



Office de la Propriété

Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2568643 A1 2005/12/29

(21) **2 568 643**

**(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2005/06/20
(87) Date publication PCT/PCT Publication Date: 2005/12/29
(85) Entrée phase nationale/National Entry: 2006/11/28
(86) N° demande PCT/PCT Application No.: NL 2005/000446
(87) N° publication PCT/PCT Publication No.: 2005/123117
(30) Priorité/Priority: 2004/06/21 (US60/580,903)

(51) Cl.Int./Int.Cl. **A61K 38/46** (2006.01),
A61K 48/00 (2006.01), **A61P 1/16** (2006.01)

(71) **Demandeurs/Applicants:**
ACADEMISCH ZIEKENHUIS BIJ DE UNIVERSITEIT
VAN AMSTERDAM, NL;
AMSTERDAM MOLECULAR THERAPEUTICS B.V., NL

(72) **Inventeur/Inventor:**
VAN DEVENTER, SANDER JAN HENDRIK, NL

(74) **Agent:** GOWLING LAFLEUR HENDERSON LLP

(54) Titre : **METHODE DE TRAITEMENT DE L'HEPATITE STEATOSIQUE NON ALCOOLIQUE (NASH)**

(54) Title: **TREATMENT OF NON ALCOHOLIC STEATOTIC HEPATITIS (NASH)**

(57) Abrégé/Abstract:

The present invention relates to a method for treating nonalcoholic steatotic hepatitis (NASH) in a subject by administering an effective amount of a lipoprotein lipase (LPL) therapeutic to the subject. The LPL therapeutic is advantageously a S447X protein or a derivative or variant thereof, or a nucleic acid encoding such a protein. The LPL therapeutic may be used in a gene therapy vector.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date
29 December 2005 (29.12.2005)

PCT

(10) International Publication Number
WO 2005/123117 A1

(51) International Patent Classification⁷: **A61K 38/46**, 48/00, A61P 1/16

(21) International Application Number: PCT/NL2005/000446

(22) International Filing Date: 20 June 2005 (20.06.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 60/580,903 21 June 2004 (21.06.2004) US

(71) Applicants (for all designated States except US): **ACADEMISCH ZIEKENHUIS BIJ DE UNIVERSITEIT VAN AMSTERDAM** [NL/NL]; Meibergdreef 9, NL-1105 AZ Amsterdam-Zuidoost (NL). **AMSTERDAM MOLECULAR THERAPEUTICS B.V.** [NL/NL]; Meibergdreef 61, NL-1105 BA Amsterdam (NL).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **VAN DEVENTER, Sander, Jan, Hendrik** [NL/NL]; Kleine Houtweg 6, NL-2012 CH Haarlem (NL).

(74) Agent: **VAN WESTENBRUGGE, Andries**; Nederlandsch Octrooibureau, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/123117 A1

(54) Title: TREATMENT OF NON ALCOHOLIC STEATOTIC HEPATITIS (NASH)

(57) Abstract: The present invention relates to a method for treating nonalcoholic steatotic hepatitis (NASH) in a subject by administering an effective amount of a lipoprotein lipase (LPL) therapeutic to the subject. The LPL therapeutic is advantageously a S447X protein or a derivative or variant thereof, or a nucleic acid encoding such a protein. The LPL therapeutic may be used in a gene therapy vector.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND
PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 15

NOTE : Pour les tomes additionnels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE
VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 15

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

TREATMENT OF NON ALCOHOLIC STEATOTIC HEPATITIS (NASH)

Field of the invention

5

The present invention is in the field of protein and nucleic acid therapeutics for the treatment of non-alcoholic steatotic hepatitis.

10 **Background of the invention**

Non-alcoholic steatotic hepatitis (NASH) is part of a spectrum of nonalcoholic fatty liver disease (NAFL) and refers to the development of histological changes in the liver that are comparable to those induced by excessive alcohol intake. However NASH occurs in patients 15 who are not abusing alcohol. NASH is characterized by elevated serum aminotransferases, indicating liver cell damage. Macrovesicular steatosis (i.e., intracytoplasmic vacuoles that eccentrically displace the hepatocyte nucleus), inflammation, and occasionally fibrosis that may progress to cirrhosis, characterise the disease. There is increasing evidence that NASH is a component of the metabolic insulin resistance syndrome. This is a cluster of disorders, 20 including obesity, dyslipidemia, arteriosclerosis and diabetes mellitis, which have insulin resistance as a common feature (de Sligte et al (2004) Eur J Int Med 15:10) .

Although many drugs have been tried to improve NASH and prevent further deterioration, there is no established treatment for this potentially serious condition. One of the drugs tested for efficacy in NASH is rosiglitazone (Muurling et al (2003) Metabolism 52: 1078). A loss of 25 body weight may also improve NASH. However, despite these interventions, in a significant number of patient NASH progresses to cirrhosis and it may ultimately require liver transplantation, after which the disease may recur.

Given the trend of increasing obesity and diabetes in both the U.S. and the European populations, there is an urgent need for novel treatments of NASH.

30

Detailed description

The present invention relates to a method of treating non alcoholic steatotic hepatitis in a subject. The method comprises administering to a subject an effective amount of a lipoprotein

lipase (LPL) therapeutic. As used herein, LPL therapeutic refers to a protein with LPL activity (EC 3.1.1.34) or to a nucleic acid encoding such a protein.

The protein form of LPL therapeutic

5 In one embodiment, the LPL therapeutic is an LPL protein with an amino acid sequence as shown in SEQ ID No.1, herein referred to as LPL S447X proteins or peptides. In general, these LPL S447X proteins are shorter than well-known wild type LPL, which has 448 amino acids (see for instance Wion et al Science (1987) 235: 1638 or WO 01/00220). They may include compounds such as peptide fragments, modified peptide fragments, analogues or 10 pharmacologically acceptable salts of LPL having amino acids 447-448 truncated from the carboxy terminal of a wild-type LPL. Such compounds are collectively referred to herein as LPL S447X peptides. LPL S447X peptides may include homologs of the wild-type mature LPL sequence from amino acids 1 through 446, including homologs from species other than *homo sapiens* (which may have veterinary applications). LPL S447X peptides may include 15 derivatives and naturally occurring isoforms or genetic variants of wild type LPL. The use of derivatives and variants of the LPL S447X protein is also encompassed in the invention.

Derivatives of S447X protein

Derivatives include, in particular, proteins with LPL activity which have the same amino acid 20 sequence as LPL S447X protein, but in which some N- or O-glycosylation sites have been modified or eliminated. Derivatives also include C-terminal hydroxymethyl derivatives, O-modified derivatives (e.g., C-terminal hydroxymethyl benzyl ether), and N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides.

Within an LPL therapeutic of the invention, a peptidic structure maybe coupled 25 directly or indirectly to a modifying group. The term "modifying group" is intended to include structures that are directly attached to the peptidic structure (e.g., by covalent coupling), as well as those that are indirectly attached to the peptidic structure (e.g., by a stable non-covalent association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the MCP-3 core peptidic 30 structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of an LPL therapeutic structure, or to a peptidic or peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of an amino acid residue of the LPL therapeutic, or to a peptidic or peptido-mimetic region flanking the core domain (e.g., through the epsilon amino group of a lysyl residue(s), through

the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the peptidic structure can be attached by means and using methods well known in the art for linking chemical structures, including, for example, amide, alkylamino, carbamate or urea bonds.

In some embodiments, the modifying group may comprise a cyclic, heterocyclic or polycyclic group. The term "cyclic group", as used herein, includes cyclic saturated or unsaturated (i.e., aromatic) group having from 3 to 10, 4 to 8, or 5 to 7 carbon atoms.

Exemplary cyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cyclooctyl. Cyclic groups may be unsubstituted or substituted at one or more ring positions. A cyclic group may for example be substituted with halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, sulfonates, selenoethers, ketones, aldehydes, esters, -CF₃, -CN.

The term "heterocyclic group" includes cyclic saturated, unsaturated and aromatic groups having from 3 to 10, 4 to 8, or 5 to 7 carbon atoms, wherein the ring structure includes about one or more heteroatoms. Heterocyclic groups include pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine. The heterocyclic ring may be substituted at one or more positions with such substituents as, for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, other heterocycles, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF₃, -CN. Heterocycles may also be bridged or fused to other cyclic groups as described below.

The term "polycyclic group" as used herein is intended to refer to two or more saturated, unsaturated or aromatic cyclic rings in which two or more carbons are common to two adjoining rings, so that the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycyclic group may be substituted with such substituents as described above, as for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF₃, or -CN.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl

substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In some embodiments, a straight chain or branched chain alkyl has 20 or fewer carbon atoms in its backbone (C₁-C₂₀ for straight chain, C₃-C₂₀ for branched chain), or 10 or fewer carbon atoms. In some embodiments, cycloalkyls may have from 4-10 carbon atoms in their ring structure, such as 5, 5 6 or 7 carbon rings. Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, having from one to ten carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have chain lengths of ten or less carbons.

The term "alkyl" (or "lower alkyl") as used throughout the specification and claims is 10 intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, halogen, hydroxyl, carbonyl (such as carboxyl, ketones (including alkylcarbonyl and arylcarbonyl groups), and esters (including alkyloxycarbonyl and aryloxycarbonyl groups)), thiocarbonyl, acyloxy, 15 alkoxy, phosphoryl, phosphonate, phosphinate, amino, acylamino, amido, amidine, imino, cyano, nitro, azido, sulfhydryl, alkylthio, sulfate, sulfonate, sulfamoyl, sulfonamido, heterocycl, aralkyl, or an aromatic or heteroaromatic moiety. The moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of aminos, 20 azidos, iminos, amidos, phosphoryls (including phosphonates and phosphinates), sulfonyls (including sulfates, sulfonamidos, sulfamoyls and sulfonates), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF₃, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxy, alkylthios, aminoalkyls, carbonyl-substituted 25 alkyls, -CF₃, -CN, and the like.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

The term "aralkyl", as used herein, refers to an alkyl or alkenyl group substituted 30 with at least one aryl group. Exemplary aralkyls include benzyl (i.e., phenylmethyl), 2-naphthylethyl, 2-(2-pyridyl)propyl, 5-dibenzosuberyl, and the like.

The term "alkylcarbonyl", as used herein, refers to -C(O)-alkyl. Similarly, the term "arylcarbonyl" refers to -C(O)-aryl. The term "alkyloxycarbonyl", as used herein, refers to the group -C(O)-O-alkyl, and the term "aryloxycarbonyl" refers to -C(O)-O-aryl. The term

"acyloxy" refers to $-O-C(O)-R_7$, in which R_7 is alkyl, alkenyl, alkynyl, aryl, aralkyl or heterocyclyl.

The term "amino", as used herein, refers to $-N(R_\alpha)(R_\beta)$, in which R_α and R_β are each independently hydrogen, alkyl, alkyenyl, alkynyl, aralkyl, aryl, or in which R_α and R_β together with the nitrogen atom to which they are attached form a ring having 4-8 atoms. Thus, the term "amino", as used herein, includes unsubstituted, monosubstituted (e.g., monoalkylamino or monoaryl amino), and disubstituted (e.g., dialkylamino or alkylarylamino) amino groups. The term "amido" refers to $-C(O)-N(R_8)(R_9)$, in which R_8 and R_9 are as defined above. The term "acylamino" refers to $-N(R'_8)C(O)-R_7$, in which R_7 is as defined above and R'_8 is alkyl.

As used herein, the term "nitro" means $-NO_2$; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulphydryl" means $-SH$; and the term "hydroxyl" means $-OH$.

The term "aryl" as used herein includes 5-, 6- and 7-membered aromatic groups that may include from zero to four heteroatoms in the ring, for example, phenyl, pyrrolyl, furyl, thiophenyl, imidazolyl, oxazole, thiazolyl, triazolyl, pyrazolyl, pyridyl, pyrazinyl, pyridazinyl and pyrimidinyl, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with such substituents as described above, as for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulphydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-CF_3$, $-CN$, or the like. Aryl groups can also be part of a polycyclic group. For example, aryl groups include fused aromatic moieties such as naphthyl, anthracenyl, quinolyl, indolyl, and the like.

Modifying groups may include groups comprising biotinyl structures, fluorescein-containing groups, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, a N-acetylneuraminy group, a cholyl structure or an iminobiotinyl group. An LPL therapeutic may be modified at its carboxy terminus with a cholyl group according to methods known in the art (for example see: Wess, G. et al. (1993) *Tetrahedron Letters*, 34:817-822; Cholyl derivatives and analogues may also be used as modifying groups, such as Aic (3-(O-aminoethyl-iso)-cholyl), which has a free amino group that can be used to further modify the LPL therapeutic. A modifying group may be a "biotinyl structure", which includes biotinyl groups and analogues and derivatives thereof (such as a 2-iminobiotinyl group). In another embodiment, the modifying group may comprise a "fluorescein-containing group", such as a group derived from reacting an LPL therapeutic peptide with 5-(and 6)-carboxyfluorescein,

succinimidyl ester or fluorescein isothiocyanate. In various other embodiments, the modifying group(s) may comprise an N-acetylneuramyl group, a trans-4-cotininecarboxyl group, a 2-imino-1-imidazolidineacetyl group, an (S)-(-)-indoline-2-carboxyl group, a (-)-menthoxyacetyl group, a 2-norbornaneacetyl group, a gamma-oxo-5-acenaphthenebutyryl, a 5 (-)-2-oxo-4-thiazolidinecarboxyl group, a tetrahydro-3-furoyl group, a 2-iminobiotinyl group, a diethylenetriaminepentaacetyl group, a 4-morpholinecarbonyl group, a 2-thiopheneacetyl group or a 2-thiophenesulfonyl group.

Variants of LPL S447X protein

10 Variants LPL polypeptides include polypeptides having substantial sequence similarity to LPL S447X protein, such as 90%, 95% or 99% sequence identity to a corresponding portion of SEQ ID No.1, the corresponding portion being any contiguous sequence of any length, such as 10, 20, 30, 40, 50 or more amino acids. Such proteins typically have LPL activity, or another LPL-like property, equal to or greater than LPL S447X protein. In some 15 embodiments, chemically similar amino acids may be substituted for amino acids in the LPL S447X protein sequence (to provide conservative amino acid substitutions). Amino acid substitutions that reduce LPL activity, of which more than 50 have been disclosed, such as the substitution of a Ser residue for Asn at position 291 (Asn291Ser), the substitution of Asn for Asp at position 9 (Asp9Asn), the substitution of Glu for Gly at position 188 (Gly188Glu, see 20 Monsalve et al., *J.Clin.Invest.* 1990, 86(3):728-734) or Asp250Asn (Ma et al., *Genomics*. 1992, 13:649-653) should be avoided in preferred embodiments.

The nucleic acid form of LPL therapeutic

25 In yet another embodiment, the LPL therapeutic is a nucleic acid encoding the wild type LPL protein or encoding a LPL S447X protein. In an alternative embodiment, the nucleic acid may comprise a DNA coding sequence encoding an RNA having at least 90% sequence identity to nucleotides 256 through 1599 of SEQ ID NO:2, which stretch of nucleotides encodes the mature wild type LPL peptide.

30 Yet alternatively, the LPL therapeutic may comprise a nucleic acid which comprises a DNA coding sequence that hybridizes under stringent conditions to nucleotides 256 through 1599 of SEQ ID NO:2.

Sequence identity

Two nucleic acid or protein sequences are considered substantially identical if, when optimally aligned, they share at least about 70% sequence identity. In alternative

5 embodiments, sequence identity may for example be at least 75%, at least 90% or at least

95%. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981,

Adv. Appl. Math. 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970,

J. Mol. Biol. 48:443, the search for similarity method of Pearson and Lipman, 1988, *Proc.*

10 *Natl. Acad. Sci. USA* 85: 2444, and the computerised implementations of these algorithms

(such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence alignment may also be carried

15 out using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10 (using the published default settings). Software for performing BLAST analysis may be

15 available through the National Center for Biotechnology Information (through the internet at <http://www.ncbi.nlm.nih.gov/>). The BLAST algorithm involves first identifying high scoring

sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score

20 threshold. Initial neighbourhood word hits act as seeds for initiating searches to find longer

HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is

25 halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due

to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity

and speed of the alignment. The BLAST programs may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992, *Proc. Natl. Acad. Sci. USA*

89: 10915-10919) alignments (B) of 50, expectation (E) of 10 (which may be changed in

30 alternative embodiments to 1 or 0.1 or 0.01 or 0.001 or 0.0001; although E values much

higher than 0.1 may not identify functionally similar sequences, it is useful to examine hits with lower significance, E values between 0.1 and 10, for short regions of similarity), M=5,

N=4, for nucleic acids a comparison of both strands. For protein comparisons, BLASTP may be used with defaults as follows: G=11 (cost to open a gap); E=1 (cost to extend a gap); E=10 (expectation value, at this setting, 10 hits with scores equal to or better than the defined alignment score, S, are expected to occur by chance in a database of the same size as the one being searched; the E value can be increased or decreased to alter the stringency of the search.); and W=3 (word size, default is 11 for BLASTN, 3 for other blast programs).

The BLOSUM matrix assigns a probability score for each position in an alignment that is based on the frequency with which that substitution is known to occur among consensus blocks within related proteins. The BLOSUM62 (gap existence cost = 11; per residue gap cost = 1; lambda ratio = 0.85) substitution matrix is used by default in BLAST 2.0. A variety of other matrices may be used as alternatives to BLOSUM62, including: PAM30 (9,1,0.87); PAM70 (10,1,0.87) BLOSUM80 (10,1,0.87); BLOSUM62 (11,1,0.82) and BLOSUM45 (14,2,0.87). One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Modifications

It is well known in the art that some modifications and changes can be made in the structure 25 of a polypeptide without substantially altering the biological function of that peptide, to obtain a biologically equivalent polypeptide. In one aspect of the invention, LPL therapeutics may include peptides that differ from a portion of the wild-type LPL sequence by conservative amino acid substitutions. As used herein, the term "conserved amino acid substitutions" refers to the substitution of one amino acid for another at a given location in the peptide, where the 30 substitution can be made without loss of function. In making such changes, substitutions of like amino acid residues can be made, for example, on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like, and such substitutions may be assayed for their effect on the function of the peptide by

routine testing.

In some embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydrophilicity value (e.g., within a value of plus or minus 2.0), where the following hydrophilicity values are assigned to amino acid residues (as detailed in United States Patent No. 4,554,101, incorporated herein by reference): Arg (+3.0); Lys (+3.0); Asp (+3.0); Glu (+3.0); Ser (+0.3); Asn (+0.2); Gln (+0.2); Gly (0); Pro (-0.5); Thr (-0.4); Ala (-0.5); His (-0.5); Cys (-1.0); Met (-1.3); Val (-1.5); Leu (-1.8); Ile (-1.8); Tyr (-2.3); Phe (-2.5); and Trp (-3.4).

10

In alternative embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydropathic index (e.g., within a value of plus or minus 2.0). In such embodiments, each amino acid residue may be assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics, as follows: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Gly (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5).

In alternative embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another in the same class, where the amino acids are divided into non-polar, acidic, basic and neutral classes, as follows: non-polar: Ala, Val, Leu, Ile, Phe, Trp, Pro, Met; acidic: Asp, Glu; basic: Lys, Arg, His; neutral: Gly, Ser, Thr, Cys, Asn, Gln, Tyr.

25

Gene therapy

In one embodiment of the invention, the LPL therapeutic is administered to a subject in a gene therapy vector. Vectors may be prepared from different type of viruses, including adenoviruses, adeno-associated viruses (AAV), herpes viruses (HSV), lentiviruses and 30 retroviruses. In one embodiment, an AAV serotype selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7 and AAV8 is used.

It is within the technical skills of the skilled person to select the most appropriate virus or virus subtype. Some subtypes may be more appropriate than others for a certain type of tissue. For example, muscle-specific over-expression of LPL may advantageously be induced by

adeno-associated virus (AAV)-mediated transduction of muscle cells. Muscle is amenable to AAV-mediated transduction, and different serotypes may be used (AAV1, AAV6, AAV7, AAV8). Transduction of muscle is accomplished by intramuscular injection of AAV-LPL in multiple sites. Multiple sites, keeping the local viral dose low, will help to prevent LPL-induced myopathy or vector-induced immune responses. This has been an effective method for long-term transduction of muscle using serotype 1, however intravenous administration using other serotypes may also be applicable (AAV6, AAV8).

General methods for gene therapy are known in the art. See for example, U.S. Pat. No. 10 5,399,346 by Anderson *et al.* (incorporated herein by reference). A biocompatible capsule for delivering genetic material is described in PCT Publication WO 95/05452 by Baetge *et al.* Methods of gene transfer into hematopoietic cells have also previously been reported (see Clapp, D. W., et al., Blood 78: 1132-1139 (1991); Anderson, Science 288:627-9 (2000); and , Cavazzana-Calvo *et al.*, Science 288:669-72 (2000), all of which are incorporated herein by 15 reference).

Effective amounts

In the treatment according to the invention, non-alcoholic steatotic hepatitis is treated by 20 administering to a subject an effective amount of an LPL therapeutic. As used herein, non-alcoholic steatotic hepatitis refers to the development of histological changes in the liver that are comparable to those induced by excessive alcohol intake, but in the absence of alcohol abuse.

The LPL therapeutic will typically be included in a pharmaceutical composition, optionally 25 in combination with a pharmaceutical carrier, diluent and/or adjuvant. Such compositions include the LPL therapeutic in an effective amount, sufficient to provide a desired therapeutic or prophylactic effect, and a pharmaceutically acceptable carrier or excipient. An "effective amount" includes a therapeutically effective amount or a prophylactically effective amount.

30 A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as alteration of parameters in lipid metabolism, such as elevation of LPL activity, elevation of HDL-cholesterol or reduction of triglyceride levels. A therapeutically effective amount of an LPL therapeutic may vary according to factors such as the disease state, age, sex, and weight of the individual, and

the ability of the LPL therapeutic to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also typically one in which any toxic or detrimental effects of the LPL therapeutic are outweighed by the therapeutically beneficial effects.

5 A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting various conditions, including LPL responsive conditions, such as coronary heart disease, cardiovascular disease, coronary artery disease, high triglycerides and/or low HDL. A prophylactic dose may be used in subjects prior to or at an earlier stage of disease, and a
10 prophylactically effective amount may be more or less than a therapeutically effective amount in some cases.

In particular embodiments, a range for therapeutically or prophylactically effective amounts of LPL therapeutic may be 0.01 nM-0.1M, 0.1 nM-0.1M, 0.1 nM-0.05M, 0.05 nM-15 μ M or 0.01 nM-10 μ M. It is to be noted that dosage values may vary with the severity of the
15 condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that may be selected by medical practitioners.

20 For gene therapy vectors, the dosage to be administered may depend to a large extent on the condition and size of the subject being treated as well as the therapeutic formulation, frequency of treatment and the route of administration. Regimens for continuing therapy, including dose, formulation, and frequency may be guided by the initial response and clinical judgment. The parenteral route of injection into the interstitial space of tissue may be
25 preferred, although other parenteral routes, such as inhalation of an aerosol formulation, may be required in specific administration. In some protocols, a formulation comprising the gene and gene delivery system in an aqueous carrier is injected into tissue in appropriate amounts. The tissue target may be specific, for example the muscle or liver tissue, or it may be a combination of several tissues, for example the muscle and liver tissues. Exemplary tissue
30 targets may include liver, skeletal muscle, heart muscle, adipose deposits, kidney, lung, vascular endothelium, epithelial and/or hematopoietic cells.

In one embodiment, the effective dose range for small animals (mice), following intramuscular injection, is between 1×10^{12} and 1×10^{13} genome copy (gc)/kg, and for larger animals (cats) and possibly human subjects, between 1×10^{11} and 1×10^{12} gc/kg. Again in mice,

levels of transgene expression, as measured by LPL in post-heparin plasma, reach up to 300 ng/ml (measured using a commercial ELISA from DaiNippon), and expression is long-term (>1 year) following a one-time administration.

- 5 The amount of active compound in the compositions of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation.
- 10 It may be advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification
- 15 for the dosage unit forms of the invention may be dictated by the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and by the limitations inherent in the art of compounding such an active compound for the treatment of a condition in individuals.
- 20 As used herein "pharmaceutically acceptable carrier" or "exipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration, which includes intravenous, intraperitoneal or intramuscular administration. Alternatively, the carrier may be suitable for
- 25 sublingual or oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of
- 30 the invention is contemplated.

Supplementary active compounds can also be incorporated into the pharmaceutical compositions of the invention. Guidance on co-administration of additional therapeutics may

for example be found in the Compendium of Pharmaceutical and Specialties (CPS) of the Canadian Pharmacists Association.

Pharmaceutical compositions are typically sterile and stable under the conditions of manufacture and storage. Pharmaceutical compositions may be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to accommodate high drug concentration. The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. The LPL therapeutic may be administered in a time or controlled release formulation, for example in a composition which includes a slow release polymer or other carriers that will protect the compound against rapid release, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers may for example be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Short description of the figures

25 Figure 1: Protein sequence of one embodiment of an LPL therapeutic according to the invention, i.e. an S447X protein.

Figure 2: LPL mRNA sequence. Nucleotides 256 through 1599 encode the mature wild type LPL peptide.

EXAMPLE**Example 1 LPL gene therapy causes triglyceride redistribution**

This example investigates the effects of AAV1 with LPL S447X as transgene on diet-induced

5 hyperlipidemia in male APOE3-Leiden transgenic mice. For this purpose we injected 6 mice with 1×10^{13} gc's/kg AAV1- LPL S447X and 6 mice with PBS (intramuscular; 4 sites). One week later the mice were started on a Western type diet. At 28 weeks after starting the diet, the mice were sacrificed.

The results show that mice that received LPL gene therapy showed a gradual increase in post-

10 heparin LPL concentrations up to 300 ng/ml at 25 weeks after injection, but total post-heparin lipase activity was not significantly changed. In addition, no effects on plasma TG, HDL-c and TC after 4 hrs fasting could be detected. Despite this absence of an effect on circulating lipoprotein concentrations, LPL gene therapy proved effective in that the treated mice displayed a faster clearance of TG and FFA after a bolus of intravenously administered 15 Intralipid ($p < 0.05$). Other significant findings included a significantly reduced gain of weight of the AAV1- LPL S447X treated animals compared to the controls ($p < 0.05$), an effect that could not be attributed to a difference in abdominal fat mass. Muscle homogenates of the AAV1- S447X treated mice indicated significantly increased triglyceride content ($p < 0.05$) while liver homogenates indicated significantly decreased triglyceride content ($p < 0.05$) 20 compared to controls.

This example demonstrates that in the absence of an effect on fasting lipids, expression of human LPL in the mouse skeletal muscle induces a redistribution of triglycerides and a reduced gain of body weight.

25

Table 1

Human LPL concentration post heparin plasma after AAV1-LPLS447X injection

	Week 0	Week 1	Week 11	Week 25
Mean (ng/ml)	0	107	187	293
Stdev	0	42	59	250

30

Table 2**Fat tolerance test of peak triglycerides, % of value after injection of intra lipid**

5

Time after injection intra lipid (min.)	Mean		Mean		P (T- test)
	PBS group	STdev	AAV1-LPL	STdev	
<3	100.0	0.0	100.0	0.0	
30	40.7	13.1	21.2	4.7	<0.05
60	9.3	5.3	5.1	2.3	
120	3.6	1.1	2.5	0.9	

Table 3**Fat tolerance test of peak free fatty acids, % of value after injection of intra lipid**10
15

Time after injection intra lipid (min.)	Mean		Mean		P (T- test)
	PBS group	Stdev	AAV1-LPL	Stdev	
<3	100.0	0.0	100.0	0.0	
30	56.9	13.7	33.8	6.4	<0.05
60	14.0	5.9	12.8	4.6	
120	9.3	2.8	11.5	3.1	

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND
PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 15

NOTE : Pour les tomes additionnels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE
VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 15

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

5

1. The use of an LPL therapeutic for the preparation of a medicament for the treatment of non-alcoholic steatotic hepatitis in a subject.

10 2. The use according to claim 1 wherein the LPL therapeutic is a member selected from the group consisting of:

a) an S447X protein with an amino acid sequence as shown in SEQ ID No.1, or a derivative thereof;

b) an LPL protein with an amino acid sequence which comprises a contiguous segment having at least 90% sequence identity to SEQ ID NO:1 when optimally aligned which has 15 equal or greater LPL activity than the protein under a), or a derivative thereof; or

c) a nucleic acid encoding a) or b), or a derivative thereof.

20 3. The use according to claim 1 or 2, wherein the LPL therapeutic comprises a nucleic acid which comprises a DNA coding sequence encoding an RNA having at least 90%

sequence identity to nucleotides 256 through 1599 of SEQ ID NO: 2.

25 4. The use according to claims 1-3, wherein the LPL therapeutic is a nucleic acid which comprises a DNA coding sequence that hybridizes under stringent conditions to nucleotides 256 through 1599 of SEQ ID NO:2.

25

5. The use according to claim 1 or 2, wherein the LPL therapeutic is an LPL S447X protein with a contiguous segment of at least 95% sequence identity to SEQ ID NO:1.

30 6. The use according to claims 1-5, wherein the LPL therapeutic is administered to the subject in a gene therapy vector.

7. The use according to claim 6, wherein the gene therapy vector comprises a viral vector.

8. The use according to claim 7, wherein the viral vector comprises adeno-associated virus (AAV).

9. The use according to claims 1-8, wherein the subject is a human.

5

10. The use according to claims 1-9, wherein the LPL therapeutic is administered parenterally.

11. A method for treating non-alcoholic steatotic hepatitis in a subject, comprising

10 administering to a subject an effective amount of an LPL therapeutic.

12. The method of claim 11, wherein the LPL therapeutic is a member selected from the group consisting of:

a) an S447X protein with an amino acid sequence as shown in SEQ ID No.1, or a derivative

15 thereof;

b) an LPL protein with an amino acid sequence which comprises a contiguous segment having at least 90% sequence identity to SEQ ID NO:1 when optimally aligned which has equal or greater LPL activity than the protein under a), or a derivative thereof; or

c) a nucleic acid encoding a) or b), or a derivative thereof.

20

13. The method of claim 11, wherein the LPL therapeutic comprises an LPL nucleic acid which comprises a DNA coding sequence encoding an RNA having at least 90% sequence identity to nucleotides 256 through 1599 of SEQ ID NO:2.

25

14. The method of claim 11, wherein the LPL therapeutic is a nucleic acid which comprises a DNA coding sequence that hybridizes under stringent conditions to nucleotides 256 through 1599 of SEQ ID NO:2.

FIG. 1

Ala Asp Gln Arg Arg Asp Phe Ile Asp Ile Glu Ser Lys Phe Ala Leu
 1 5 10 15

Arg Thr Pro Glu Asp Thr Ala Glu Asp Thr Cys His Leu Ile Pro Gly
 20 25 30

Val Ala Glu Ser Val Ala Thr Cys His Phe Asn His Ser Ser Lys Thr
 35 40 45

Phe Met Val Ile His Gly Trp Thr Val Thr Gly Met Tyr Glu Ser Trp
 50 55 60

Val Pro Lys Leu Val Ala Ala Leu Tyr Lys Arg Glu Pro Asp Ser Asn
 65 70 75 80

Val Ile Val Val Asp Trp Leu Ser Arg Ala Gln Glu His Tyr Pro Val
 85 90 95

Ser Ala Gly Tyr Thr Lys Leu Val Gly Gln Asp Val Ala Arg Phe Ile
 100 105 110

Asn Trp Met Glu Glu Glu Phe Asn Tyr Pro Leu Asp Asn Val His Leu
 115 120 125

Leu Gly Tyr Ser Leu Gly Ala His Ala Ala Gly Ile Ala Gly Ser Leu
 130 135 140

Thr Asn Lys Lys Val Asn Arg Ile Thr Gly Leu Asp Pro Ala Gly Pro
 145 150 155 160

Asn Phe Glu Tyr Ala Glu Ala Pro Ser Arg Leu Ser Pro Asp Asp Ala
 165 170 175

Asp Phe Val Asp Val Leu His Thr Phe Thr Arg Gly Ser Pro Gly Arg
 180 185 190

Ser Ile Gly Ile Gln Lys Pro Val Gly His Val Asp Ile Tyr Pro Asn
 195 200 205

Gly Gly Thr Phe Gln Pro Gly Cys Asn Ile Gly Glu Ala Ile Arg Val
 210 215 220

Ile Ala Glu Arg Gly Leu Gly Asp Val Asp Gln Leu Val Lys Cys Ser
 225 230 235 240

His Glu Arg Ser Ile His Leu Phe Ile Asp Ser Leu Leu Asn Glu Glu
 245 250 255

Asn Pro Ser Lys Ala Tyr Arg Cys Ser Ser Lys Glu Ala Phe Glu Lys
 260 265 270

Gly Leu Cys Leu Ser Cys Arg Lys Asn Arg Cys Asn Asn Leu Gly Tyr
 275 280 285

Glu Ile Asn Lys Val Arg Ala Lys Arg Ser Ser Lys Met Tyr Leu Lys
 290 295 300

Thr Arg Ser Gln Met Pro Tyr Lys Val Phe His Tyr Gln Val Lys Ile
 305 310 315 320

His Phe Ser Gly Thr Glu Ser Glu Thr His Thr Asn Gln Ala Phe Glu
 325 330 335

Ile Ser Leu Tyr Gly Thr Val Ala Glu Ser Glu Asn Ile Pro Phe Thr
 340 345 350

Leu Pro Glu Val Ser Thr Asn Lys Thr Tyr Ser Phe Leu Ile Tyr Thr
 355 360 365

Glu Val Asp Ile Gly Glu Leu Leu Met Leu Lys Leu Lys Trp Lys Ser
 370 375 380

Asp Ser Tyr Phe Ser Trp Ser Asp Trp Trp Ser Ser Pro Gly Phe Ala
 385 390 395 400

Ile Gln Lys Ile Arg Val Lys Ala Gly Glu Thr Gln Lys Lys Val Ile
 405 410 415

Phe Cys Ser Arg Glu Lys Val Ser His Leu Gln Lys Gly Lys Ala Pro
 420 425 430

Ala Val Phe Val Lys Cys His Asp Lys Ser Leu Asn Lys Lys
 435 440 445

FIG. 2

ccctcttcc tcctcctcaa	ggaaagctg cccacttcta	gctgccctgc	catccccttt	60		
aaaggcgac ttgctcagcg	ccaaaccgcg	gctccagccc	tctccagcct	ccggctcagc	120	
cggctcatca	gtcggtccgc	gccttgcagc	tcctccagag	ggacgcgccc	cgagatggag	180
agcaaagccc	tgctcgtgct	gactctggcc	gtgtggctcc	agagtctgac	cgcctccgc	240
ggaggggtgg	ccgcccggca	ccaaagaaga	gattttatcg	acatcgaaag	taaatttgcc	300
ctaaggaccc	ctgaagacac	agctgaggac	acttgccacc	tcattccgg	agtagcagag	360
tccgtggcta	cctgtcattt	caatcacagc	agcaaaacct	tcatgggtat	ccatggctgg	420
acgtaacag	aatgttatga	gagttgggtg	ccaaaacttg	tggccgccc	gtacaagaga	480
gaaccagact	ccaatgtcat	tgtggtgac	tggctgtcac	gggctcagga	gcattaccca	540
gtgtccgcgg	gctacaccaa	actggtgaa	caggatgtgg	cccggtttat	caactggatg	600
gaggaggagt	ttaactaccc	tctggacaat	gtccatctct	tggatacag	ccttggagcc	660
catgctgctg	gcattgcagg	aagtctgacc	aataagaaag	tcaacagaat	tactggcctc	720
gatccagctg	gacctaactt	tgagtatgca	gaagccccga	gtcgctttc	tcctgatgat	780
gcagattttg	tagacgtctt	acacacattc	accagagggt	ccctggtcg	aagcattgga	840
atccagaaac	cagttggca	tgttgacatt	tacccgaatg	gaggtacttt	tcagccagga	900
tgtaacattg	gagaagctat	ccgcgtgatt	gcagagagag	gacttgaga	tgtggaccag	960
ctagtgaagt	gctcccacga	gcgcctccatt	catctttca	tcgactctct	gttgaatgaa	1020
gaaaatccaa	gtaaggccta	caggtgcagt	tccaaggaag	ccttgagaa	agggctctgc	1080
ttgagttgta	gaaagaaccg	ctgcaacaat	ctgggctatg	agatcaataa	agtcagagcc	1140
aaaagaagca	gcaaaatgta	cctgaagact	cgttctcaga	tgcctacaa	agtcttccat	1200
taccaagtaa	agattcattt	ttctggact	gagagtgaaa	cccataccaa	tcaggccttt	1260
gagatttctc	tgtatggcac	cgtggccgag	agtgagaaca	tcccattcac	tctgcctgaa	1320
gtttccacaa	ataagaccta	ctccttccta	atttacacag	aggtagatat	tggagaacta	1380
ctcatgttga	agctcaaatg	gaagagtat	tcatacttta	gctggtcaga	ctggtgagc	1440
agtcccggct	tcgccattca	gaagatcaga	gtaaaagcag	gagagactca	aaaaaagggtg	1500
atcttctgtt	ctagggagaa	agtgtctcat	ttgcagaaag	gaaaggcacc	tgcggattt	1560
gtgaaatgcc	atgacaagtc	tctgaataag	aagtcaggct	gaaactggc	aatctacag	1620

aacaaagaac	ggcatgtgaa	ttctgtgaag	aatgaagtgg	aggaagtaac	ttttacaaaa	1680
cataccagt	gttgggggtg	tttcaaaagt	ggattttcct	gaatattaat	cccagcccta	1740
cccttggtag	ttattttagg	agacagtctc	aagcactaaa	aagtggctaa	ttcaatttat	1800
gggtatagt	ggccaaatag	cacatcctcc	aacgttaaaa	gacagtggat	catgaaaagt	1860
gctgtttgt	ccttgagaa	agaaataatt	gtttgagcgc	agagtaaaat	aaggctcctt	1920
catgtggcgt	attgggccat	agcctataat	tggtagaac	ctcctattt	aattggaatt	1980
ctggatctt	cggactgagg	ccttctcaa	cttactcta	agtctccaag	aatacagaaa	2040
atgctttcc	gcggcacgaa	tcagactcat	ctacacagca	gtatgaatga	tgttttagaa	2100
tgattccctc	ttgctattgg	aatgtggtcc	agacgtcaac	caggaacatg	taacttggag	2160
aggcacgaag	aaagggtctg	ataaacacag	aggtttaaa	cagtccctac	cattggcctg	2220
catcatgaca	aagttacaaa	ttcaaggaga	tataaaatct	agatcaatta	attcttaata	2280
ggcttatcg	tttattgctt	aatccctctc	tcccccttct	ttttgtctc	aagattatat	2340
tataataatg	ttctctgggt	aggtgttcaa	aatgagcctg	taatcctcag	ctgacacata	2400
atttgaatgg	tgcagaaaaaa	aaaaagatac	cgtaattttt	ttatttagatt	ctccaaatga	2460
tttcatcaa	tttaaaatca	ttcaatatct	gacagttact	cttcagttt	aggcttacct	2520
tggcatgct	tcaagggtac	ttccagtgcg	tctttttgt	tcctggcttt	gacatgaaaa	2580
gataggttt	agttcaaatt	ttgcattgtg	tgagcttcta	cagattttag	acaaggaccg	2640
ttttactaa	gtaaaagggt	ggagaggttc	ctggggtgga	ttcctaagca	gtgcttgtaa	2700
accatcgct	gcaatgagcc	agatggagta	ccatgagggt	tgttatttgc	tgttttaac	2760
aactaatcaa	gagtggatga	acaactattt	ataaaactaga	tctcctattt	ttcagaatgc	2820
tcttctacgt	ataaatatga	aatgataaaag	atgtcaaata	tctcagaggc	tatagctgg	2880
aacccgactg	tgaaagtatg	tgatatctga	acacatacta	gaaagctctg	catgtgtgtt	2940
gtccttcagc	ataattcgg	aggaaaaaca	gtcgatcaag	ggatgtattt	gaacatgtcg	3000
gagtagaaat	tgttcctgat	gtgccagaac	ttcgaccctt	tctctgagag	agatgatcgt	3060
gcctataaaat	agtaggacca	atgttgcgtat	taacatcatc	aggcttggaa	tgaattctct	3120
ctaaaaataa	aatgatgtat	gatttgcgtat	tggcatcccc	tttatttttt	cattaaattt	3180
ctggatttgg	gttgcgtaccc	agggtgcatt	aactaaaag	attcactaaa	gcagcacata	3240
gcactggaa	ctctggctcc	gaaaaacttt	gttatata	tcaaggatgt	tctggcttta	3300
cattttattt	attagctgt	aatacatgtg	tggatgtgt	aatggagctt	gtacatattg	3360

gaaaggtcat tgtggctatc tgcatatata aatgtgtggt gctaactgta tgtgtcttta 3420
tcagtgatgg tctcacagag ccaactcact cttatgaaat gggctttaac aaaacaagaa 3480
agaaaacgtac ttaactgtgt gaagaaatgg aatcagcttt taataaaatt gacaacattt 3540
tattaccac 3549