophin protein and/or mRNA and/or at least in part decreases the production of an aberrant or less functional dystrophin protein and/or mRNA in an individual.

Preferably, a method of the disclosure increases production of a more functional dystrophin protein and/or mRNA and/or decreases the production of an aberrant or less functional dystrophin protein and/or mRNA in a patient, by inducing and/or promoting skipping of at least exon 44 of the DMD pre-mRNA as identified herein in one or more cells, preferably muscle cells of said patient. Increasing the production of a more functional dystrophin protein and/or mRNA and/or decreasing the production of an aberrant dystrophin protein and/or mRNA in a patient is typically applied in a DMD patient. Increasing the production of a more functional or functional dystrophin and/or mRNA is typically applied in a BMD patient.

Therefore a preferred method is a method, wherein in a patient or in one or more cells of said patient, production of a more functional or functional dystrophin protein and/or mRNA is increased and/or the production of an aberrant dystrophin protein and/or mRNA in said patient is decreased, wherein the level of said aberrant or more functional dystrophin protein and/or mRNA is assessed by comparison to the level of said dystrophin and/or mRNA in said patient at the onset of the method.

As defined herein, a functional dystrophin is preferably a wild type dystrophin corresponding to a protein having the amino acid sequence as identified in SEQ ID NO: 55. A functional dystrophin is preferably a dystrophin, which has an actin binding domain in its N terminal part (first 240 amino acids at the N terminus), a cystein-rich domain (amino acid 3361 till 3685) and a C terminal domain (last 325 amino acids at the C terminus) each of these domains being present in a wild type dystrophin as known to the skilled person. The amino acids indicated herein correspond to amino acids of the wild type dystrophin being represented by SEQ ID NO: 55. In another embodiment, a functional dystrophin is a dystrophin, which exhibits at least to some extent an activity of a wild type dystrophin. "At least to some extent" preferably means at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of a corresponding activity of a wild type functional dystrophin. In this context, an activity of a wild type dystrophin is preferably binding to actin and to the dystrophin-associated glycoprotein complex (DGC) (Aartsma-Rus A et al, (2006), Entries in the Leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve, 34: 135-144.). Binding of dystrophin to actin and to the DGC complex may be visualized

by either co-immunoprecipitation using total protein extracts or immunofluorescence analysis of cross-sections, from a biopsy of a muscle suspected to be dystrophic, as known to the skilled person. Individuals suffering from Duchenne muscular dystrophy typically have a mutation in the gene encoding dystrophin that prevents synthesis of the complete protein, i.e. a premature stop prevents the synthesis of the C-terminus of the protein. In Becker muscular dystrophy the dystrophin gene also comprises a mutation compared to the wild type but the mutation does typically not include a premature stop and the C-terminus of the protein is typically synthesized. As a result a functional dystrophin protein is synthesized that has at least the same activity in kind as a wild type protein, although not necessarily the same amount of activity. In a preferred embodiment, a functional dystrophin protein means an in frame dystrophin gene. The genome of a BMD individual typically encodes a dystrophin protein comprising the N terminal part (first 240 amino acids at the N terminus), a cystein-rich domain (amino acid 3361 till 3685) and a C terminal domain (last 325 amino acids at the C terminus) but its central rod shaped domain may be shorter than the one of a wild type dystrophin (Aartsma-Rus A et al, (2006), Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve, 34: 135-144). The amino acids indicated herein correspond to amino acids of the wild type dystrophin being represented by SEQ ID NO: 55. Exon-skipping for the treatment of DMD is preferably but not exclusively directed to overcome a premature stop in the pre-mRNA by skipping an exon in the rod-domain shaped domain to correct the reading frame and allow synthesis of remainder of the dystrophin protein including the C-terminus, albeit that the protein is somewhat smaller as a result of a smaller rod domain. In a preferred embodiment, an individual having DMD and being treated using an oligonucleotide as defined herein will be provided a dystrophin, which exhibits at least to some extent an activity of a wild type dystrophin. More preferably, if said individual is a Duchenne patient or is suspected to be a Duchenne patient, a functional dystrophin is a dystrophin comparable in functionality to a dystrophin from an individual having BMD: preferably said dystrophin is able to interact with both actin and the DGC, but its central rod shaped domain may be shorter than the one of a wild type dystrophin (Aartsma-Rus A et al, (2006), Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve, 34: 135-144). The central rod domain of wild type dystrophin comprises 24 spectrin-like repeats (Aartsma-Rus A et al, (2006), Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve, 34: 135-144). For example, a central rod shaped domain of a dystrophin as provided herein may comprise 5 to 23, 10 to 22 or 12 to 18 spectrin-like repeats as long as it can bind to actin and to DGC.

Decreasing the production of an aberrant dystrophin in said patient or in a cell of said patient may be assessed at the mRNA level and preferably means that 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or less of the initial amount of aberrant dystrophin mRNA, is still detectable by RT PCR. An aberrant dystrophin mRNA or protein is also referred to herein as a non-functional or less to non-functional or semi-functional dystrophin mRNA or protein. A non-functional pre-mRNA dystrophin is preferably leads to an out of frame dystrophin protein, which means that no dystrophin protein will be produced and/or detected. A non functional dystrophin protein is preferably a dystrophin protein which is not able to bind actin and/or

members of the DGC protein complex. A non-functional dystrophin protein or dystrophin mRNA does typically not have, or does not encode a dystrophin protein with an intact C-terminus of the protein.

[0022] Increasing the production of a functional dystrophin in a patient or in a cell of said patient may be assessed at the mRNA level (by RT-PCR analysis) and preferably means that a detectable amount of a functional or in frame dystrophin mRNA is detectable by RT PCR. In another embodiment, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the detectable dystrophin mRNA is a functional or in frame dystrophin mRNA.

Increasing the production of a functional dystrophin in a patient or in a cell of said patient may be assessed at the protein level (by immunofluorescence and western blot analyses) and preferably means that a detectable amount of a functional dystrophin protein is detectable by immunofluorescence or western blot analysis. In another embodiment, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the detectable dystrophin protein is a functional dystrophin protein.

[0023] An increase or a decrease is preferably assessed in a muscular tissue or in a muscular cell of an individual or a patient by comparison to the amount present in said individual or patient before treatment with said molecule or composition of the invention. Alternatively, the comparison can be made with a muscular tissue or cell of said individual or patient, which has not yet been treated with said oligonucleotide or composition in case the treatment is local.

[0024] In a further aspect, there is provided a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual or alleviate one or more characteristic(s) of a myogenic or muscle cell of said individual, the method comprising administering to said individual an oligonucleotide or a composition as defined herein.

[0025] There is further provided a method for enhancing, inducing or promoting skipping of an exon from a dystrophin pre-mRNA in a cell expressing said pre-mRNA in an individual suffering from Duchenne Muscular Dystrophy or Becker Muscular Dystrophy, the method comprising administering to said individual an oligonucleotide or a composition as defined herein.

Further provided is a method for increasing the production of a functional dystrophin protein and/or decreasing the production of an aberrant dystrophin protein in a cell, said cell comprising a pre-mRNA of a dystrophin gene encoding an aberrant dystrophin protein, the method comprising providing said cell with an oligonucleotide or composition of the invention and allowing translation of mRNA produced from splicing of said pre-mRNA. In one embodiment, said method is performed *in vivo*, for instance using a cell culture. Preferably, said method is *in vivo* in said individual.

[0026] In this context, increasing the production of a functional dystrophin protein has been defined herein.

[0027] Alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker

Muscular Dystrophy in an individual using a molecule or a composition of the invention may be assessed by any of the following assays: prolongation of time to loss of walking, improvement of muscle strength, improvement of the ability to lift weight, improvement of the time taken to rise from the floor, improvement in the nine-meter walking time, improvement in the time taken for four-stairs climbing, improvement of the leg function grade, improvement of the pulmonary function, improvement of cardiac function, improvement of the quality of life. Each of these assays is known to the skilled person. As an example, the publication of Manzur et al (Manzur AY et al, (2008), Glucocorticoid corticosteroids for Duchenne muscular dystrophy (review), Wiley publishers, The Cochrane collaboration.) gives an extensive explanation of each of these assays. For each of these assays, as soon as a detectable improvement or prolongation of a parameter measured in an assay has been found, it will preferably mean that one or more symptoms of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy has been alleviated in an individual using a molecule or composition of the invention. Detectable improvement or prolongation is preferably a statistically significant improvement or prolongation as described in Hodgetts et al (Hodgetts S., et al, (2006), Neuromuscular Disorders, 16: 591-602).

[0028] Alternatively, the alleviation of one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy may be assessed by measuring an improvement of a characteristic of a muscle fiber relating to its function, integrity and/or survival, said characteristic being assessed on the patient self. Such characteristics may be assessed at the cellular, tissue level of a given patient. An alleviation of one or more characteristics may be assessed by any of the following assays on a myogenic cell or muscle cell from a patient: reduced calcium uptake by muscle cells, decreased collagen synthesis, altered morphology, altered lipid biosynthesis, decreased oxidative stress, and/or improved muscle fiber function, integrity, and/or survival. These parameters are usually assessed using immunofluorescence and/or histochemical analyses of cross sections of muscle biopsies.

[0029] An oligonucleotide as used herein preferably comprises an antisense oligonucleotide or antisense oligoribonucleotide. In a preferred embodiment an exon skipping technique is applied. Exon skipping interferes with the natural splicing processes occurring within a eukaryotic cell. In higher eukaryotes the genetic information for proteins in the DNA of the cell is encoded in exons which are separated from each other by intronic sequences. These introns are in some cases very long. The transcription machinery of eukaryotes generates a pre-mRNA which contains both exons and introns, while the splicing machinery, often already during the production of the pre-mRNA, generates the actual coding region for the protein by splicing together the exons present in the pre-mRNA.

Exon-skipping results in mature mRNA that lacks at least one skipped exon. Thus, when said exon codes for amino acids, exon skipping leads to the expression of an altered product. Technology for exon-skipping is currently directed towards the use of antisense oligonucleotides (AONs). Much of this work is done in the *mdx* mouse model for Duchenne muscular dystrophy. The *mdx* mouse carries a nonsense mutation in exon 23. Despite the *mdx* mutation, which should preclude the synthesis of a functional dystrophin protein, rare, naturally occurring dystrophin positive fibers have been observed in *mdx* muscle tissue. These

dystrophin-positive fibers are thought to have arisen from an apparently naturally occurring exon-skipping mechanism, either due to somatic mutations or through alternative splicing. AONs directed to, respectively, the 3' and/or 5' splice sites of introns 22 and 23 in dystrophin pre-mRNA, have been shown to interfere with factors normally involved in removal of intron 23 so that also exon 23 was removed from the mRNA (Alter J, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med* 2006;12(2):175-7, Lu QL, et al. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med* 2003;6:6, Lu QL, et al. Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. *Proc Natl Acad Sci U S A* 2005;102(1):198-203, Mann CJ, et al, Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J Gene Med* 2002;4(6):644-54 or Graham IR, et al, Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligoribonucleotides (splcomers): target sequence optimisation using oligonucleotide arrays. *J Gene Med* 2004;6(10):1149-58).

[0030] By the targeted skipping of a specific exon, a DMD phenotype is converted into a milder BMD phenotype. The skipping of an exon is preferably induced by the binding of AONs targeting exon-internal sequences. An oligonucleotide directed toward an exon internal sequence typically exhibits no overlap with non-exon sequences. It preferably does not overlap with the splice sites at least not insofar, as these are present in the intron. An oligonucleotide directed toward an exon internal sequence preferably does not contain a sequence complementary to an adjacent intron. Further provided is thus an oligonucleotide according to the invention, wherein said oligonucleotide, or a functional equivalent thereof, is for inhibiting inclusion of an exon of a dystrophin pre-mRNA into mRNA produced from splicing of said pre-mRNA. An exon skipping technique is preferably applied such that the absence of an exon from mRNA produced from dystrophin pre-mRNA generates a coding region for a more functional - albeit shorter - dystrophin protein. In this context, inhibiting inclusion of an exon preferably means that the detection of the original, aberrant dystrophin mRNA and/or protein is decreased as earlier defined herein.

[0031] Within the context of the invention, a functional equivalent of an oligonucleotide preferably means an oligonucleotide as defined herein wherein one or more nucleotides have been substituted and wherein an activity of said functional equivalent is retained to at least some extent. Preferably, an activity of said functional equivalent is providing a functional dystrophin protein. Said activity of said functional equivalent is therefore preferably assessed by quantifying the amount of a functional dystrophin protein or by quantifying the amount of a functional dystrophin mRNA. A functional dystrophin protein (or a functional dystrophin mRNA) is herein preferably defined as being a dystrophin protein (or a dystrophin protein encoded by said mRNA) able to bind actin and members of the DGC protein. The assessment of said activity of an oligonucleotide is preferably done by RT-PCR (m-RNA) or by immunofluorescence or Western blot analyses (protein). Said activity is preferably retained to at least some extent when it represents at least 50%, or at least 60%, or at least 70% or at least 80% or at least 90% or at least 95% or more of corresponding activity of said

oligonucleotide the functional equivalent derives from. Such activity may be measured in a muscular tissue or in a muscular cell of an individual or in vitro in a cell by comparison to an activity of a corresponding oligonucleotide of said oligonucleotide the functional equivalent derives from. Throughout this application, when the word oligonucleotide is used it may be replaced by a functional equivalent thereof as defined herein.

[0032] In a preferred embodiment, an oligonucleotide of the invention, which comprises a sequence that binds and/or is complementary to a sequence of exon 44 of dystrophin pre-mRNAs as earlier defined herein is such that the complementary part is at least 50% of the length of the oligonucleotide of the invention, more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90% or even more preferably at least 95%, or even more preferably 98% or even more preferably at least 99%, or even more preferably 100%. In a most preferred embodiment, an oligonucleotide of the invention consists of a sequence that is complementary to part of dystrophin pre-mRNA as defined herein. As an example, an oligonucleotide may comprise a sequence that is complementary to part of dystrophin pre-mRNA as defined herein and additional flanking sequences. In a more preferred embodiment, the length of said complementary part of said oligonucleotide is of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides. Preferably, additional flanking sequences are used to modify the binding of a protein to the oligonucleotide, or to modify a thermodynamic property of the oligonucleotide, more preferably to modify target RNA binding affinity.

[0033] It is thus not absolutely required that all the bases in the region of complementarity are capable of pairing with bases in the opposing strand. For instance, when designing the oligonucleotide one may want to incorporate for instance a residue that does not base pair with the base on the complementary strand. Mismatches may, to some extent, be allowed, if under the circumstances in the cell, the stretch of nucleotides is sufficiently capable of hybridising to the complementary part. In this context, "sufficiently" preferably means that using a gel mobility shift assay as described in example 1 of EP 1 619 249, binding of an oligonucleotide is detectable. Optionally, said oligonucleotide may further be tested by transfection into muscle cells of patients. Skipping of the targeted exon may be assessed by RT-PCR (as described in EP 1 619 249). The complementary regions are preferably designed such that, when combined, they are specific for the exon in the pre-mRNA. Such specificity may be created with various lengths of complementary regions as this depends on the actual sequences in other (pre-)mRNA in the system. The risk that also one or more other pre-mRNA will be able to hybridise to the oligonucleotide decreases with increasing size of the oligonucleotide. It is clear that oligonucleotides comprising mismatches in the region of complementarity but that retain the capacity to hybridise and/or bind to the targeted region(s) in the pre-mRNA, can be used in the present invention. However, preferably at least the complementary parts do not comprise such mismatches as these typically have a higher efficiency and a higher specificity, than oligonucleotides having such mismatches in one or more complementary regions. It is thought, that higher hybridisation strengths, (i.e. increasing number of interactions with the opposing

strand) are favourable in increasing the efficiency of the process of interfering with the splicing machinery of the system. Preferably, the complementarity is between 90 and 100%. In general this allows for 1 or 2 mismatch(es) in an oligonucleotide of 20 nucleotides or 1, 2, 3 or 4 mismatches in an oligonucleotide of 40 nucleotides, or 1, 2, 3, 4, 5 or 6 mismatches in an oligonucleotide of 60 nucleotides.

[0034] A preferred molecule of the invention comprises or consists of a nucleotide-based sequence that is antisense to a sequence selected from exon 44 of the DMD pre-mRNA. The sequence of the DMD pre-mRNA is preferably selected from SEQ ID NO 1: 5'-GUGGCUAACAGAAGCU; SEQ ID NO 2: 5'-GGGAACAUGCUAAAUAC, SEQ ID NO 3: 5'-AGACACAAAUUCCUGAGA, and SEQ ID NO 4: 5'-CUGUUGAGAAA.

[0035] A molecule of the invention is preferably an isolated molecule.

[0036] A molecule of the invention is preferably a nucleic acid molecule or a nucleotide-based molecule or an oligonucleotide or an antisense oligonucleotide which binds and/or is complementary to a sequence of exon 44 selected from SEQ ID NO:1, 2, 3 or 4.

[0037] A preferred molecule of the invention comprises or consists of from about 8 to about 60 nucleotides, more preferred from about 10 to about 50 nucleotides, more preferred from about 17 to about 40 nucleotides, more preferred from about 18 to about 30 nucleotides, more preferred from about 18 to about 24 nucleotides, most preferred about 20 nucleotides, such as 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides or 23 nucleotides.

[0038] A preferred molecule of the invention comprises or consists of from 8 to 60 nucleotides, more preferred from 10 to 50 nucleotides, more preferred from 17 to 40 nucleotides, more preferred from 18 to 30 nucleotides, more preferred from 21 to 60, more preferred from 22 to 55, more preferred from 23 to 53, more preferred from 24 to 50, more preferred from 25 to 45, more preferred from 26 to 43, more preferred from 27 to 41, more preferred from 28 to 40, more preferred from 29 to 40, more preferred from 18 to 24 nucleotides, or preferably comprises or consists of 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0039] In certain embodiments, the invention provides a molecule comprising or consisting of an antisense nucleotide sequence selected from the antisense nucleotide sequences depicted in Table 1A.

A molecule or nucleic acid molecule of the invention that binds and/or is complementary and/or is antisense to a nucleotide having nucleotide sequence: SEQ ID NO 1: 5'-GUGGCUAACAGAAGCU preferably comprises or consists of the antisense nucleotide sequence of SEQ ID NO 5; SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 21, SEQ ID

NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34, SEQ ID NO 35, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:49 or SEQ ID NO: 54. A preferred molecule that targets this region of the DMD pre-mRNA comprises or consists of the antisense nucleotide sequence of SEQ ID NO:5, SEQ ID NO 49, or SEQ ID NO 54. Most preferred oligonucleotide comprises or consists of the antisense nucleotide sequence of SEQ ID NO:5.

[0040] In a more preferred embodiment, the invention provides a molecule comprising or consisting of the antisense nucleotide sequence SEQ ID NO 5: 5'-UCAGCUUCUGUUAGCCACUG. It was found that this molecule is very efficient in modulating splicing of exon 44 of the DMD gene in muscle cells. This preferred molecule of the invention comprising SEQ ID NO:5 comprises from 21 to 60, more preferred from 22 to 55, more preferred from 23 to 53, more preferred from 24 to 50, more preferred from 25 to 45, more preferred from 26 to 43, more preferred from 27 to 41, more preferred from 28 to 40, more preferred from 29 to 40, or preferably comprises or consists of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0041] In another preferred embodiment, the disclosure provides a molecule comprising or consisting of the antisense nucleotide sequence SEQ ID NO 49 or 54. These preferred molecules of the invention comprising either SEQ ID NO:49 or SEQ ID NO:54 further comprise from 18 to 60, more preferred from 18 to 55, more preferred from 20 to 53, more preferred from 24 to 50, more preferred from 25 to 45, more preferred from 26 to 43, more preferred from 27 to 41, more preferred from 28 to 40, more preferred from 29 to 40, or preferably comprises or consists of 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0042] In a further embodiment, a molecule of the disclosure that is antisense to SEQ ID NO 2: 5'-GGGAACAUGCUAAAUAC preferably comprises or consists of the antisense nucleotide sequence of SEQ ID NO 43 or SEQ ID NO 44. These preferred molecules of the disclosure comprising either SEQ ID NO: 43 or SEQ ID NO:44, further comprise from 17 to 60 nucleotides, more preferred from 18 to 30 nucleotides, more preferred from 21 to 60, more preferred from 22 to 55, more preferred from 23 to 53, more preferred from 24 to 50, more preferred from 25 to 45, more preferred from 26 to 43, more preferred from 27 to 41, more preferred from 28 to 40, more preferred from 29 to 40, more preferred from 18 to 24 nucleotides, or preferably comprises or consists of 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0043] In yet a further embodiment, a molecule of the disclosure that is antisense to SEQ ID NO 3: 5'-AGACACAAAUCCUGAGA preferably comprises or consists of the antisense nucleotide sequence of SEQ ID NO 47 or SEQ ID NO 48. These preferred molecules of the

disclosure comprising either SEQ ID NO:47 or SEQ ID NO:48 further comprise from 17 to 60 nucleotides, more preferred from 18 to 30 nucleotides, more preferred from 17 to 60, more preferred from 22 to 55, more preferred from 23 to 53, more preferred from 24 to 50, more preferred from 25 to 45, more preferred from 26 to 43, more preferred from 27 to 41, more preferred from 28 to 40, more preferred from 29 to 40, more preferred from 18 to 24 nucleotides, or preferably comprises or consists of 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0044] In still a further embodiment, a molecule of the disclosure that is antisense to SEQ ID NO 4: 5'-CUGUUGAGAAA preferably comprises or consists of the antisense nucleotide sequence of SEQ ID NO 45 or SEQ ID NO 46. These preferred molecules of the disclosure comprising either SEQ ID NO:45 or SEQ ID NO:46 further comprise from 11 to 60 nucleotides, more preferred from 11 to 30 nucleotides, more preferred from 11 to 60, or preferably comprises or consists of 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides.

[0045] A nucleotide sequence of a molecule of the invention may contain RNA residues, or one or more DNA residues, and/or one or more nucleotide analogues or equivalents, as will be further detailed herein below.

[0046] It is preferred that a molecule of the invention comprises one or more residues that are modified to increase nuclease resistance, and/or to increase the affinity of the antisense nucleotide for the target sequence. Therefore, in a preferred embodiment, the antisense nucleotide sequence comprises at least one nucleotide analogue or equivalent, wherein a nucleotide analogue or equivalent is defined as a residue having a modified base, and/or a modified backbone, and/or a non-natural internucleoside linkage, or a combination of these modifications.

[0047] In a preferred embodiment, the nucleotide analogue or equivalent comprises a modified backbone. Examples of such backbones are provided by morpholino backbones, carbamate backbones, siloxane backbones, sulfide, sulfoxide and sulfone backbones, formacetyl and thioformacetyl backbones, methyleneformacetyl backbones, riboacetyl backbones, alkene containing backbones, sulfamate, sulfonate and sulfonamide backbones, methyleneimino and methylenehydrazino backbones, and amide backbones. Phosphorodiamidate morpholino oligomers are modified backbone oligonucleotides that have previously been investigated as antisense agents. Morpholino oligonucleotides have an uncharged backbone in which the deoxyribose sugar of DNA is replaced by a six membered ring and the phosphodiester linkage is replaced by a phosphorodiamidate linkage. Morpholino oligonucleotides are resistant to enzymatic degradation and appear to function as antisense agents by arresting translation or interfering with pre-mRNA splicing rather than by activating RNase H. Morpholino oligonucleotides have been successfully delivered to tissue culture cells by methods that physically disrupt the cell membrane, and one study comparing several of

these methods found that scrape loading was the most efficient method of delivery; however, because the morpholino backbone is uncharged, cationic lipids are not effective mediators of morpholino oligonucleotide uptake in cells. A recent report demonstrated triplex formation by a morpholino oligonucleotide and, because of the non-ionic backbone, these studies showed that the morpholino oligonucleotide was capable of triplex formation in the absence of magnesium.

[0048] It is further preferred that the linkage between the residues in a backbone do not include a phosphorus atom, such as a linkage that is formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages.

[0049] Peptide Nucleic Acid (PNA)-based molecules (Nielsen, et al. (1991) Science 254, 1497-1500) are true mimics of DNA molecules in terms of base-pair recognition. The backbone of the PNA is composed of N-(2-aminoethyl)-glycine units linked by peptide bonds, wherein the nucleobases are linked to the backbone by methylene carbonyl bonds. An alternative backbone comprises a one-carbon extended pyrrolidine PNA monomer (Govindaraju and Kumar (2005) Chem. Commun, 495-497). Since the backbone of a PNA molecule contains no charged phosphate groups, PNA-RNA hybrids are usually more stable than RNA-RNA or RNA-DNA hybrids, respectively (Egholm et al (1993) Nature 365, 566-568).

[0050] A further preferred backbone comprises a morpholino nucleotide analog or equivalent, in which the ribose or deoxyribose sugar is replaced by a 6-membered morpholino ring.

[0051] In yet a further embodiment, a nucleotide analogue or equivalent of the invention comprises a substitution of one of the non-bridging oxygens in the phosphodiester linkage. This modification slightly destabilizes base-pairing but adds significant resistance to nuclease degradation. A preferred nucleotide analogue or equivalent comprises phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, H-phosphonate, methyl and other alkyl phosphonate including 3'-alkylene phosphonate, 5'-alkylene phosphonate and chiral phosphonate, phosphinate, phosphoramidate including 3'-amino phosphoramidate and aminoalkylphosphoramidate, thionophosphoramidate, thionoalkylphosphonate, thionoalkylphosphotriester, selenophosphate or boranophosphate.

[0052] A further preferred nucleotide analogue or equivalent of the invention comprises one or more sugar moieties that are mono- or disubstituted at the 2', 3' and/or 5' position such as a -OH; -F; substituted or unsubstituted, linear or branched lower (C1-C10) alkyl, alkenyl, alkynyl, alkaryl, allyl, or aralkyl, that may be interrupted by one or more heteroatoms; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; O-, S-, or N-allyl; O-alkyl-O-alkyl, -methoxy, -aminopropoxy; methoxyethoxy; - dimethylaminoethoxy; and -dimethylaminoethoxyethoxy. The sugar moiety can be a pyranose or derivative thereof, or a deoxypyranose or derivative thereof, preferably ribose or derivative thereof, or deoxyribose or derivative of. A preferred derivatized sugar moiety is a Locked Nucleic Acid (LNA), in which the 2'-carbon atom is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. A preferred LNA comprises 2'-O,4'-C-ethylene-bridged nucleic acid (Morita et al. 2001. Nucleic

Acid Res Supplement No. 1: 241-242). These substitutions render the nucleotide analogue or equivalent RNase H and nuclease resistant and increase the affinity for the target RNA.

[0053] In another embodiment, a nucleotide analogue or equivalent of the invention comprises one or more base modifications or substitutions. Modified bases comprise synthetic and natural bases such as inosine, xanthine, hypoxanthine and other -aza, deaza, -hydroxy, -halo, -thio, thiol, -alkyl, -alkenyl, -alkynyl, thioalkyl derivatives of pyrimidine and purine bases that are or will be known in the art.

[0054] It is understood by a skilled person that it is not necessary for all positions in an antisense oligonucleotide to be modified uniformly. In addition, more than one of the aforementioned analogues or equivalents may be incorporated in a single antisense oligonucleotide or even at a single position within an antisense oligonucleotide. In certain embodiments, an antisense oligonucleotide of the invention has at least two different types of analogues or equivalents.

[0055] A preferred antisense oligonucleotide according to the invention comprises a 2'-O alkyl phosphorothioate antisense oligonucleotide, such as 2'-O-methyl modified ribose (RNA), 2'-O-ethyl modified ribose, 2'-O-propyl modified ribose, and/or substituted derivatives of these modifications such as halogenated derivatives.

[0056] A most preferred antisense oligonucleotide according to the invention comprises a 2'-O-methyl phosphorothioate ribose.

[0057] It will also be understood by a skilled person that different antisense oligonucleotides can be combined for efficiently skipping of exon 44. In a preferred embodiment, a combination of at least two antisense oligonucleotides are used in a method of the invention, such as two different antisense oligonucleotides, three different antisense oligonucleotides, four different antisense oligonucleotides, or five different antisense oligonucleotides.

[0058] An antisense oligonucleotide can be linked to a moiety that enhances uptake of the antisense oligonucleotide in cells, preferably myogenic cells or muscle cells. Examples of such moieties are cholesterol, carbohydrates, vitamins, biotin, lipids, phospholipids, cell-penetrating peptides including but not limited to antennapedia, TAT, transportan and positively charged amino acids such as oligoarginine, poly-arginine, oligolysine or polylysine, antigen-binding domains such as provided by an antibody, a Fab fragment of an antibody, or a single chain antigen binding domain such as a cameloid single domain antigen-binding domain.

[0059] A preferred antisense oligonucleotide comprises a peptide-linked phosphorodiamidate morpholino oligomer (PMO).

[0060] An oligonucleotide of the invention may be indirectly administered using suitable means known in the art. As part of the disclosure, an oligonucleotide may for example be provided to an individual or a cell, tissue or organ of said individual in the form of an expression

vector wherein the expression vector encodes a transcript comprising said oligonucleotide. The expression vector is preferably introduced into a cell, tissue, organ or individual via a gene delivery vehicle. In a preferred embodiment, there is provided a viral-based expression vector comprising an expression cassette or a transcription cassette that drives expression or transcription of a molecule as identified herein. A cell can be provided with a molecule capable of interfering with essential sequences that result in highly efficient skipping of exon 44 by plasmid-derived antisense oligonucleotide expression or viral expression provided by adenovirus- or adeno-associated virus-based vectors. Expression is preferably driven by a polymerase III promoter, such as a U1, a U6, or a U7 RNA promoter. A preferred delivery vehicle is a viral vector such as an adeno-associated virus vector (AAV), or a retroviral vector such as a lentivirus vector (Goyenvallé A, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 2004;306(5702):1796-9, De Angelis FG, et al. Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. *Proc Natl Acad Sci U S A* 2002;99(14):9456-61 or Denti MA, et al. Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice. *Hum Gene Ther* 2006;17(5):565-74) and the like. Also, plasmids, artificial chromosomes, plasmids usable for targeted homologous recombination and integration in the human genome of cells may be suitably applied for delivery of an oligonucleotide as defined herein. Preferred for the current disclosure are those vectors wherein transcription is driven from PolIII promoters, and/or wherein transcripts are in the form fusions with U1 or U7 transcripts, which yield good results for delivering small transcripts. It is within the skill of the artisan to design suitable transcripts. Preferred are PolIII driven transcripts. Preferably, in the form of a fusion transcript with an U1 or U7 transcript (see the same Goyenvallé A et al, De Angelis FG et al or Denti MA et al). Such fusions may be generated as described (Gorman L, et al, Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. *Proc Natl Acad Sci U S A* 1998;95(9):4929-34 or Suter D, et al, Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations. *Hum Mol Genet* 1999;8(13):2415-23).

The oligonucleotide may be delivered as is. However, the oligonucleotide may also be encoded by the viral vector. Typically, this is in the form of an RNA transcript that comprises the sequence of the oligonucleotide in a part of the transcript.

[0061] One preferred antisense oligonucleotide expression system is an adenovirus associated virus (AAV)-based vector. Single chain and double chain AAV-based vectors have been developed that can be used for prolonged expression of small antisense nucleotide sequences for highly efficient skipping of exon 44 of DMD.

[0062] A preferred AAV-based vector comprises an expression cassette that is driven by a polymerase III-promoter (Pol III). A preferred Pol III promoter is, for example, a U1, a U6, or a U7 RNA promoter.

[0063] The disclosure therefore also provides a viral-based vector, comprising a Pol III-

promoter driven expression cassette for expression of an antisense oligonucleotide of the invention for inducing skipping of exon 44 of the DMD gene.

[0064] Improvements in means for providing an individual or a cell, tissue, organ of said individual with an oligonucleotide and/or an equivalent thereof, are anticipated considering the progress that has already thus far been achieved. Such future improvements may of course be incorporated to achieve the mentioned effect on restructuring of mRNA using a method of the disclosure. An oligonucleotide and/or an equivalent thereof can be delivered as is to an individual, a cell, tissue or organ of said individual. When administering an oligonucleotide and/or an equivalent thereof, it is preferred that an oligonucleotide and/or an equivalent thereof is dissolved in a solution that is compatible with the delivery method. As part of the disclosure, muscle or myogenic cells can be provided with a plasmid for antisense oligonucleotide expression by providing the plasmid in an aqueous solution. Alternatively, a plasmid can be provided by transfection using known transfection agentia. For intravenous, subcutaneous, intramuscular, intrathecal and/or intraventricular administration it is preferred that the solution is a physiological salt solution. Particularly preferred in the invention is the use of an excipient or transfection agentia that will aid in delivery of each of the constituents as defined herein to a cell and/or into a cell, preferably a muscle cell. Preferred are excipients or transfection agentia capable of forming complexes, nanoparticles, micelles, vesicles and/or liposomes that deliver each constituent as defined herein, complexed or trapped in a vesicle or liposome through a cell membrane. Many of these excipients are known in the art. Suitable excipients or transfection agentia comprise polyethylenimine (PEI; ExGen500 (MBI Fermentas)), LipofectAMINE™ 2000 (Invitrogen) or derivatives thereof, or similar cationic polymers, including polypropyleneimine or polyethylenimine copolymers (PECs) and derivatives, synthetic amphiphils (SAINT-18), lipofectin™, DOTAP and/or viral capsid proteins that are capable of self assembly into particles that can deliver each constituent as defined herein to a cell, preferably a muscle cell. Such excipients have been shown to efficiently deliver an oligonucleotide such as antisense nucleic acids to a wide variety of cultured cells, including muscle cells. Their high transfection potential is combined with an excepted low to moderate toxicity in terms of overall cell survival. The ease of structural modification can be used to allow further modifications and the analysis of their further (*in vivo*) nucleic acid transfer characteristics and toxicity.

[0065] Lipofectin represents an example of a liposomal transfection agent. It consists of two lipid components, a cationic lipid N-[1-(2,3 dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (cp. DOTAP which is the methylsulfate salt) and a neutral lipid dioleoylphosphatidylethanolamine (DOPE). The neutral component mediates the intracellular release. Another group of delivery systems are polymeric nanoparticles.

[0066] Polycations such like diethylaminoethylaminoethyl (DEAE)-dextran, which are well known as DNA transfection reagent can be combined with butylcyanoacrylate (BCA) and hexylcyanoacrylate (PHCA) to formulate cationic nanoparticles that can deliver each constituent as defined herein, preferably an oligonucleotide across cell membranes into cells.

[0067] In addition to these common nanoparticle materials, the cationic peptide protamine offers an alternative approach to formulate an oligonucleotide with colloids. This colloidal nanoparticle system can form so called proticles, which can be prepared by a simple self-assembly process to package and mediate intracellular release of an oligonucleotide. The skilled person may select and adapt any of the above or other commercially available alternative excipients and delivery systems to package and deliver an oligonucleotide for use in the current invention to deliver it for the treatment of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in humans.

[0068] In addition, an oligonucleotide could be covalently or non-covalently linked to a targeting ligand specifically designed to facilitate the uptake in to the cell, cytoplasm and/or its nucleus. Such ligand could comprise (i) a compound (including but not limited to peptide(-like) structures) recognising cell, tissue or organ specific elements facilitating cellular uptake and/or (ii) a chemical compound able to facilitate the uptake in to cells and/or the intracellular release of an oligonucleotide from vesicles, e.g. endosomes or lysosomes.

[0069] Therefore, in a preferred embodiment, an oligonucleotide is formulated in a composition or a medicament or a composition, which is provided with at least an excipient and/or a targeting ligand for delivery and/or a delivery device thereof to a cell and/or enhancing its intracellular delivery. Accordingly, the invention also encompasses a pharmaceutically acceptable composition comprising an oligonucleotide and further comprising at least one excipient and/or a targeting ligand for delivery and/or a delivery device of said oligonucleotide to a cell and/or enhancing its intracellular delivery.

It is to be understood that if a composition comprises an additional constituent such as an adjunct compound as later defined herein, each constituent of the composition may not be formulated in one single combination or composition or preparation. Depending on their identity, the skilled person will know which type of formulation is the most appropriate for each constituent as defined herein. In a preferred embodiment, the invention provides a composition or a preparation which is in the form of a kit of parts comprising an oligonucleotide and a further adjunct compound as later defined herein.

[0070] A preferred oligonucleotide is for preventing or treating Duchenne Muscular Dystrophy (DMD) or Becker Muscular Dystrophy (BMD) in an individual. An individual, which may be treated using an oligonucleotide of the invention may already have been diagnosed as having a DMD or a BMD. Alternatively, an individual which may be treated using an oligonucleotide of the invention may not have yet been diagnosed as having a DMD or a BMD but may be an individual having an increased risk of developing a DMD or a BMD in the future given his or her genetic background. A preferred individual is a human being.

[0071] If required, a molecule or a vector expressing an antisense oligonucleotide of the invention can be incorporated into a pharmaceutically active mixture by adding a pharmaceutically acceptable carrier.

[0072] Therefore, the invention also provides a pharmaceutical composition comprising a

molecule comprising an antisense oligonucleotide according to the invention, or a viral-based vector expressing the antisense oligonucleotide according to the invention.

[0073] In a further aspect, there is provided a composition comprising an oligonucleotide as defined herein. Preferably, said composition comprises at least two distinct oligonucleotides as defined herein. More preferably, these two distinct oligonucleotides are designed to skip one or two or more exons. Multi-skipping is encompassed by the present invention, wherein an oligonucleotide of the invention inducing the skipping of exon 44 is used in combination with another oligonucleotide inducing the skipping of another exon. In this context, another exon may be exon 43, 45 or 52. Multi exon skipping has been already disclosed in EP 1 619 249. The DMD gene is a large gene, with many different exons. Considering that the gene is located on the X-chromosome, it is mostly boys that are affected, although girls can also be affected by the disease, as they may receive a bad copy of the gene from both parents, or are suffering from a particularly biased inactivation of the functional allele due to a particularly biased X chromosome inactivation in their muscle cells. The protein is encoded by a plurality of exons (79) over a range of at least 2.4 Mb. Defects may occur in any part of the DMD gene. Skipping of a particular exon or particular exons can, very often, result in a restructured mRNA that encodes a shorter than normal but at least partially functional dystrophin protein. A practical problem in the development of a medicament based on exon-skipping technology is the plurality of mutations that may result in a deficiency in functional dystrophin protein in the cell. Despite the fact that already multiple different mutations can be corrected for by the skipping of a single exon, this plurality of mutations, requires the generation of a series of different pharmaceuticals as for different mutations different exons need to be skipped. An advantage of an oligonucleotide or of a composition comprising at least two distinct oligonucleotide as later defined herein capable of inducing skipping of two or more exons, is that more than one exon can be skipped with a single pharmaceutical. This property is not only practically very useful in that only a limited number of pharmaceuticals need to be generated for treating many different DMD or particular, severe BMD mutations. Another option now open to the person skilled in the art is to select particularly functional restructured dystrophin proteins and produce compounds capable of generating these preferred dystrophin proteins. Such preferred end results are further referred to as mild phenotype dystrophins.

[0074] In a preferred embodiment, said composition being preferably a pharmaceutical composition said pharmaceutical composition comprising a pharmaceutically acceptable carrier, adjuvant, diluent and/or excipient. Such a pharmaceutical composition may comprise any pharmaceutically acceptable carrier, filler, preservative, adjuvant, solubilizer, diluent and/or excipient is also provided. Such pharmaceutically acceptable carrier, filler, preservative, adjuvant, solubilizer, diluent and/or excipient may for instance be found in Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, MD: Lippincott Williams & Wilkins, 2000. Each feature of said composition has earlier been defined herein.

If several oligonucleotides are used, concentration or dose already defined herein may refer to the total concentration or dose of all oligonucleotides used or the concentration or dose of each oligonucleotide used or added. Therefore in one embodiment, there is provided a composition wherein each or the total amount of oligonucleotide used is dosed in an amount

ranged between 0.5 mg/kg and 10 mg/kg.

[0075] The invention further provides the use of an antisense oligonucleotide according to the invention, or a viral-based vector that expresses an antisense oligonucleotide according to the invention, for modulating splicing of the DMD mRNA. The splicing is preferably modulated in human myogenic cells or muscle cells *in vitro*. More preferred is that splicing is modulated in human myogenic cells or muscle cells *in vivo*.

[0076] A preferred antisense oligonucleotide comprising one or more nucleotide analogs or equivalents of the invention modulates splicing in one or more muscle cells, including heart muscle cells, upon systemic delivery. In this respect, systemic delivery of an antisense oligonucleotide comprising a specific nucleotide analog or equivalent might result in targeting a subset of muscle cells, while an antisense oligonucleotide comprising a distinct nucleotide analog or equivalent might result in targeting of a different subset of muscle cells. Therefore, in one embodiment it is preferred to use a combination of antisense oligonucleotides comprising different nucleotide analogs or equivalents for modulating skipping of exon 44 of the DMD mRNA.

[0077] The invention furthermore provides the use of an antisense oligonucleotide according to the invention, or of a viral-based vector expressing the antisense oligonucleotide according to the invention, for the preparation of a medicament for the treatment of a DMD or BMD patient.

Therefore in a further aspect, there is provided the use of a oligonucleotide or of a composition as defined herein for the manufacture of a medicament for preventing or treating Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual. Each feature of said use has earlier been defined herein.

A treatment in a use or in a method according to the invention is at least one week, at least one month, at least several months, at least one year, at least 2, 3, 4, 5, 6 years or more. Each molecule or oligonucleotide or equivalent thereof as defined herein for use according to the invention may be suitable for direct administration to a cell, tissue and/or an organ *in vivo* of individuals affected by or at risk of developing DMD or BMD, and may be administered directly *in vivo*, *ex vivo* or *in vitro*. The frequency of administration of an oligonucleotide, composition, compound or adjunct compound of the invention may depend on several parameters such as the age of the patient, the mutation of the patient, the number of molecules (i.e. dose), the formulation of said molecule. The frequency may be ranged between at least once in two weeks, or three weeks or four weeks or five weeks or a longer time period.

[0078] Dose ranges of oligonucleotide according to the invention are preferably designed on the basis of rising dose studies in clinical trials (*in vivo* use) for which rigorous protocol requirements exist. A molecule or an oligonucleotide as defined herein may be used at a dose which is ranged between 0.1 and 20 mg/kg, preferably 0.5 and 10 mg/kg.

In a preferred embodiment, a concentration of an oligonucleotide as defined herein, which is ranged between 0.1 nM and 1 μ M is used. Preferably, this range is for *in vitro* use in a cellular model such as muscular cells or muscular tissue. More preferably, the concentration used is

ranged between 0.3 to 400 nM, even more preferably between 1 to 200 nM. If several oligonucleotides are used, this concentration or dose may refer to the total concentration or dose of oligonucleotides or the concentration or dose of each oligonucleotide added. The ranges of concentration or dose of oligonucleotide(s) as given above are preferred concentrations or doses for *in vitro* or *ex vivo* uses. The skilled person will understand that depending on the oligonucleotide(s) used, the target cell to be treated, the gene target and its expression levels, the medium used and the transfection and incubation conditions, the concentration or dose of oligonucleotide(s) used may further vary and may need to be optimised any further.

An oligonucleotide as defined herein for use according to the invention may be suitable for administration to a cell, tissue and/or an organ *in vivo* of individuals affected by or at risk of developing DMD or BMD, and may be administered *in vivo*, *ex vivo* or *in vitro*. Said oligonucleotide may be directly or indirectly administered to a cell, tissue and/or an organ *in vivo* of an individual affected by or at risk of developing DMD or BMD, and may be administered directly or indirectly *in vivo*, *ex vivo* or *in vitro*. As Duchenne and Becker muscular dystrophy have a pronounced phenotype in muscle cells, it is preferred that said cells are muscle cells, it is further preferred that said tissue is a muscular tissue and/or it is further preferred that said organ comprises or consists of a muscular tissue. A preferred organ is the heart. Preferably, said cells comprise a gene encoding a mutant dystrophin protein. Preferably, said cells are cells of an individual suffering from DMD or BMD.

[0079] Unless otherwise indicated each embodiment as described herein may be combined with another embodiment as described herein.

[0080] In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a compound or adjunct compound as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the invention.

[0081] In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

[0082] The word "approximately" or "about" when used in association with a numerical value (approximately 10, about 10) preferably means that the value may be the given value of 10 more or less 1% of the value.

[0083] The expression "in vivo" as used herein may mean in a cellular system which may be isolated from the organism the cells derive from. Preferred cells are muscle cells. In vivo may also mean in a tissue or in a multicellular organism which is preferably a patient as defined herein. Throughout the invention, in vivo is opposed to in vitro which is generally associated

with a cell free system.

[0084] Each embodiment as identified herein may be combined together unless otherwise indicated.

[0085] The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention.

Figure legends

[0086]

Figure 1. Evaluation of AONs designed to induce the skipping of exon 44 from the DMD gene in transfected muscle cells from healthy control or a DMD patient with an exon 45 deletion.

1. **(A)** In differentiated muscle cells (myotubes) from a patient with an exon 45 deletion, all tested (transfected) AONs induced exon 44 skipping at a concentration of 150 nM, with PS188 (SEQ ID NO:5), PS190 (previously published as h44AON2; Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71), PS191 (SEQ ID NO: 47), PS193 (SEQ ID NO: 48), PS194 (SEQ ID NO: 46), and PS196 (SEQ ID NO: 51) demonstrating highest efficiencies (between 84% and 94%).
2. **(B)** The majority of AONs was also tested by transfection into healthy human control cells at 150 and 400 nM concentrations. The results are summarized in this column chart. PS188 (SEQ ID NO:5), PS190, PS191 (SEQ ID NO: 47), PS193 (SEQ ID NO: 48), PS194 (SEQ ID NO: 46), and PS196 (SEQ ID NO: 51) were confirmed to be most efficient in inducing exon 44 skipping. Note that the exon 44 skipping levels in patient cells are typically higher than in control cells as a result of the fact that, in contrast to healthy cells, in patient cells exon 44 skipping is frame-restoring and giving rise to a more functional and stable. No exon 44 skipping was observed in non-transfected muscle cells in all experiments (data not shown).
3. **(C)** Examples of PS197 (SEQ ID NO 52) and three additional AONs, PS199 (SEQ ID NO 44), PS200 (SEQ ID NO 49), and PS201 (SEQ ID NO 50), similarly tested in control muscle cells, at transfection concentrations 150 nM and 400 nM. The exon 44 skipping percentages varied between 1% (PS199) and 44% (PS200). M: DNA size marker (100 bp ladder).

Figure 2. Further evaluation of PS188 (SEQ ID NO:5) by transfection of human control muscle cells or peripheral blood mononuclear cells (PB-MNCs).

1. **(A)** Dose-response experiment. In human control muscle cells, PS188 showed increasing levels of exon 44 skipping at transfection doses increasing from 50 nM to 400 nM (*in triplo*), up to 45% at 400 nM.
2. **(B)** PB-MNCs of a healthy individual were transfected with 200 nM PS188. Despite the fact that dystrophin is only expressed at low levels in this type of cells, exon 44 skipping

was clearly observed. These results confirm the efficiency of PS188 in inducing exon 44 skipping from the DMD gene. M: DNA size marker.

Figure 3. Further evaluation of PS188 (SEQ ID NO:5) by administration to transgenic hDMD mice expressing the full length human DMD gene, and to cynomolgus monkeys included in extensive toxicity studies.

1. **(A)** Following intramuscular injection of 2x40 µg PS188 into both gastrocnemius muscles (G1 and G2) of an hDMD mouse, exon 44 skipping was observed, albeit at low levels. This confirms the capacity of PS188 to induce human exon 44 skipping in muscle tissue *in vivo*. The low levels were expected given the fact that this mouse model has healthy muscle fibers typically showing lower levels of AON uptake when compared to dystrophic muscle fibers. NT: in non-treated hDMD muscle no exon 44 skipping was observed. M: DNA size marker
2. **(B)** In monkeys included in toxicity studies on PS188, exon 44 skipping was observed in peripheral blood mononuclear cells (PB-MNCs) after 1-hour intravenous infusions every fourth day for 29 days at a dose-level of 6mg/kg PS188. No exon 44 skipping was observed in non-treated monkeys (NT). M: DNA size marker.

Examples

Example 1

Material and Methods

[0087] AON design was based on (partly) overlapping open secondary structures of the target exon RNA as predicted by the m-fold program (Mathews et al., J Mol Biol 1999; 288(5): 911-40), on (partly) overlapping putative SR-protein binding sites as predicted by the ESE-finder software (rulai.cshl.edu/tools/ESE/) (Cartegni et al., Nucleic Acids Res 2003; 31(13): 3568-71), and on avoiding G-stretches of 3 or more nucleotides or CpG pairs. AONs (see Table 1) were synthesized by Eurogentec (Belgium) and Prosensa Therapeutics BV (Leiden, Netherlands), and contain 2'-O-methyl RNA and full-length phosphorothioate backbones.

Tissue culturing, transfection and RT-PCR analysis

[0088] Myotube cultures derived from a healthy individual ("human control") or a DMD patient with an exon 45 deletion were processed as described previously (Aartsma-Rus et al. Hum Mol Genet 2003; 12(8): 907-14; Havenga et al. J Virol 2002; 76(9): 4612-20). For the screening of

AONs, myotube cultures were transfected with 150 and/or 400 nM of each AON. Transfection reagent polyethylenimine (PEI, ExGen500 MBI Fermentas) or a derivative (UNIFectylin, Prosensa Therapeutics BV, Netherlands) was used, with 2 µl ExGen500 or UNIFectylin per µg AON. A control AON with a fluorescein label was used to confirm optimal transfection efficiencies (typically over 90% fluorescent nuclei were obtained). RNA was isolated 24 to 48 hours after transfection as described (Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71). Exon skipping efficiencies were determined by nested RT-PCR analysis using primers in the exons flanking exon 44 (Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71). PCR fragments were isolated from agarose gels (using the QIAquick Gel Extraction Kit (QIAGEN) for sequence verification (by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), and ABI 3700 Sequencer (PE Applied Biosystems). For quantification, the PCR products were analyzed using the DNA 1000 LabChips Kit on the Agilent 2100 bioanalyzer (Agilent Technologies, USA).

Results

[0089] A series of AONs targeting sequences within exon 44 were designed and tested both in healthy control and patient-derived myotube cultures, by transfection and subsequent RT-PCR and sequence analysis of isolated RNA. In myotubes derived from a DMD patient with a deletion of exon 45, specific exon 44 skipping was induced at 150 nM for every AON (PS187 to PS201) tested, with PS188 (SEQ ID NO:5), PS190 (previously published as h44AON2, Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71), PS191 (SEQ ID NO: 47), PS193 (SEQ ID NO: 48), PS194 (SEQ ID NO: 46), and PS196 (SEQ ID NO: 51) demonstrating highest levels of skipping (between 84% and 94% at 150 nM) (Fig.1A).

[0090] Similar transfection experiments were done in control cells from a healthy individual. Percentages of exon 44 skipping were assessed and compared to those in the patient cell cultures (Fig. 1B). Inherent to nonsense-mediated RNA decay of the control transcript after exon 44 skipping, the control percentages were typically lower than those in the patient cells (see for instance results with PS197 in fig. 1A (patient cells) vs Fig. 1C (control cells)).

[0091] Three additional AONs (PS199 (SEQ ID NO 44), PS200 (SEQ ID NO 49), and PS201 (SEQ ID NO 50) were tested in control muscle cells, at concentrations of 150 nM and 400 nM. The exon 44 skipping percentages varied between 1% (PS199) and 44% (PS200) (Fig. 1C). Based on all transfection experiments, the AONs PS187, PS188, PS190, PS191, PS192, PS193, PS194, PS196 and PS200 were considered most efficient, and AONs PS189, PS197, PS198, PS199, and PS201 least efficient.

[0092] PS188 (SEQ ID NO 5) was further tested in dose-response experiments in healthy human control muscle cells, applying increasing doses from 50 to 400 nM *in triplo*. Increasing levels of exon 44 skipping were accordingly observed, up to 45% at 400 nMPS188 (Figure 2A).

Example 2

Materials and methods

[0093] A fresh healthy human control blood sample, collected in an EDTA tube, was layered on top of a HistoPaque gradient. Upon centrifugation, the second layer (of the four layers, from top to bottom) with the mononuclear cells was collected, washed, and centrifuged again. The cell pellet was resuspended in proliferation culturing medium and counted. In a 6-wells plate, 8×10^6 cells per well were plated and incubated at 37°C, 5% CO₂ for 3 hrs. The cells were then transfected with 0 or 200 nM PS188 (SEQ ID NO:5; 2'OMePS RNA; Prosensa Therapeutics BV), *in duplo*, per dish. RNA was isolated 72 hrs after transfection, and analysed by RT-PCR analysis using DMD-gene specific primers flanking exon 44 (Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71). Sequence analysis (by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), and ABI 3700 Sequencer (PE Applied Biosystems) was performed on isolated PCR products (using the QIAquick Gel Extraction Kit (QIAGEN) to confirm the specific exon 44 skipping on RNA level.

Results

[0094] In transfected peripheral blood mononuclear cells (PB-MNCs) from a healthy control individual, PS188 induced the production of a novel shorter transcript fragment when applied at 200 nM (Fig. 2B). This fragment was isolated and sequenced and confirmed due to the specific skipping of exon 44. In non-transfected PB-MNCs no exon 44 skipping was observed. These results indicate that PS188 is an efficient compound inducing human exon 44 skipping *in vitro*.

Example 3

Materials and methods

Antisense oligoribonucleotides (AONs).

[0095] Normal and mdx mice (Sicinski et al. (1989). Science 244: 1578-1580) were injected with the mouse-specific m46AON4 (van Deutekom et al. (2001) Hum Mol Genet 10: 1547-1554), whereas the hDMD mice with the human-specific PS196 (SEQ ID NO 51) or PS188 (SEQ ID NO 5). Both AONs contained a full-length phosphorothioate backbone and 2'-O-methyl modified ribose molecules (PS196: Eurogentec, Belgium; PS188: Prosensa

Therapeutics BV).

Normal, mdx and transgenic hDMD mice

[0096] Normal mice (C57Bl/6NCrL) and mdx mice (C57Bl/10ScSn-mdx/J) were obtained from Charles River Laboratories (The Netherlands). Transgenic hDMD mice were engineered in our own LUMC laboratories. Briefly, embryonic stem (ES) cells were genetically modified through fusions with yeast spheroplasts carrying a YAC of 2.7 Mb that contained the full-length (2.4 Mb) human DMD gene. This YAC was previously reconstructed by homologous recombination of smaller overlapping YACs in yeast (Den Dunnen et al. (1992). Hum Mol Genet 1: 19-28). ES-cells showing integration of one copy of the full-size YAC, as assessed by PFGE mapping, exon-PCR analysis across the entire gene, and metaphase FISH analysis, were then used to generate homozygous hDMD mice ('t Hoen et al., J. Biol. Chem. 2008). Transgenic hDMD mice do not appear to be physically affected by the genetic modification. Appropriate expression of the human DMD gene could be demonstrated in muscle, both at RNA and protein level. The engineering of these mice was authorised by the Dutch Ministry of Agriculture (LNV); project nr. WA/BD01.284 (E21).

Administration of AONs.

[0097] The experiments on intramuscular AON-injections in mice were authorised by the animal experimental commission (UDEEC) of the Medical Faculty of the Leiden University (project no. 00095, 03027). AONs were injected, either pure, or complexed to the cationic polymer polyethylenimine (PEI; ExGen 500 (20x), MBI Fermentas) at ratios of 1 ml PEI per nmol AON in a 5% w/v glucose solution, or to 15 nmol SAINT-18TM (Synvolux Therapeutics B.V., The Netherlands), according to the manufacturers' instructions. The SAINT-18TM delivery system is based on a cationic pyridinium head group and allows nontoxic delivery of antisense oligonucleotides. Mice were anaesthetised by intraperitoneal injection of a 1:1 (v/v) Hypnorm/Dormicum solution (Janssen Pharmaceutica, Belgium/Roche, The Netherlands). Pure AON (PS188) was administered in a final injection volume of 40 µl by intramuscular injection into both gastrocnemius muscles of the mice using a Hamilton syringe with a 22-Gauge needle. The mice received two injections of 40 µg at a 24 h interval. They were sacrificed at different time-points post-injection; for PS188-injected hDMD mice ten days after the last injection. Muscles were isolated and frozen in liquid nitrogen-cooled 2-methylbutane.

RT-PCR analysis.

[0098] Muscle samples were homogenized in RNA-Bee solution (Campro Scientific, The Netherlands). Total RNA was isolated and purified according to the manufacturer's instructions. For cDNA synthesis with the reverse transcriptase C. therm polymerase or Transcriptor (Roche

Diagnostics, The Netherlands), 300 ng of RNA was used in a 20 µl reaction at 60°C for 30 min, reverse primed with either mouse- or human-specific primers. First PCRs were performed with outer primer sets (flanking exons 43-45 for PS188-injected mice), for 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). One µl of this reaction (diluted 1:10) was then re-amplified using nested primer combinations in the exons directly flanking the target exon (exon 44 for PS188-injected mice), with 30 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). PCR products were analysed on 2% agarose gels. Skipping efficiencies were determined by quantification of PCR products using the DNA 1000 LabChip® Kit and the Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands). Primer sets and sequences were described previously (Aartsma-Rus et al. (2002) *Neuromuscul Disord* 12 Suppl: S71.8,17; van Deutekom et al. (2001) *Hum Mol Genet* 10: 1547-1554).

Sequence analysis.

[0099] RT-PCR products were isolated from 2% agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). Direct DNA sequencing was carried out by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), and analyzed on an ABI 3700 Sequencer (PE Applied Biosystems).

MALDI-TOF mass-spectrometry.

[0100] RNA-Bee muscle homogenates were purified using a nucleic acid purification kit (Nucleic Acid Purification Kit for Sequazyme™ Pinpoint SNP Kit, Applied Biosystems) with 96 well spin plates (Applied Biosystems) following the manufacturer's instructions. Matrix solution (50 mg/ml 3-hydroxy picolinic acid and 25 mM dibasic ammonium citrate in 50% acetonitrile) was applied in 1 ml aliquots to an AnchorChip™ sample target (Bruker Daltonics, Germany) and air-dried. Samples were spotted in 0.5 ml aliquots onto the matrix crystals and air-dried. Mass determinations were performed on a Reflex III MALDI-TOF mass-spectrometer (Bruker Daltonics, Germany). Spectra were acquired in reflector mode and accumulated for approximately 900 laser shots. Samples of labelled and unlabelled m46AON4 were analyzed for comparison.

Results

Exon Skipping in Wild-type Muscle

[0101] We first set up targeted exon skipping in mouse muscle in vivo and optimised different parameters of administration. Initial experiments were performed in wild type mice, and, while nonsense-mediated RNA decay will cause underestimation of the exon skipping efficiencies,

the effect of the AONs was monitored on mRNA level only. We injected increasing dosages from 0.9 nmol to 5.4 nmol of each antisense oligonucleotide. RT-PCR analysis of total muscle RNA demonstrated the occurrence of a novel shorter transcript fragment in all samples injected. Sequence analysis confirmed the precise skipping of exon 44 in this product (data not shown).

[0102] Cross-sections of the contra-lateral injected muscles were analysed for dispersion and persistence of a fluorescein-labelled control AON. Following injection of pure AON, we observed fluorescent signals within some fibres for up to one week. At later time points only weak signals were observed, and mainly within the interstitial spaces. The use of PEI clearly enhanced both dispersion and persistence of the fluorescent signal, even after 3 weeks. However, it also induced fibre degeneration and monocyte infiltration absorbing most fluorescence. Using SAINT, most of the signal was detected in the interstitial spaces for up to one week, indicating that this reagent did not efficiently deliver the AON into the muscle fibres. Since the fluorescent signal may not correspond to the presence of intact and functional AONs, we performed MALDI-TOF mass-spectrometry of injected muscle samples. The analyses indicated that the fluorescent label was removed from the AON within 24 hours. The labelled AON was only detectable for up to two weeks when using PEI. The interstitial AONs were probably more vulnerable to degradation than the intracellular AONs. The unlabelled AON was observed for three to four weeks post-injection in all three series, but it may only be functional when present intracellularly, i.e. in the PEI series.

Human-specific Exon Skipping in hDMD Muscle

[0103] Since the exon skipping strategy is a sequence-specific therapeutic approach, the ideal pre-clinical validation would be a target human DMD gene, in a mouse experimental background. We have engineered such transgenic, "humanised" DMD (hDMD) mice carrying an integrated and functional copy of the full-length human DMD gene. Expression of human dystrophin in hDMD mouse muscle was specifically detected by immunohistochemical analysis of cross-sections, using a human-specific antibody (MANDYS106). On muscle RNA level, RT-PCR analyses using either mouse- or human-specific primers demonstrated correct transcription of the human DMD gene. Furthermore, upon crossing with mdx mice, the hDMD construct showed to complement the dystrophic defect, as was assessed by histological and cDNA microarray analysis ('t Hoen et al., J. Biol. Chem. 2008). hDMD mice have healthy muscle fibers typically exhibiting a limited uptake of naked AONs. We injected the human-specific AON PS196 (SEQ ID NO 51) complexed to PEI, or PS188 (SEQ ID NO 5) without PEI, into the gastrocnemius muscles of the hDMD mice (2x40 µg injections within 24 hrs). At 7 to 10 days post-injection we clearly observed the skipping of the targeted exon 44 from the human DMD transcript (Fig. 3A). Although the human-specific AONs are highly homologous to the corresponding mouse sequences, with only 2 or 3 mismatches in the respective 20-mers, the mouse endogenous transcripts were not affected to any detectable level. PS188 induced exon 44 skipping, as confirmed by sequence analysis. No exon 44 skipping was observed in non-treated hDMD muscle. These results indicate that PS188 is an efficient compound inducing

human exon 44 skipping in muscle tissue.

Example 4

Material and Methods

[0104] As part of an extensive toxicity program for PS188, non-fasted cynomolgus monkeys were treated by 1-hour intravenous infusion (5 mL/kg/ h) every fourth day for 29 days at the dose-level of 6mg/kg PS188 (SEQ ID NO 5; 2'OMePS RNA; Agilent Life Sciences, USA). The PS188 formulations were freshly prepared on each treatment day (on test days 1, 5, 9, 13, 17, 21, 25 and 29) shortly before initiation of the administration (as soon as possible before, at the most within one hour before start of administration). Formulations were prepared by dissolving PS188 in phosphate buffer; the purity and water content were taken into account as provided in the Certificate of Analysis of the drug substance. The amount of PS188 was adjusted to each animal's current body weight. The animals were sacrificed 96 hours after the last administration (day 33). Whole blood samples (10 ml) were collected in EDTA tubes, and (after overnight shipment at room temperature) layered on top of a HistoPaque gradient. Upon centrifugation, the second layer (of the four layers, from top to bottom) with the mononuclear cells was collected, washed, and centrifuged again. RNA was isolated from the resulting cell pellet and analysed by RT-PCR analysis using DMD-gene specific primers flanking exon 44 (Aartsma-Rus et al. Neuromuscul Disord 2002; 12 Suppl: S71). Sequence analysis (by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), and ABI 3700 Sequencer (PE Applied Biosystems) was performed on isolated PCR products (using the QIAquick Gel Extraction Kit (QIAGEN) to confirm the specific exon 44 skipping on RNA level.

Results

[0105] In monkeys treated by 1-hour intravenous infusions every fourth day for 29 days at the dose-level of 6mg/kg PS188, exon 44 skipping was observed in peripheral blood mononuclear cells (Fig. 3B), despite the fact that these cells express only low levels of dystrophin. The human and monkey DMD sequence targeted by PS188 is in fact 100% identical. No exon 44 skipping was observed in non-treated monkeys. These results indicate that PS188 is an efficient compound inducing exon 44 skipping *in vivo*.

[0106] Table 1 Antisense oligonucleotide sequences.

Table 1A

1 (PS188)	UCAGCUUCUGUUAGCCACUG	SEQ ID NO 5
2	UUCAGCUUCUGUUAGCCACU	SEQ ID NO 6

3	UUCAGCUUCUGUUAGCCACUG	SEQ ID NO 7
4	UCAGCUUCUGUUAGCCACUGA	SEQ ID NO 8
5	UUCAGCUUCUGUUAGCCACUGA	SEQ ID NO 9
6	UCAGCUUCUGUUAGCCACUGA	SEQ ID NO 10
7	UUCAGCUUCUGUUAGCCACUGA	SEQ ID NO 11
8	UCAGCUUCUGUUAGCCACUGAU	SEQ ID NO 12
9	UUCAGCUUCUGUUAGCCACUGAU	SEQ ID NO 13
10	UCAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 14
11	UUCAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 15
12	UCAGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 16
13	UUCAGCUUCUGUUAGCCACUGAUA	SEQ ID NO 17
14	UCAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 18
15	UUCAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 19
16	UCAGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 20
17	UUCAGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 21
18	CAGCUUCUGUUAGCCACUG	SEQ ID NO 22
19	CAGCUUCUGUUAGCCACUGAU	SEQ ID NO 23
20	AGCUUCUGUUAGCCACUGAUU	SEQ ID NO 24
21	CAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 25
22	AGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 26
23	CAGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 27
24	AGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 28
25	CAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 29
26	AGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 30
27	CAGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 31
28	AGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 32
29	AGCUUCUGUUAGCCACUGAU	SEQ ID NO 33
30	GCUUCUGUUAGCCACUGAUU	SEQ ID NO 34
31	AGCUUCUGUUAGCCACUGAUU	SEQ ID NO 35
32	GCUUCUGUUAGCCACUGAUUA	SEQ ID NO 36
33	AGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 37
34	GCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 38
35	AGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 39
36	GCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 40
37	AGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 41
38	GCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 42

39 (PS 192)	CCAUUUGUAUUUAGCAUGUUCCC	SEQ ID NO 43
40 (PS 199)	AGAUACCAUUUGUAUUUAGC	SEQ ID NO 44
41 (PS 187)	GCCAUUUCUCAACAGAUCU	SEQ ID NO 45
42 (PS 194)	GCCAUUUCUCAACAGAUCUGUCA	SEQ ID NO 46
43 (PS 191)	AUUCUCAGGAAUUUGUGUCUUUC	SEQ ID NO 47
44 (PS 193)	UCUCAGGAAUUUGUGUCUUUC	SEQ ID NO 48
45 (PS 200)	GUUCAGCUUCUGUUAGCC	SEQ ID NO 49
46 (PS 201)	CUGAUUAAAUAUCUUUAU C	SEQ ID NO 50

Table 1B

47 (PS196)	GCCGCCAUUUCUCAACAG	SEQ ID NO 51
48 (PS 197)	GUAUUUAGCAUGUUCCCA	SEQ ID NO 52
49 (PS 198)	CAGGAUUUGUGUCUUUC	SEQ ID NO 53
50 (PS189)	UCUGUUAGCCACUGAUUAAU	SEQ ID NO 54

SEQ ID NO:55 homo sapiens DMD amino acid sequence

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SEQUENCE LISTING

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Academisch Ziekenhuis Leiden

Platenburg, Gerardus J

De Kimpe, Josephus J

van Deutekom, Judith CT

van Ommen, Garrit-Jan B

Aartsma-Rus, Annemieke

<120> Method for efficient exon (44) skipping in Duchenne Muscular Dystrophy and associate means

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<141> 2009-05-14

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Lys	Leu	Ser	Ile	Pro	Gln	Leu	Ser	Val	Thr	Asp	Tyr	Glu	Ile	Met	Glu				
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Gln	Arg	Leu	Gly	Glu	Leu	Gln	Ala	Leu	Gln	Ser	Ser	Leu	Gln	Glu	Gln				
							980					985					990		
Gln	Ser	Gly	Leu	Tyr	Tyr	Leu	Ser	Thr	Thr	Val	Lys	Glu	Met	Ser	Lys				
							995					1000					1005		
Lys	Ala	Pro	Ser	Glu	Ile	Ser	Arg	Lys	Tyr	Gln	Ser	Glu	Phe	Glu					
						1010					1015					1020			
Glu	Ile	Glu	Gly	Arg	Trp	Lys	Lys	Leu	Ser	Ser	Gln	Leu	Val	Glu					
							1025					1030					1035		
His	Cys	Gln	Lys	Leu	Glu	Glu	Gln	Met	Asn	Lys	Leu	Arg	Lys	Ile					
							1040					1045					1050		
Gln	Asn	His	Ile	Gln	Thr	Leu	Lys	Lys	Trp	Met	Ala	Glu	Val	Asp					
							1055					1060					1065		
Val	Phe	Leu	Lys	Glu	Glu	Trp	Pro	Ala	Leu	Gly	Asp	Ser	Glu	Ile					
							1070					1075					1080		
Leu	Lys	Lys	Gln	Leu	Lys	Gln	Cys	Arg	Leu	Leu	Val	Ser	Asp	Ile					
							1085					1090					1095		
Gln	Thr	Ile	Gln	Pro	Ser	Leu	Asn	Ser	Val	Asn	Glu	Gly	Gly	Gln					
							1100					1105					1110		
Lys	Ile	Lys	Asn	Glu	Ala	Glu	Pro	Glu	Phe	Ala	Ser	Arg	Leu	Glu					
							1115					1120					1125		
Thr	Gln	Leu	Leu	Gln	Leu	Asn	Thr	Gln	Thr	Asn	His	Met	Ser	Gln					
							1130					1135					1140		

Thr	Glu	Leu	Lys	Glu	Leu	Asn	Thr	Gln	Trp	Asp	His	Met	Cys	Gln
1130						1135					1140			
Gln	Val	Tyr	Ala	Arg	Lys	Glu	Ala	Leu	Lys	Gly	Gly	Leu	Glu	Lys
1145						1150					1155			
Thr	Val	Ser	Leu	Gln	Lys	Asp	Leu	Ser	Glu	Met	His	Glu	Trp	Met
1160						1165					1170			
Thr	Gln	Ala	Glu	Glu	Glu	Tyr	Leu	Glu	Arg	Asp	Phe	Glu	Tyr	Lys
1175						1180					1185			
Thr	Pro	Asp	Glu	Leu	Gln	Lys	Ala	Val	Glu	Glu	Met	Lys	Arg	Ala
1190						1195					1200			
Lys	Glu	Glu	Ala	Gln	Gln	Lys	Glu	Ala	Lys	Val	Lys	Leu	Leu	Thr
1205						1210					1215			
Glu	Ser	Val	Asn	Ser	Val	Ile	Ala	Gln	Ala	Pro	Pro	Val	Ala	Gln
1220						1225					1230			
Glu	Ala	Leu	Lys	Lys	Glu	Leu	Glu	Thr	Leu	Thr	Thr	Asn	Tyr	Gln
1235						1240					1245			
Trp	Leu	Cys	Thr	Arg	Leu	Asn	Gly	Lys	Cys	Lys	Thr	Leu	Glu	Glu
1250						1255					1260			
Val	Trp	Ala	Cys	Trp	His	Glu	Leu	Leu	Ser	Tyr	Leu	Glu	Lys	Ala
1265						1270					1275			
Asn	Lys	Trp	Leu	Asn	Glu	Val	Glu	Phe	Lys	Leu	Lys	Thr	Thr	Glu
1280						1285					1290			
Asn	Ile	Pro	Gly	Gly	Ala	Glu	Glu	Ile	Ser	Glu	Val	Leu	Asp	Ser
1295						1300					1305			
Leu	Glu	Asn	Leu	Met	Arg	His	Ser	Glu	Asp	Asn	Pro	Asn	Gln	Ile
1310						1315					1320			
Arg	Ile	Leu	Ala	Gln	Thr	Leu	Thr	Asp	Gly	Gly	Val	Met	Asp	Glu
1325						1330					1335			
Leu	Ile	Asn	Glu	Glu	Leu	Glu	Thr	Phe	Asn	Ser	Arg	Trp	Arg	Glu
1340						1345					1350			
Leu	His	Glu	Glu	Ala	Val	Arg	Arg	Gln	Lys	Leu	Leu	Glu	Gln	Ser
1355						1360					1365			
Ile	Gln	Ser	Ala	Gln	Glu	Thr	Glu	Lys	Ser	Leu	His	Leu	Ile	Gln
1370						1375					1380			
Glu	Ser	Leu	Thr	Phe	Ile	Asp	Lys	Gln	Leu	Ala	Ala	Tyr	Ile	Ala
1385						1390					1395			
Asp	Lys	Val	Asp	Ala	Ala	Gln	Met	Pro	Gln	Glu	Ala	Gln	Lys	Ile
1400						1405					1410			

1400		1405		1410
Gln Ser Asp Leu Thr Ser His	Glu Ile Ser Leu Glu	Glu Met Lys		
1415	1420	1425		
Lys His Asn Gln Gly Lys Glu	Ala Ala Gln Arg Val	Leu Ser Gln		
1430	1435	1440		
Ile Asp Val Ala Gln Lys Lys	Leu Gln Asp Val Ser	Met Lys Phe		
1445	1450	1455		
Arg Leu Phe Gln Lys Pro Ala	Asn Phe Glu Gln Arg	Leu Gln Glu		
1460	1465	1470		
Ser Lys Met Ile Leu Asp Glu	Val Lys Met His Leu	Pro Ala Leu		
1475	1480	1485		
Glu Thr Lys Ser Val Glu Gln	Glu Val Val Gln Ser	Gln Leu Asn		
1490	1495	1500		
His Cys Val Asn Leu Tyr Lys	Ser Leu Ser Glu Val	Lys Ser Glu		
1505	1510	1515		
Val Glu Met Val Ile Lys Thr	Gly Arg Gln Ile Val	Gln Lys Lys		
1520	1525	1530		
Gln Thr Glu Asn Pro Lys Glu	Leu Asp Glu Arg Val	Thr Ala Leu		
1535	1540	1545		
Lys Leu His Tyr Asn Glu Leu	Gly Ala Lys Val Thr	Glu Arg Lys		
1550	1555	1560		
Gln Gln Leu Glu Lys Cys Leu	Lys Leu Ser Arg Lys	Met Arg Lys		
1565	1570	1575		
Glu Met Asn Val Leu Thr Glu	Trp Leu Ala Ala Thr	Asp Met Glu		
1580	1585	1590		
Leu Thr Lys Arg Ser Ala Val	Glu Gly Met Pro Ser	Asn Leu Asp		
1595	1600	1605		
Ser Glu Val Ala Trp Gly Lys	Ala Thr Gln Lys Glu	Ile Glu Lys		
1610	1615	1620		
Gln Lys Val His Leu Lys Ser	Ile Thr Glu Val Gly	Glu Ala Leu		
1625	1630	1635		
Lys Thr Val Leu Gly Lys Lys	Glu Thr Leu Val Glu	Asp Lys Leu		
1640	1645	1650		
Ser Leu Leu Asn Ser Asn Trp	Ile Ala Val Thr Ser	Arg Ala Glu		
1655	1660	1665		
Glu Trp Leu Asn Leu Leu Leu	Glu Tyr Gln Lys His	Met Glu Thr		
1670	1675	1680		
Phe Asp Gln Asn Val Asp His	Ile Thr Lys Trp Ile	Ile Gln Ala		
1685	1690	1695		

1685	1690	1695
Asp Thr Leu Leu Asp Glu Ser 1700	Glu Lys Lys Lys 1705	Pro Gln Gln Lys 1710
Glu Asp Val Leu Lys Arg Leu 1715	Lys Ala Glu Leu 1720	Asn Asp Ile Arg 1725
Pro Lys Val Asp Ser Thr Arg 1730	Asp Gln Ala Ala 1735	Asn Leu Met Ala 1740
Asn Arg Gly Asp His Cys Arg 1745	Lys Leu Val Glu 1750	Pro Gln Ile Ser 1755
Glu Leu Asn His Arg Phe Ala 1760	Ala Ile Ser His 1765	Arg Ile Lys Thr 1770
Gly Lys Ala Ser Ile Pro Leu 1775	Lys Glu Leu Glu 1780	Gln Phe Asn Ser 1785
Asp Ile Gln Lys Leu Leu Glu 1790	Pro Leu Glu Ala 1795	Glu Ile Gln Gln 1800
Gly Val Asn Leu Lys Glu Glu 1805	Asp Phe Asn Lys 1810	Asp Met Asn Glu 1815
Asp Asn Glu Gly Thr Val Lys 1820	Glu Leu Leu Gln 1825	Arg Gly Asp Asn 1830
Leu Gln Gln Arg Ile Thr Asp 1835	Glu Arg Lys Arg 1840	Glu Glu Ile Lys 1845
Ile Lys Gln Gln Leu Leu Gln 1850	Thr Lys His Asn 1855	Ala Leu Lys Asp 1860
Leu Arg Ser Gln Arg Arg Lys 1865	Lys Ala Leu Glu 1870	Ile Ser His Gln 1875
Trp Tyr Gln Tyr Lys Arg Gln 1880	Ala Asp Asp Leu 1885	Leu Lys Cys Leu 1890
Asp Asp Ile Glu Lys Lys Leu 1895	Ala Ser Leu Pro 1900	Glu Pro Arg Asp 1905
Glu Arg Lys Ile Lys Glu Ile 1910	Asp Arg Glu Leu 1915	Gln Lys Lys Lys 1920
Glu Glu Leu Asn Ala Val Arg 1925	Arg Gln Ala Glu 1930	Gly Leu Ser Glu 1935
Asp Gly Ala Ala Met Ala Val 1940	Glu Pro Thr Gln 1945	Ile Gln Leu Ser 1950
Lys Arg Trp Arg Glu Ile Glu 1955	Ser Lys Phe Ala 1960	Gln Phe Arg Arg 1965
Leu Asn Phe Ala Gln Ile His 1970	Thr Val Arg Glu 1975	Glu Thr Met Met 1980

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Val Met Thr Glu Asp Met Pro Leu Glu Ile Ser Tyr Val Pro Ser
1985 1990 1995

Thr Tyr Leu Thr Glu Ile Thr His Val Ser Gln Ala Leu Leu Glu
2000 2005 2010

Val Glu Gln Leu Leu Asn Ala Pro Asp Leu Cys Ala Lys Asp Phe
2015 2020 2025

Glu Asp Leu Phe Lys Gln Glu Glu Ser Leu Lys Asn Ile Lys Asp
2030 2035 2040

Ser Leu Gln Gln Ser Ser Gly Arg Ile Asp Ile Ile His Ser Lys
2045 2050 2055

Lys Thr Ala Ala Leu Gln Ser Ala Thr Pro Val Glu Arg Val Lys
2060 2065 2070

Leu Gln Glu Ala Leu Ser Gln Leu Asp Phe Gln Trp Glu Lys Val
2075 2080 2085

Asn Lys Met Tyr Lys Asp Arg Gln Gly Arg Phe Asp Arg Ser Val
2090 2095 2100

Glu Lys Trp Arg Arg Phe His Tyr Asp Ile Lys Ile Phe Asn Gln
2105 2110 2115

Trp Leu Thr Glu Ala Glu Gln Phe Leu Arg Lys Thr Gln Ile Pro
2120 2125 2130

Glu Asn Trp Glu His Ala Lys Tyr Lys Trp Tyr Leu Lys Glu Leu
2135 2140 2145

Gln Asp Gly Ile Gly Gln Arg Gln Thr Val Val Arg Thr Leu Asn
2150 2155 2160

Ala Thr Gly Glu Glu Ile Ile Gln Gln Ser Ser Lys Thr Asp Ala
2165 2170 2175

Ser Ile Leu Gln Glu Lys Leu Gly Ser Leu Asn Leu Arg Trp Gln
2180 2185 2190

Glu Val Cys Lys Gln Leu Ser Asp Arg Lys Lys Arg Leu Glu Glu
2195 2200 2205

Gln Lys Asn Ile Leu Ser Glu Phe Gln Arg Asp Leu Asn Glu Phe
2210 2215 2220

Val Leu Trp Leu Glu Glu Ala Asp Asn Ile Ala Ser Ile Pro Leu
2225 2230 2235

Glu Pro Gly Lys Glu Gln Gln Leu Lys Glu Lys Leu Glu Gln Val
2240 2245 2250

Lys Leu Leu Val Glu Glu Leu Pro Leu Arg Gln Gly Ile Leu Lys
2255 2260 2265

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2255	2260	2265
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2270	2275	2280
Ser Pro Glu Glu Gln Asp Lys	Leu Glu Asn Lys Leu	Lys Gln Thr
2285	2290	2295
Asn Leu Gln Trp Ile Lys Val	Ser Arg Ala Leu Pro	Glu Lys Gln
2300	2305	2310
Gly Glu Ile Glu Ala Gln Ile	Lys Asp Leu Gly Gln	Leu Glu Lys
2315	2320	2325
Lys Leu Glu Asp Leu Glu Glu	Gln Leu Asn His Leu	Leu Leu Trp
2330	2335	2340
Leu Ser Pro Ile Arg Asn Gln	Leu Glu Ile Tyr Asn	Gln Pro Asn
2345	2350	2355
Gln Glu Gly Pro Phe Asp Val	Gln Glu Thr Glu Ile	Ala Val Gln
2360	2365	2370
Ala Lys Gln Pro Asp Val Glu	Glu Ile Leu Ser Lys	Gly Gln His
2375	2380	2385
Leu Tyr Lys Glu Lys Pro Ala	Thr Gln Pro Val Lys	Arg Lys Leu
2390	2395	2400
Glu Asp Leu Ser Ser Glu Trp	Lys Ala Val Asn Arg	Leu Leu Gln
2405	2410	2415
Glu Leu Arg Ala Lys Gln Pro	Asp Leu Ala Pro Gly	Leu Thr Thr
2420	2425	2430
Ile Gly Ala Ser Pro Thr Gln	Thr Val Thr Leu Val	Thr Gln Pro
2435	2440	2445
Val Val Thr Lys Glu Thr Ala	Ile Ser Lys Leu Glu	Met Pro Ser
2450	2455	2460
Ser Leu Met Leu Glu Val Pro	Ala Leu Ala Asp Phe	Asn Arg Ala
2465	2470	2475
Trp Thr Glu Leu Thr Asp Trp	Leu Ser Leu Leu Asp	Gln Val Ile
2480	2485	2490
Lys Ser Gln Arg Val Met Val	Gly Asp Leu Glu Asp	Ile Asn Glu
2495	2500	2505
Met Ile Ile Lys Gln Lys Ala	Thr Met Gln Asp Leu	Glu Gln Arg
2510	2515	2520
Arg Pro Gln Leu Glu Glu Leu	Ile Thr Ala Ala Gln	Asn Leu Lys
2525	2530	2535

Asn Lys Thr Ser Asn Gln Glu	Ala Arg Thr Ile Ile Thr Asp Arg
2540	2545 2550
Ile Glu Arg Ile Gln Asn Gln	Trp Asp Glu Val Gln Glu His Leu
2555	2560 2565
Gln Asn Arg Arg Gln Gln Leu	Asn Glu Met Leu Lys Asp Ser Thr
2570	2575 2580
Gln Trp Leu Glu Ala Lys Glu	Glu Ala Glu Gln Val Leu Gly Gln
2585	2590 2595
Ala Arg Ala Lys Leu Glu Ser	Trp Lys Glu Gly Pro Tyr Thr Val
2600	2605 2610
Asp Ala Ile Gln Lys Lys Ile	Thr Glu Thr Lys Gln Leu Ala Lys
2615	2620 2625
Asp Leu Arg Gln Trp Gln Thr	Asn Val Asp Val Ala Asn Asp Leu
2630	2635 2640
Ala Leu Lys Leu Leu Arg Asp	Tyr Ser Ala Asp Asp Thr Arg Lys
2645	2650 2655
Val His Met Ile Thr Glu Asn	Ile Asn Ala Ser Trp Arg Ser Ile
2660	2665 2670
His Lys Arg Val Ser Glu Arg	Glu Ala Ala Leu Glu Glu Thr His
2675	2680 2685
Arg Leu Leu Gln Gln Phe Pro	Leu Asp Leu Glu Lys Phe Leu Ala
2690	2695 2700
Trp Leu Thr Glu Ala Glu Thr	Thr Ala Asn Val Leu Gln Asp Ala
2705	2710 2715
Thr Arg Lys Glu Arg Leu Leu	Glu Asp Ser Lys Gly Val Lys Glu
2720	2725 2730
Leu Met Lys Gln Trp Gln Asp	Leu Gln Gly Glu Ile Glu Ala His
2735	2740 2745
Thr Asp Val Tyr His Asn Leu	Asp Glu Asn Ser Gln Lys Ile Leu
2750	2755 2760
Arg Ser Leu Glu Gly Ser Asp	Asp Ala Val Leu Leu Gln Arg Arg
2765	2770 2775
Leu Asp Asn Met Asn Phe Lys	Trp Ser Glu Leu Arg Lys Lys Ser
2780	2785 2790
Leu Asn Ile Arg Ser His Leu	Glu Ala Ser Ser Asp Gln Trp Lys
2795	2800 2805
Arg Leu His Leu Ser Leu Gln	Glu Leu Leu Val Trp Leu Gln Leu
2810	2815 2820

Lys	Asp	Asp	Glu	Leu	Ser	Arg	Gln	Ala	Pro	Ile	Gly	Gly	Asp	Phe
2825						2830					2835			
Pro	Ala	Val	Gln	Lys	Gln	Asn	Asp	Val	His	Arg	Ala	Phe	Lys	Arg
2840						2845					2850			
Glu	Leu	Lys	Thr	Lys	Glu	Pro	Val	Ile	Met	Ser	Thr	Leu	Glu	Thr
2855						2860					2865			
Val	Arg	Ile	Phe	Leu	Thr	Glu	Gln	Pro	Leu	Glu	Gly	Leu	Glu	Lys
2870						2875					2880			
Leu	Tyr	Gln	Glu	Pro	Arg	Glu	Leu	Pro	Pro	Glu	Glu	Arg	Ala	Gln
2885						2890					2895			
Asn	Val	Thr	Arg	Leu	Leu	Arg	Lys	Gln	Ala	Glu	Glu	Val	Asn	Thr
2900						2905					2910			
Glu	Trp	Glu	Lys	Leu	Asn	Leu	His	Ser	Ala	Asp	Trp	Gln	Arg	Lys
2915						2920					2925			
Ile	Asp	Glu	Thr	Leu	Glu	Arg	Leu	Gln	Glu	Leu	Gln	Glu	Ala	Thr
2930						2935					2940			
Asp	Glu	Leu	Asp	Leu	Lys	Leu	Arg	Gln	Ala	Glu	Val	Ile	Lys	Gly
2945						2950					2955			
Ser	Trp	Gln	Pro	Val	Gly	Asp	Leu	Leu	Ile	Asp	Ser	Leu	Gln	Asp
2960						2965					2970			
His	Leu	Glu	Lys	Val	Lys	Ala	Leu	Arg	Gly	Glu	Ile	Ala	Pro	Leu
2975						2980					2985			
Lys	Glu	Asn	Val	Ser	His	Val	Asn	Asp	Leu	Ala	Arg	Gln	Leu	Thr
2990						2995					3000			
Thr	Leu	Gly	Ile	Gln	Leu	Ser	Pro	Tyr	Asn	Leu	Ser	Thr	Leu	Glu
3005						3010					3015			
Asp	Leu	Asn	Thr	Arg	Trp	Lys	Leu	Leu	Gln	Val	Ala	Val	Glu	Asp
3020						3025					3030			
Arg	Val	Arg	Gln	Leu	His	Glu	Ala	His	Arg	Asp	Phe	Gly	Pro	Ala
3035						3040					3045			
Ser	Gln	His	Phe	Leu	Ser	Thr	Ser	Val	Gln	Gly	Pro	Trp	Glu	Arg
3050						3055					3060			
Ala	Ile	Ser	Pro	Asn	Lys	Val	Pro	Tyr	Tyr	Ile	Asn	His	Glu	Thr
3065						3070					3075			
Gln	Thr	Thr	Cys	Trp	Asp	His	Pro	Lys	Met	Thr	Glu	Leu	Tyr	Gln
3080						3085					3090			
Ser	Leu	Ala	Asp	Leu	Asn	Asn	Val	Arg	Phe	Ser	Ala	Tyr	Arg	Thr
3095						3100					3105			

Ala Met Lys Leu Arg Arg Leu Gln Lys Ala Leu Cys Leu Asp Leu
3110 3115 3120

Leu Ser Leu Ser Ala Ala Cys Asp Ala Leu Asp Gln His Asn Leu
3125 3130 3135

Lys Gln Asn Asp Gln Pro Met Asp Ile Leu Gln Ile Ile Asn Cys
3140 3145 3150

Leu Thr Thr Ile Tyr Asp Arg Leu Glu Gln Glu His Asn Asn Leu
3155 3160 3165

Val Asn Val Pro Leu Cys Val Asp Met Cys Leu Asn Trp Leu Leu
3170 3175 3180

Asn Val Tyr Asp Thr Gly Arg Thr Gly Arg Ile Arg Val Leu Ser
3185 3190 3195

Phe Lys Thr Gly Ile Ile Ser Leu Cys Lys Ala His Leu Glu Asp
3200 3205 3210

Lys Tyr Arg Tyr Leu Phe Lys Gln Val Ala Ser Ser Thr Gly Phe
3215 3220 3225

Cys Asp Gln Arg Arg Leu Gly Leu Leu Leu His Asp Ser Ile Gln
3230 3235 3240

Ile Pro Arg Gln Leu Gly Glu Val Ala Ser Phe Gly Gly Ser Asn
3245 3250 3255

Ile Glu Pro Ser Val Arg Ser Cys Phe Gln Phe Ala Asn Asn Lys
3260 3265 3270

Pro Glu Ile Glu Ala Ala Leu Phe Leu Asp Trp Met Arg Leu Glu
3275 3280 3285

Pro Gln Ser Met Val Trp Leu Pro Val Leu His Arg Val Ala Ala
3290 3295 3300

Ala Glu Thr Ala Lys His Gln Ala Lys Cys Asn Ile Cys Lys Glu
3305 3310 3315

Cys Pro Ile Ile Gly Phe Arg Tyr Arg Ser Leu Lys His Phe Asn
3320 3325 3330

Tyr Asp Ile Cys Gln Ser Cys Phe Phe Ser Gly Arg Val Ala Lys
3335 3340 3345

Gly His Lys Met His Tyr Pro Met Val Glu Tyr Cys Thr Pro Thr
3350 3355 3360

Thr Ser Gly Glu Asp Val Arg Asp Phe Ala Lys Val Leu Lys Asn
3365 3370 3375

Ile Phe Arg Thr Lys Arg Thr Phe Ala Lys His Pro Arg Met Glu

Lys Phe Arg Thr Lys Arg Tyr Phe Ala Lys His Phe Arg Met Gly
 3380 3385 3390
 Tyr Leu Pro Val Gln Thr Val Leu Glu Gly Asp Asn Met Glu Thr
 3395 3400 3405
 Pro Val Thr Leu Ile Asn Phe Trp Pro Val Asp Ser Ala Pro Ala
 3410 3415 3420
 Ser Ser Pro Gln Leu Ser His Asp Asp Thr His Ser Arg Ile Glu
 3425 3430 3435
 His Tyr Ala Ser Arg Leu Ala Glu Met Glu Asn Ser Asn Gly Ser
 3440 3445 3450
 Tyr Leu Asn Asp Ser Ile Ser Pro Asn Glu Ser Ile Asp Asp Glu
 3455 3460 3465
 His Leu Leu Ile Gln His Tyr Cys Gln Ser Leu Asn Gln Asp Ser
 3470 3475 3480
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 3485 3490 3495
 Glu Ser Glu Glu Arg Gly Glu Leu Glu Arg Ile Leu Ala Asp Leu
 3500 3505 3510
 Glu Glu Glu Asn Arg Asn Leu Gln Ala Glu Tyr Asp Arg Leu Lys
 3515 3520 3525
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 3530 3535 3540
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 3545 3550 3555
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 3560 3565 3570
 Ala Arg Met Gln Ile Leu Glu Asp His Asn Lys Gln Leu Glu Ser
 3575 3580 3585
 Gln Leu His Arg Leu Arg Gln Leu Leu Glu Gln Pro Gln Ala Glu
 3590 3595 3600
 Ala Lys Val Asn Gly Thr Thr Val Ser Ser Pro Ser Thr Ser Leu
 3605 3610 3615
 Gln Arg Ser Asp Ser Ser Gln Pro Met Leu Leu Arg Val Val Gly
 3620 3625 3630
 Ser Gln Thr Ser Asp Ser Met Gly Glu Glu Asp Leu Leu Ser Pro
 3635 3640 3645
 Pro Gln Asp Thr Ser Thr Gly Leu Glu Glu Val Met Glu Gln Leu
 3650 3655 3660
 Asn Asn Ser Phe Pro Ser Ser Arg Glu Arg Asn Thr Pro Glu Lys

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Met Met Arg Glu Asp Thr Met
3665                               3670                               3675
Pro Met Arg Glu Asp Thr Met
3680                               3685

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- [NL0100697W](#) [0013]
- [US6875736B](#) [0013]
- [EP1619249A](#) [0013] [0033] [0033] [0073]
- [NL2009050258W](#) [0107]
- [EP08156193A](#) [0107]
- [US61128010B](#) [0107]

Non-patent literature cited in the description

- **VAN DEUTEKOMVAN OMMEN** Nat. Rev. Genet., 2003, vol. 4, 10774-83 [0004]
- **AARTSMA-RUSVAN OMMEN** RNA, 2007, vol. 13, 101609-24 [0004]
- **FRISENDARBY** Nature Structural Biology, 1998, vol. 5, 543-546 [0013]
- **DORNKIPPENBERGER** Curr Opin Mol Ther, 2008, vol. 10, 110-20 [0015]
- **CHENGVAN DYKE** Gene, 1997, vol. 197, 1-2253-60 [0016]
- **AARTSMA-RUS A et al.** Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule Muscle Nerve, 2006, vol. 34, 135-144 [0021] [0021] [0021] [0021]
- **MANZUR AY et al.** Glucocorticoid corticosteroids for Duchenne muscular dystrophy (review) Wiley publishers 20080000 [0027]
- **HODGETTS S. et al.** Neuromuscular Disorders, 2006, vol. 16, 591-602 [0027]
- **ALTER et al.** Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology Nat Med, 2006, vol. 12, 2175-

7 [0029]

- **LU QL et al.** Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse *Nat Med*, 2003, vol. 6, 6- [0029]
- **LU QL et al.** Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles *Proc Natl Acad Sci U S A*, 2005, vol. 102, 1198-203 [0029]
- **MANN CJ et al.** Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy *J Gene Med*, 2002, vol. 4, 6644-54 [0029]
- **GRAHAM IR et al.** Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligoribonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays *J Gene Med*, 2004, vol. 6, 101149-58 [0029]
- **NIELSEN et al.** *Science*, 1991, vol. 254, 1497-1500 [0049]
- **GOVINDARAJUKUMAR** *Chem. Commun*, 2005, 495-497 [0049]
- **EGHOLM et al.** *Nature*, 1993, vol. 365, 566-568 [0049]
- **MORITA et al.** *Nucleic Acid Res*, 2001, vol. 1, 241-242 [0052]
- **GOYENVALLE A et al.** Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping *Science*, 2004, vol. 306, 57021796-9 [0060]
- **DE ANGELIS FG et al.** Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells *Proc Natl Acad Sci U S A*, 2002, vol. 99, 149456-61 [0060]
- **DENTI MA et al.** Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice *Hum Gene Ther*, 2006, vol. 17, 5565-74 [0060]
- **GORMAN L et al.** Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs *Proc Natl Acad Sci U S A*, 1998, vol. 95, 94929-34 [0060]
- **SUTER D et al.** Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations *Hum Mol Genet*, 1999, vol. 8, 132415-23 [0060]
- **REMINGTON** *The Science and Practice of Pharmacy* Lippincott Williams & Wilkins 20000000 [0074]
- **MATHEWSETJ** *Mol Biol*, 1999, vol. 288, 5911-40 [0087]
- **CARTEGNI et al.** *Nucleic Acids Res*, 2003, vol. 31, 133568-71 [0087]
- **AARTSMA-RUS et al.** *Hum Mol Genet*, 2003, vol. 12, 8907-14 [0088]
- **HAVENGA et al.** *J Virol*, 2002, vol. 76, 94612-20 [0088]
- **AARTSMA-RUS et al.** *Neuromuscul Disord*, 2002, vol. 12, S71- [0088] [0088] [0104]
- **SICINSKI et al.** *Science*, 1989, vol. 244, 1578-1580 [0095]
- **VAN DEUTEKOM et al.** *Hum Mol Genet*, 2001, vol. 10, 1547-1554 [0095] [0098]
- **DEN DUNNEN et al.** *Hum Mol Genet*, 1992, vol. 1, 19-28 [0096]
- **T HOEN et al.** *J. Biol. Chem.*, 2008, [0096] [0103]
- **AARTSMA-RUS et al.** *Neuromuscul Disord*, 2002, vol. 12, S71.817- [0098]

Patentkrav

- 5 **1.** Antisense-oligonukleotid bestående af sekvensen 5' ucagcuucuguuagccacug 3' (SEQ ID NO:5), hvor mindst ét af nukleotiderne er en nukleotidanalogue.
- 2.** Oligonukleotid ifølge krav 1, omfattende RNA-rester eller en eller flere DNA-rester.
- 10 **3.** Oligonukleotid ifølge krav 1, hvor nukleotidanalogen har en modificeret base og/eller en ikke-naturlig internukleosid-binding eller en kombination af disse modifikationer.
- 15 **4.** Oligonukleotid ifølge krav 3, hvor bindingen mellem resterne i et skelet ikke omfatter et phosphoratom.
- 20 **5.** Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3 eller 4, hvor oligonukleotidet omfatter mindst én nukleotidanalogue med en sukkerenhed, som er mono- eller disubstitueret ved den 2', 3' og/eller 5' position.
- 25 **6.** Oligonukleotid ifølge krav 3, hvor nukleotidanalogen har en modificeret base.
- 30 **7.** Oligonukleotid ifølge krav 6, hvor den modificerede base er en syntetisk eller naturlig base såsom inosin, xanthin, hypoxanthin og andre aza-, deaza-, hydroxy-, halo-, thio-, thiol-, alkyl-, alkenyl-, alkynyl-, thioalkylderivater af pyrimidin- og purinbaser.
- 30 **8.** Oligonukleotid ifølge et hvilket som helst af kravene 1-7, omfattende mindst to forskellige typer nukleotidaloger.

9. Oligonukleotid ifølge krav 5, hvor oligonukleotidet omfatter mindst én nukleotidanalogue med én sukkerenhed, som er monosubstitueret ved den 2', 3' og/eller 5' position, fortrinsvis ved den 2'-position, og mere foretrukket er sukkerenheden 2'-methoxy substitueret.

5

10. Oligonukleotid ifølge krav 5, hvor nukleotidanalogen er en låst nukleinsyre (LNA).

10

11. Oligonukleotid ifølge krav 4, hvor det modificerede skelet er valgt fra gruppen bestående af et morpholino-skelet, et carbamat-skelet, et siloxan-skelet, et sulfid-skelet, et sulfoxid-skelet, et sulfon-skelet, et formacetyl-skelet, et thioformacetyl-skelet, et methylenformacetyl-skelet, et riboacetyl-skelet, et alken-holdigt skelet, et sulfamat-skelet, et sulfonat-skelet, et sulfonamid-skelet, et methylenimino-skelet, et methylenhydrazino-skelet og et amid-skelet.

15

12. Farmaceutisk sammensætning omfattende et oligonukleotid som defineret i et hvilket som helst af kravene 1-11, og et farmaceutisk acceptabelt bærestof.

20

13. Oligonukleotid som defineret i et hvilket som helst af kravene 1-11, eller den farmaceutiske sammensætning ifølge krav 12 til anvendelse som et lægemiddel til behandling af en DMD- eller BMD-patient.

DRAWINGS

Fig. 1a

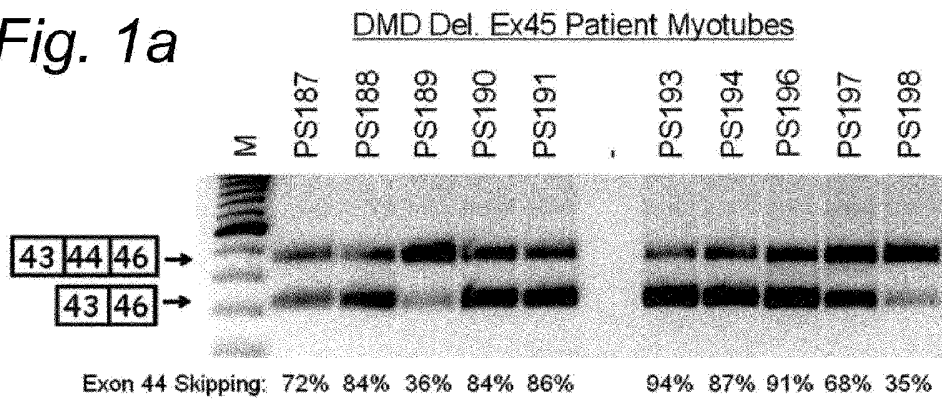


Fig. 1b

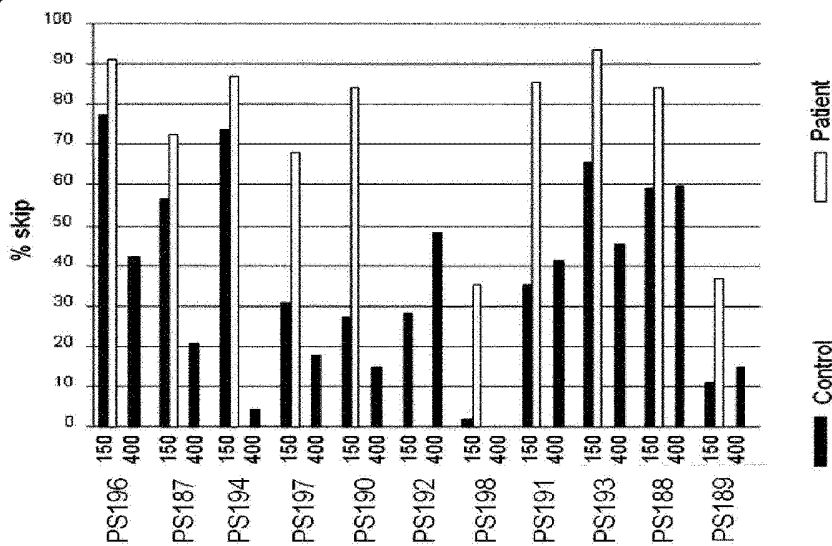


Fig. 1c

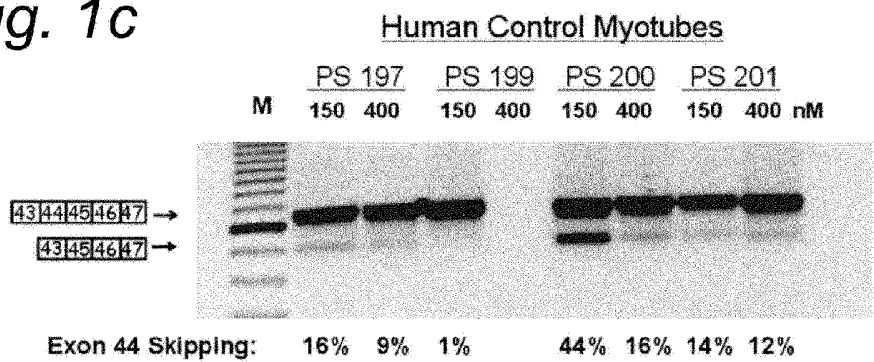


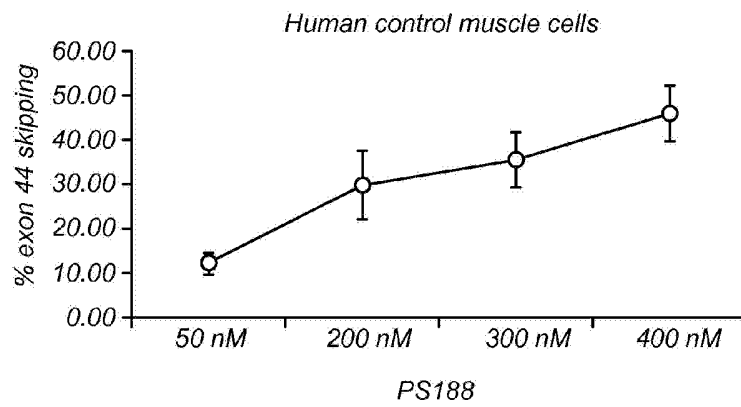
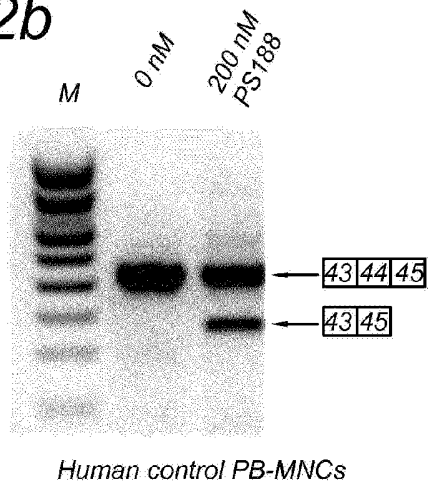
Fig. 2a*Fig. 2b*

Fig. 3a

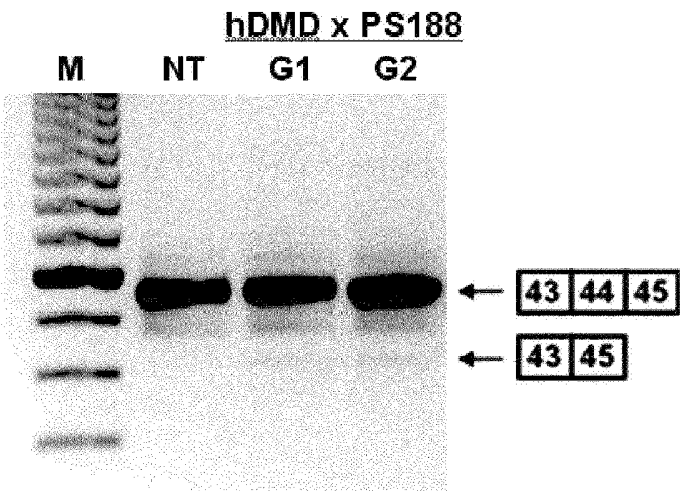


Fig. 3b

