MATERIALS AND METHODS FOR THE TREATMENT OF LYSOSOMAL ACID LIPASE DEFICIENCY (LAL-D)

[0001] This application contains, as a separate part of the disclosure, a Sequence Listing in computer-readable form which is incorporated by reference in its entirety and identified as follows: 54743_Seqlisting.txt; Size: 14,266 bytes; Created: January 21, 2022.

FIELD OF THE INVENTION

[0002] The disclosure provides gene therapy vectors, such as adeno-associated virus (AAV), designed for treatment of Lysosomal Acid Lipase Deficiency (LAL-D) disorders such as Wolman Disease and cholesterol ester storage disease (CESD). The disclosed rAAV provide a wild type human lipase A (LIPA) cDNA to a subject in need which results in expression of the wild type human LAL protein.

BACKGROUND

[0003] Lysosomal Acid Lipase Deficiency, LAL-D, is a lysosomal storage disorder caused by recessive mutations in the Lipase A (*LIPA*) gene that result in a failure of the lysosomal acid lipase (LAL) protein to sufficiently hydrolyze cholesterol esters into free cholesterol and triglycerides into free fatty acids in the lysosome. LAL occupies a critical and essential position in the control of plasma lipoprotein levels and in the prevention of cellular lipid overload, especially in the liver and spleen (Li et al., *Arterioscler Thromb Vasc Biol* **39**: 850-856, 2019; Aguisanda et al. *Curr Chem Genom Transl Med* **11**: 1-18, 2017). The *LIPA* gene is the only gene with this lysosomal function in the human genome. LAL-D is a rare genetic disease, with prevalence ranging from 1 in 40,000 to 1 in 300,000, though disease incidence may be underestimated through failed diagnosis in some instances (Pastores et al., Lysosomal Acid Lipase Deficiency: Therapeutic Options. *Drug Des Devel Ther* **14**: 591-601, 2020).

[0004] Null *LIPA* gene mutations cause Wolman disease (WD), a fatal disease of infancy named after Moshe Wolman, who reported one of the first cases (Abromov et al., *AMA J Dis Child* 91: 282-286, 1956). WD is characterized by hepatomegaly with liver dysfunction, dyslipidemia (elevated serum triglycerides and LDL-cholesterol with reduced HDL-cholesterol), hepatosplenomegaly, pulmonary fibrosis, and adrenal calcification and insufficiency. Infants manifest disease in the first month of life and fail to thrive, most likely due to liver disease combined with a failure to absorb nutrients through the intestinal lining. Median lifespan of untreated WD infants is 3.7 months. Partial loss of function *LIPA* mutations, usually with 1-12% of normal activity, give rise to cholesterol-ester storage disease (CESD), a later onset, less severe disease form. While CESD need not result in

premature death, it is associated with significant morbidity, including liver fibrosis and cirrhosis (and also liver failure). Chronic dyslipidemia in LAL-D may also cause accelerated atherosclerosis and high risk of cardiac disease, including myocardial infarction, and cerebrovascular complications, including stroke. Liver biopsy in LAL-D patients typically demonstrate micro- and macro-vascular steatosis involving Kuppfer cells and hepatocytes, accompanied by fibrosis and cirrhosis as the disease progresses. Unlike other lysosomal storage disorders such as Gaucher disease and Niemann-Pick disease, there appears to be no primary CNS involvement (though histological studies are lacking).

[0005] While LAL-D is a rare genetic disorder, the pathology findings in LAL-D speak to larger and far more common significant health issues that are found in the general population. For example, reduced LAL-D activity is a biomarker for non-alcoholic fatty liver disease, a disorder affecting many millions of American adults and children. Such reductions in LAL activity are progressive with the increasing severity of liver disease; LIPA enzyme activity decreases from simple liver steatosis to non-alcoholic steatohepatitis to cryptogenic liver cirrhosis. Additionally, there is a LIPA haplotype strongly associated with coronary artery disease [10, 11]. The buildup of fatty acids in LAL-D mimics human conditions such as morbid obesity and obesity related to type II diabetes. Given how easily AAV transduces the liver, and how important the liver is in controlling lipoprotein-based metabolism, it is conceivable that LIPA gene therapy could be applied to these other genetic, and even non-genetic, human diseases related to obesity, based on the relationship of LAL-D to fat absorption in these other disorders.

SUMMARY

[0006] The disclosure provides for a clinical AAV vector used in gene replacement therapy for LAL-D including those disorders caused by mutations in the LIPA gene and non-genetic disorders that are associated with lipid accumulation and storage.

[0007] In one embodiment, described herein is a polynucleotide comprising (a) one or more regulatory control elements and (b) LIPA cDNA sequence. In some embodiments, the regulatory control element is a miniCMV promoter comprising a nucleotide sequence set forth in SEQ ID NO: 3, or fragments thereof which retain regulatory control or promoter activity. In some embodiments, the vector comprises a late SV40 poly adenylation sequence having the nucleotide sequence of SEQ ID NO: 5. In some embodiments, the LIPA cDNA is the LIPA variant 1 cDNA, and the LIPA cDNA comprises the polynucleotide sequence set forth in SEQ ID NO: 1.

[0007a] Disclosed herein is a polynucleotide comprising (a) a regulatory control element comprising the nucleotide sequence of SEQ ID NO: 3; and (b) a lipase A (LIPA) cDNA sequence comprising the nucleotide sequence set forth in SEQ ID NO: 1.

[0008] In one embodiment, the disclosure provides for a rAAV comprising a nucleotide sequence that encodes a functional lysosomal acid lipase (LAL) protein, wherein the nucleotide has, e.g., at least 65%, at least 70%, at least 75%, at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically at least 90%, 91%, 92%, 93%, or 94% and even more typically at least 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 1, wherein the protein retains LAL activity, such as the activity to hydrolyze cholesterol esters into free cholesterol and triglycerides into free fatty acids in the lysosome. For example, the nucleotide sequence that encodes a functional LAL protein may comprise one or more base pair substitutions, deletions or insertions which do affect the function of the LIPA protein. Furthermore, the nucleotide sequence that encodes a functional LIPA protein may comprise one or more base pair substitutions, deletions or insertions may increase or reduce expression of the LAL protein, and this change in expression pattern may be desired for treatment of the LAL-D or the disorder related to lipid storage and accumulation.

[0009] In another embodiment, the disclosure provides for a rAAV comprising a nucleotide sequence that encodes a functional LAL protein, wherein the protein comprises an amino acid sequence that has, e.g., at least 65%, at least 70%, at least 75%, at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically at least 90%, 91%, 92%, 93%, or 94% and even more typically at least 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 2, wherein the protein retains LAL activity, such as the activity to hydrolyze cholesterol esters into free cholesterol and triglycerides into fatty acids in the lysosome. For example, the nucleotide sequence that encodes a functional LAL protein may comprise one or more amino acid substitutions, deletions or insertions which do affect the function of the LAL protein.

[0010] The term “sequence identity”, “percent sequence identity”, or “percent identical” in the context of nucleic acid or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, e.g. of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. The percent identity of the sequences can be

determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs such as ALIGN, ClustalW2 and BLAST. In one embodiment, when BLAST is used as the alignment tool, the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR.

[0011] In another embodiment, the disclosure provides for an rAAV construct contained in the plasmid comprising the nucleotide sequence of SEQ ID NO: 4. For example, the rscrAAVrh74.miniCMV.LIPA vector comprises the nucleotide sequence within and inclusive of the ITR's of SEQ ID NO: 4. The rAAV vector comprises the 5' ITR, miniCMV promoter, the coding sequence for the human LIPA gene, SV40 late polyA, and 3' ITR. In this embodiment, the 3'ITR contains a deletion of the terminal resolution site (dTR), which inhibits Rep protein nicking of the single stranded viral genome. The presence of the dTR in the 3' ITR increases self-complementary binding of the viral genome to itself, which it may do because of its small (2.2kB) size that allows for a double-stranded viral genome to be packaged within the viral capsid. The self-complementary nature of rscAAVrh74.mCMV.LIPA facilitates both the speed and the extent of gene expression relative to constructs that remain as a single-stranded viral genome. In one embodiment, the vector comprises nucleotides 1853-3906 of SEQ ID NO: 4. The nucleotides within the ITRs may be in forward or reverse orientation. For example, the miniCMV promoter sequence, human LIPA gene sequence, and SV40 late polyA sequence and may be in forward or reverse orientation. In another embodiment, the vector comprises a nucleotide sequence that has about at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the nucleotides of 1-4397 of SEQ ID NO: 4. The plasmid set forth in SEQ ID NO 4 further comprises kanamycin resistance and an origin of replication.

[0012] Disclosed herein is a recombinant adeno-associated virus (rAAV) having a genome comprising a polynucleotide sequence described herein. In some embodiments, the rAAV is of the serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVRH10, AAVrh74, AAVrh, AAV11, AAV12, AAV13, or Anc80, AAV7m8 or their derivatives.

[0013] In some embodiments, the genome of the rAAV comprises a miniCMV promoter and LIPA cDNA. An exemplary genome comprises the miniCMV promoter, and the LIPA cDNA such as the rscrAAVrh74.miniCMV.LIPA, the rAAV set out as nucleotides 1853-3906

of SEQ ID NO: 4. The miniaturized CMV promoter allows for AAV packaging of the self-complementary double-stranded viral genome, which is not allowed with promoters that are of a larger size.

[0014] Disclosed herein is an rAAV particle comprising an rAAV described herein.

[0015] Compositions comprising any of the rAAV described herein or any of the viral particles described herein. The disclosed composition may be formulated for any means of delivery, such as direct injection into the cerebrospinal fluid, intracerebroventricular delivery, intrathecal delivery, intraperitoneal delivery, intraarterial delivery, or intravenous delivery.
[0015a] Disclosed herein is a composition comprising the rAAV as defined herein or the viral particle as defined herein.

[0016] In addition, the disclosed composition is formulated for intravenous delivery or intraperitoneal delivery and comprises a dose of rAAV or rAAV particles of about 1e13 vg/kg to about 2e14 vg/kg, e.g. $8x10^{13}$vg/kg.

[0017] Methods of treating LAL-D or a disorder related to lipid storage or accumulation in a subject in need thereof comprising administering a polynucleotide, an rAAV or an rAAV particle described herein are specifically contemplated. In some embodiments, the methods further comprise administering an immunosuppressing agent prior to, after or simultaneously with the polynucleotide, rAAV or rAAV particle. The LAL-D includes a disorder or disease caused by a mutation in the LIPA gene, such as Wolman disease or cholesterol ester storage disease. The disorder related to lipid storage or accumulation include coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

[0018] The disclosure also provides for methods of treating dyslipidemia or hypercholesterolemia in a subject in need thereof comprising administering a polynucleotide, an rAAV or an rAAV particle described herein are specifically contemplated. In some embodiments, the methods further comprise administering an immunosuppressing agent prior to, after or simultaneously with the polynucleotide, rAAV or rAAV particle.

[0019] The disclosure also provides for method of decreasing triglycerides, cholesterol, and/or fatty acids in a subject in need thereof comprising administering a polynucleotide, an rAAV or an rAAV particle described herein are specifically contemplated. In some embodiments, the methods further comprise administering an immunosuppressing agent prior to, after or simultaneously with the polynucleotide, rAAV or rAAV particle.

[0020] In any of the disclosed methods, the polynucleotide, rAAV, rAAV particle or composition are intravenously delivered to the subject. In some embodiments, the method further comprises a step of administering an immunosuppressing agent. For example, the

polynucleotide, rAAV, rAAV particle or composition is administered simultaneously, prior to or after administration of an immunosuppressing agent, such as prednisone, prednisolone, rapamycin, methotrexate, myophenolate mofetil, tacrolimus, mycophenolate, or rituximab. In any of the methods, the subject has a mutation in the LIPA gene. These mutations include those currently known, such as those set out in Table 1 herein, or a mutation(s) in the LIPA gene identified in the future that is associated with LAL-D.

[0021] A "subject," as used herein, can be any animal, and may also be referred to as the patient. Preferably the subject is a vertebrate animal, and more preferably the subject is a mammal, such as a domesticated farm animal (e.g., cow, horse, pig) or pet (e.g., dog, cat). in some embodiments, the subject is a human. In some embodiments, the subject is a pediatric subject. In some embodiments, the subject is a pediatric subject, such as a subject ranging in age from 1 to 10 years or the subject is an infant ranging in age for one month to 12 months. In some embodiments, the subject is 4 to 15 years of age. The subject, in on embodiment, is an adolescent subject, such as a subject ranging in age from 10 to 19 years. In other embodiments, the subject is an adult (18 years or older).

[0022] In another embodiment, described herein is the use of a polynucleotide, an rAAV or an rAAV particle described herein in the preparation of a medicament for the treatment of an LAL-D or a disorder related to lipid storage or accumulation. In some embodiments, the LAL-D is Wolman disease or cholesterol ester storage disease. In additional embodiments, the disorder related to lipid storage or accumulation is coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

[0022a] Disclosed herein is the use of a polynucleotide as defined herein, an rAAV as defined herein, a rAAV particle as defined herein or a composition as defined herein for the preparation of a medicament for the treatment of a Lysosomal Acid Lipase Deficiency (LAL-D) disorder in a subject in need thereof, wherein the LAL-D disorder is Wolman Disease or cholesterol ester storage disease (CESD).

[0022b] Disclosed herein is the use of a polynucleotide as defined herein, an rAAV as defined herein, a rAAV particle as defined herein or a composition as defined herein for the preparation of a medicament for the treatment of a disorder related to lipid storage or accumulation in a subject in need thereof, wherein the disorder is coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

[0022c] Disclosed herein is the use of a polynucleotide as define herein, an rAAV as defined herein, a rAAV particle as defined herein or a composition as defined herein for the preparation of a medicament for treating dyslipidemia or hypercholesterolemia in a subject in need thereof.

[0023] In addition, described herein is the use of a polynucleotide, an rAAV or an rAAV particle described herein in the preparation of a medicament for the treatment dyslipidemia or hypercholesterolemia in a subject in need thereof.

[0024] The disclosure also provides for use of a polynucleotide, an rAAV or an rAAV particle described herein in the preparation of a medicament for decreasing triglycerides, cholesterol, and/or fatty acids in a subject in need thereof.

[0025] For example, any of the disclosed medicaments are formulated for intravenous or intraperitoneal delivery. In some embodiments, the medicament is administered simultaneously, prior to or after administration of an immunosuppressing agent, such as prednisone, prednisolone, rapamycin, methotrexate, myophenolate mofetil, tacrolimus, mycophenolate, or rituximab.

[0026] In another embodiment, described herein is a composition comprising a polynucleotide, an rAAV, an rAAV particle or composition described herein for the treatment of LAL-D or a disorder related to lipid storage or accumulation. In some embodiments, the LAL-D is Wolman disease or cholesterol ester storage disease. In additional embodiments, the disorder related to lipid storage or accumulation is coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

[0026a] Disclosed herein is a composition for use in treating a lysosomal acid lipase deficiency (LAL-D) or a disorder related to lipid storage or accumulation in a subject in need thereof, wherein the composition comprises a polynucleotide, an rAAV, a rAAV particle or composition described herein.

[0027] In addition, described herein is a composition comprising a polynucleotide, an rAAV, an rAAV particle or composition described herein for the treatment dyslipidemia or hypercholesterolemia in a subject in need thereof.

[0028] In a further embodiment, described herein is a composition comprising a a polynucleotide, an rAAV, an rAAV particle or composition described herein for decreasing triglycerides, cholesterol, and/or fatty acids in a subject in need thereof.

[0028a] Disclosed herein is a composition for use in treating dyslipidemia or hypercholesterolemia in a subject in need thereof or for decreasing triglycerides, cholesterol, and/or fatty acids in a subject, wherein the composition comprises a polynucleotide, an rAAV, a rAAV particle or composition described herein.

[0029] For example, any of the disclosed compositions are formulated for intravenous or intraperitoneal delivery. In some embodiments, the composition is administered simultaneously, prior to or after administration of an immunosuppressing agent. In another

embodiment, the composition further comprises an immunosuppressing agent. Exemplary , immunosuppressing agents include prednisone, prednisolone, rapamycin, methotrexate, myophenolate mofetil, tacrolimus, mycophenolate, or rituximab.

BRIEF DESCRIPTION OF THE FIGURES

[0030] Figure 1 provides a schematic to the rscrAAVrh74.miniCMV.LIPA.

[0031] Figure 2 demonstrates that serum measures liver enzymes in treated and untreated Lipa$^{-/-}$ mice at 4 months of age. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in serum from wild type (WT), mock-treated *Lipa*$^{-/-}$, and rAAVrh74.mCVM.*LIPA*-treated *Lipa*$^{-/-}$ mice (treated with 8x10^{13}vg/kg AAV IV at postnatal day 1, analyzed at 4mo). Errors are SEM for n=6(WT), 7 (*Lipa*$^{-/-}$) or 8 (*Lipa*$^{-/-}$ Treated) mice/grp, with roughly even male and female representation. ***p<0.001, ****p<0.0001.

[0032] Figure 3 demonstrates that serum LDL-cholesterol levels at 4 months of age. LDL-cholesterol level in serum from wild type (WT), mock-treated *Lipa*$^{-/-}$, and rAAVrh74.mCVM.*LIPA*-Treated *Lipa*$^{-/-}$ mice (treated with 8x10^{13}vg/kg AAV IV at postnatal day 1, analyzed at 4mo). Errors are SEM for n=6 (WT), 7 (*Lipa*$^{-/-}$) or 8 (*Lipa*$^{-/-}$ Treated) mice/grp. **p<0.01, ***p<0.001

[0033] Figure 4 provides a comparison of body and organ weights in treated and untreated *Lipa*$^{-/-}$ mice in comparison to wild type. (A) total body weight, (B) liver weight, (C) spleen weight, (D) intestine weight, (E) heart weight, (F) kidney weight, (G) brain weight, and (H) muscle weight were compared. Treated *Lipa*$^{-/-}$ mice were given an IV dose of 8x10^{13}vg/kg rscAAVrh74.mCVM.*LIPA* at postnatal day 1 (P1) and analyzed at 6 months of age. Errors are SEM for n=4-5/grp. *p<0.05, **p<0.01, ***p<0.001. Note that while *Lipa*$^{-/-}$ mice gain weight, this pales in comparison to the dramatic increases in organ weight for certain organs (liver and spleen). There are milder weight gains for other organs (kidney and heart), while there is no weight gain (brain) or reduced weight gain for others (e.g., muscle, most likely due to nutritional deficits from fat deposition in the intestine).

[0034] Figure 5 provides a representative oil red/hematoxylin staining of organs from mock-treated and AAV-treated Lipa$^{-/-}$ mice and wild type controls. *Lipa*$^{-/-}$ Treated mice were given 8x10^{13}vg/kg rscAAVrh74.mCVM.*LIPA* gene therapy IV at postnatal day 1 and tissues were stained with Oil Red O, to identify lipid overload from disease, at 6 months of age. In all instances, Oil Red O staining was very reduced by treatment, though not absent. Bar is 200 μm.

[0035] Figure 6 provides cellular triglyceride levels in liver and spleen after AAV treatment of *Lipa*$^{-/-}$ mice. (A) Liver and (B) Spleen from 6-month-old wild type (WT), *Lipa*$^{-/-}$, and *Lipa*$^{-/-}$

Treated mice (treated IV with $8x10^{13}$vg/kg of rscAAVrh74.mCVM.*LIPA* at postnatal day 1). Errors are SEM for n=4-5/grp. *p<0.05, **p<0.001, ***p<0.001, ****p<0.0001

[0036] Figure 7 provides AAV vector genome biodistribution per nucleus. Injection if *Lipa*$^{-/-}$ mice (LIPA KO) at 2 months led to increased transduction of liver lymph node, spleen and intestine relative to injection at P1 when assayed at 4 months of age. Errors are SD for n=3/group. Vgs were measured per ug of genomic DNA, then converted to vg/nucleus (or per cell).

[0037] Figure 8 demonstrates LIPA gene expression induced by rscAAVrh74.mCMV.LIPA. Injection if *Lipa*$^{-/-}$ mice (LIPA KO) at 2 months led to increased gene expression in liver lymph node, spleen and intestine relative to injection at P1 when assayed at 4 months of age. Errors are SD for n=3/group. Gene expression in treated LIPA KO mice is compared to normal gene expression in wild type mouse tissue.

[0038] Figure 9 provides images of whole livers in 4 month old mice. LIPA KO mice, untreated, showed high fat content and increased size relative to wild type (FVBn). Treatment at P1 led to reduced size, but some fat content remained, while treatment at 2mo removed all fat content.

[0039] Figure 10 provides Liver cholesterol and triglyceride content after rscAAVrh74.mCMV.LIPA treatment in 4mo LIPA KO mice compared to wild type (FVBn). Errors are SD.

[0040] Figure 11 provides organ weights at 4 months after treatment with rscAAVrh74.mCMV.LIPA gene therapy. Errors are SD for n=2-5/grp.

[0041] Figure 12 provides serum LIPA enzyme activity levels after rscAAVrh74.mCMV.LIPA gene therapy treatment at 4 months. Errors are SD

[0042] Figure 13 provides liver LIPA enzyme activity after treatment with rscAAVrh74.mCMV.LIPA at 4 months of age. Errors are SD.

[0043] Figure 14A-14H provide data demonstrating that rscAAVrh74.miniCMV.LIPA treatment reverses hepatosplenomegaly and elevated serum liver enzymes in *Lipa*$^{-/-}$ mice. (A) Schematic of treatment plan of *Lipa*$^{-/-}$ mice. (B) Gross pathology of liver and spleen from wild type, untreated *Lipa*$^{-/-}$ and treated *Lipa*$^{-/-}$ mice. Scale bar = 1 cm. (C-F) Relative weights of liver, spleen, intestines, and mesenteric lymph node. (G-H) Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. All data represented as mean ± SD (n=5-8). Statistical significance between groups is denoted by different letters, P<0.05, using one-way ANOVA with Tukey's post-hoc test. Schematic created with BioRender.com. ITR=inverted terminal repeats; pA=SV40 polyA; dTIR=mutated ITR.

[0044] Figure 15 provides Kaplan-Meier survival curve of untreated *Lipa*$^{-/-}$ vs *Lipa*$^{-/-}$ treated at P2. Treatment with gene therapy extends lifespan beyond the 224-day median survival.

[0045] Figure 16A-16H demonstrates muscle atrophy may contribute to ambulation differences in *Lipa*$^{-/-}$ mice. (A) Body weight of mice at 2, 4, 6 months. (B-C) Relative weight of gastrocnemius muscle and quadricep muscle at 6 months. (D-H) Open field analysis at 6 months. All data represented as mean ± SD (n=5-8). Statistical significance between groups is denoted by different letters, P<0.05, using one-way ANOVA with Tukey's post-hoc test.

[0046] Figure 17A-17E demonstrates *LIPA* expression after rscAAVrh74.miniCMV.*LIPA* treatment leads to increased lysosomal acid lipase enzyme activity. **(A)** Biodistribution of AAV in various organs and tissues. Vector genomes (vg) per nucleus were quantified using quantitative real-time PCR. **(B)** Relative expression of AAV-introduced human *LIPA*, relative to the endogenous mouse *Lipa,* normalized to 18S mRNA. **(C-E)** Lysosomal acid lipase enzyme activity in liver, spleen, and serum. All data represented as mean ± SD (n=5-8). Statistical significance between groups is denoted by different letters, *P<0.05*, using one-*way ANOVA with Tukey's post-hoc test.*

[0047] Figures183A-18J demonstrates that cholesterol and triglyceride content is reduced with treatment. (A-B) Cholesterol content in (A) liver and (B) spleen. (C-D) Triglyceride control in (C) liver and (D) spleen. (E-I) Serum lipid panel. (J) Oil Red O (ORO) staining of liver, spleen and intestines tissue sections. Neutral lipids are stained red with ORO. Tissue sections were counter-stained with hematoxylin (purple). Scale bar = 25 μm. All data represented as mean ± SD (n=5-8). Statistical significance between groups is denoted by different letters, P<0.05, using one-way ANOVA with Tukey's post-hoc test.

[0048] Figure 19 provides immunhistochemical staining of LIPA and CD68 in liver sections. LIPA immunostaining in treated livers (brown). Macrophages are stained with anti-CD68 (brown). Tissue sections were counter-stained with hematoxylin (purple). Scale bar = 100 μm.

[0049] Figure 20A-20E demonstrate that lower dose of rscAAVrh74.miniCMV.LIPA still show therapeutic benefits. (A) Gross pathology of the liver and spleen in the untreated WT and *Lipa*$^{-/-}$ and treated *Lipa*$^{-/}$at different doses with rscAAVrh74.miniCMV.LIPA. Scale bar = 1 cm. (B-E) Relative weight of liver (B), spleen (C), intestines (D), and lymph node (E) after treatment with different doses. (F-G) Serum ALT and AST levels with different doses. All data represented as mean ± SD (n=3-8). Statistical significance between groups is denoted by different letters, P<0.05, using one-way ANOVA with Tukey's post-hoc test.

[0050] Figures 21A-21 E demonstrate that all doses of rscAAVrh74.miniCMV.LIPA treatment results in restored LIPA expression and lysosomal acid lipase enzyme activity. (A) Biodistribution of AAV decreases with decreasing dose in the liver, spleen, intestine, lymph node, heart, and lung. (B) LIPA expression also decreases with dose in the liver, spleen, intestine, lymph node, heart, and lung. (C-E) Lysosomal acid lipase activity in the liver, spleen, and serum. All data represented as mean ± SD (n=3-8). Statistical significance between groups is denoted by different letters, $P<0.05$, using one-way ANOVA with Tukey's post-hoc test.

[0051] Figures 22A-22D demonstrate that lipoid content is reduced with treatment at all doses. (A) Cholesterol levels in the liver. (B) Cholesterol content in the spleen. (C) Triglyceride content in the liver. (D) Triglyceride content in the spleen. All data represented as mean ± SD (n=3-8). Statistical significance between groups is denoted by different letters, $P<0.05$, using one-way ANOVA with Tukey's post-hoc test.

[0052] Figure 23 provides an annotated sequence of ptrs-miniCMV.LIPA.KanR (5626 bp).

Detailed Description

[0053] While enzyme replacement protein therapy has shown clinical efficacy in WD and CESD patients and is approved by the FDA, such treatments require protein infusions every two weeks and give rise to only partial clinical correction (Burton et al. *N Engl J Med* **373**: 1010-1020, 2015). In the phase 3 enzyme replacement clinical trial of Sebelipase Alfa (also called Kanuma), for example, elevations in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and low-density lipoprotein (LDL)-cholesterol, were, on average, reduced only by 50%, at best, with a 32% overall reduction in liver fat content and a 6.8% reduction in spleen volume (Burton et al. *supra*). As demonstrated herein, AAV delivery of the LIPA gene can have far greater impacts on these measures in a LAL-D mouse model.

[0054] The disclosure provides a recombinant (r) self-complementary (sc) AAV vector, rscAAVrh74.mCMV.LIPA, for use in treating WD and CESD patients. The rhesus 74 (rh74) serotype of AAV, originally isolated from the spleen of a rhesus macaque has shown safety at high intravenous doses ($2x10^{14}$vg/kg) in clinical trials with pediatric patients. rAAVrh74 is similar to rAAV8, rAAV9, and rAAVrh.10 in that it shows a high propensity to enter tissues after intravenous (IV) delivery to the blood, allowing for systemic multi-organ perfusion of the designed gene therapy using a single dose scheme (Zygmunt et al., *Mol Ther Methods Clin Dev* **15**: 305-319, 2019). This dosing can last, in theory, at least in post-mitotic cells, for the lifetime of the animal (Chicoine et al., *Mol Ther* **22**: 713-724, 2014; Martin et al., *Am J Physiol Cell Physiol* **296**: C476-488, 2009). AAV is unique in its safety profile, as the viral

genome, once transduced into its carrier cell, remains stably expressed as an episomal DNA and only very rarely ever integrates into the host genome (Grieger et al., *Adv Biochem Eng Biotechnol* **99**: 119-145, 2005; Xiao et al. *J Virol* **72**: 2224-2232, 1998). As with almost all AAV serotypes, liver and spleen are the most highly perfused organs when rAAVrh74 is delivered intravenously (Bish et al., *Hum Gene Ther* **19**: 1359-1368, 2008) Liver and spleen typically receive logarithmically higher numbers of AAV DNA vector genomes (vg) than for other organs when adult animals are dosed, and this is true in multiple mammalian species, including humans, rhesus macaques, dogs, and rodents, including mice (Cunningham et al. *Methods Mol Biol* **1937**: 213-219., 2019, Palaschak et al., *Methods Mol Biol* **1950**: 333-360, 2019). Such features make AAV an ideal gene delivery method for treatment of genetic disorders such as LAL-D, where liver and spleen are the most affected organs (Aguisanda et al., *supra;* Burton et al. *supra.)*.

[0055] The disclosed AAV vector is optimized for therapeutic usefulness in LAL-D. The self-complementary (sc) technology allows for binding of the single-stranded viral DNA genome onto itself, thereby priming second strand DNA synthesis. This self-complimentary element both quickens and strengthens gene expression relative to constructs lacking the self-complimentary element. Use of the self-complimentary technology is important for effective treatment of LAL-D, as children with complete deficiency become severely ill within a week or two after birth. Thus, use of a single-stranded rAAV vector, which will take 3-4 week for maximal onset of gene expression, would not be ideal for preventing a disease with such an early and severe onset. Because only about 2.2kB of DNA can be packaged into self-complementary AAV vectors, a little less than half what can normally be packaged in a single-stranded vector (4.7kB), a miniaturized Cytomegalovirus (mCMV) promoter was used to allow for all of the required DNA elements to be included along with the full-length human LIPA transgene. This miniaturized mCMV promoter has been used by Fu and McCarty to drive scAAV vectors to treat other lysosomal storage disorders such as Mucopolysaccharidosis, where the mCMV promoter shows the ability to induce gene expression across a broad spectrum of cells and tissues II (Fu et al., *Mol Ther Methods Clin Dev* **10**: 327-340, 2008). The size limitation of AAV in the self-complimentary form, coupled with the size of the LIPA gene itself, make it impossible to use a full-length form of the CMV promoter to produce a self-complementary AAV form containing the LIPA gene. AAV yields were reduced 19-fold when the full-length CMV promoter was used, likely from reduced genomic packaging into viral capsids because of the increased size of the AAV genome. Packaging yields from a standard small scale AAV preparation average $1.15\pm0.15x10^{13}$vg when the miniCMV is used and average only $6.0\pm1.1x10^{11}$vg when the full-length CMV

promoter is used (n=2/group). Thus, miniCMV is an important design element of this AAV vector.

LIPA Mutations

[0056] The *LIPA* gene is located on human chromosome 10q23.2–23.3 and consists of 10 exons spread over approximately 38 kb. *LIPA* has 3 transcript variants: Variant 2 (NM_000235) lacks an internal segment in the 5' UTR compared with variant 1 (NM_001127605). The two variants encode the same protein isoform in size of 399 amino acids (AAs), which has been experimentally validated by cDNA cloning (Baratta et al., World J Gastroenterol 25: 4172-4180). The annotated variant 3 (NM_001288979) lacks two consecutive exons in the 5' region, which results in translation initiation at a downstream AUG and presumably a shorter protein isoform consists of 283 AAs. (Li and Zhang, Arterioscler Thromb Vasc Biol. 39(5): 850–856, 2019).

[0057] There are at least 59 known mutations in the LIPA gene. Examples of these mutations are provided in Table 1 below.

DNA	Protein	CItation
	p.T16P	Morris et al. Arterioscler Thromb Vasc Biol. 2017 Jun; 37(6): 1050–1057.
	p.T16W	Morris et al. Arterioscler Thromb Vasc Biol. 2017 Jun; 37(6): 1050–1057.
	p.(Trp140*)	
	p.(Arg218*)	
	p.(Gly266*),	
c.67G>A	p.Gly23Arg	
c.129C>G	p,Tyr43*	Ries et al Hum Mutat, 12 (1998), pp. 44-51
c.253C>A	p.Gln85Lys	
c.260G>T;	p.Gly87Val	
c.294C>G;	p.Asn98Lys	
c.229+3A > C		Pisciotta et al. Arterosclerosis 265:124-132, 2017
c.386A>G	p.His129Arg	Consuelo-Sanchez Ann. Hepatol. 18(4): 646-650, 2019
c.414dup;	p.Phe139llefs*7	Mayatepek et al. Hum Mutat, 12 (1998), pp. 44-51
	p.(Thr288Ile),	
	p.(Leu294Ser)	

c.822+37A>G		
c.894G>A	p.Gln298Gln	Mutoni et al. Hum Genet 95:491-494, 1995
c.894G>A	p.delS275_Q298	Consuelo-Sanchez Ann. Hepatol. 18(4): 646-650, 2019
c.1024G>A;	p.Gly342Arg	Consuelo-Sanchez Ann. Hepatol. 18(4): 646-650, 2019
	p.(Asp345Asn),	Pisciotta et al. Arterosclerosis 265:124-132, 2017

AAV Gene Therapy

[0058] The present disclosure provides for gene therapy vectors, e.g. rAAV vectors expressing the LIPA cDNA, and methods of treating Lysosomal Acid Lipase Deficiency (LAL-D), including Wolman disease and cholesterol ester storage disease. The disclosed gene therapy vectors are useful for treating disorders related to lipid storage and accumulation such as coronary artery disease such as atherosclerosis, type II diabetes, obesity and non-alcoholic fatty liver disease. In addition, the disclosed gene therapy vectors are useful for decreasing triglycerides, cholesterol, and/or fatty acids in a subject in need thereof, and for treating dyslipidemia or hypercholesterolemia in a subject in need thereof.

[0059] As used herein, the term "AAV" is a standard abbreviation for adeno-associated virus. Adeno-associated virus is a single-stranded DNA parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. There are currently at least thirteen serotypes of AAV that have been characterized. General information and reviews of AAV can be found in, for example, Carter, 1989, Handbook of Parvoviruses, Vol. 1, pp. 169-228, and Berns, 1990, Virology, pp. 1743-1764, Raven Press, (New York). However, it is fully expected that these same principles will be applicable to additional AAV serotypes since it is well known that the various serotypes are quite closely related, both structurally and functionally, even at the genetic level. (See, for example, Blacklowe, 1988, pp. 165-174 of Parvoviruses and Human Disease, J. R. Pattison, ed.; and Rose, Comprehensive Virology 3:1-61 (1974)). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous rep genes; and all bear three related capsid proteins such as those expressed in AAV2. The degree of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to "inverted terminal repeat sequences"

(ITRs). The similar infectivity patterns also suggest that the replication functions in each serotype are under similar regulatory control.

[0060] An "AAV vector" as used herein refers to a vector comprising one or more polynucleotides of interest (or transgenes) that are flanked by AAV terminal repeat sequences (ITRs). Such AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding and expressing rep and cap gene products.

[0061] An "AAV virion" or "AAV viral particle" or "AAV vector particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide AAV vector. If the particle comprises a heterologous polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "AAV vector particle" or simply an "AAV vector". Thus, production of AAV vector particle necessarily includes production of AAV vector, as such a vector is contained within an AAV vector particle.

[0062] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including an inverted terminal repeat (ITRs). Exemplary ITR sequences may be 130 base pairs in length or 141 base pairs in length, such as the ITR sequence set out in SEQ ID NOS: 6 and 7. The nucleotide SEQ ID NO: 6 is an exemplary 5' ITR, while the nucleotide sequence of SEQ ID NO: 7 is an exemplary 3' ITR, which contains a deletion of the terminal resolution site (referred to as "dTR"). Deletion of the terminal resolution site inhibits Rep protein nicking of the single stranded viral genome. The presence of the dTR in the 3' ITR increases self-complementary binding of the viral genome to itself, which it may do because of its small (2.2kB) size that allows for a double-stranded viral genome to be packaged within the viral capsid.

[0063] There are multiple serotypes of AAV. The nucleotide sequences of the genomes of the AAV serotypes are known. For example, the nucleotide sequence of the AAV serotype 2 (AAV2) genome is presented in Srivastava *et al., J Virol,* 45: 555-564 (1983) as corrected by Ruffing *et al., J Gen Virol,* 75: 3385-3392 (1994). As other examples, the complete genome of AAV-1 is provided in GenBank Accession No. NC_002077; the complete genome of AAV-3 is provided in GenBank Accession No. NC_1829; the complete genome of AAV-4 is provided in GenBank Accession No. NC_001829; the AAV-5 genome is provided in GenBank Accession No. AF085716; the complete genome of AAV-6 is provided in GenBank Accession No. NC_00 1862; at least portions of AAV-7 and AAV-8 genomes are provided in GenBank Accession Nos. AX753246 and AX753249, respectively (see also U.S. Patent Nos. 7,282,199 and 7,790,449 relating to AAV-8); the AAV-9 genome is provided in

Gao *et al., J. Virol., 78*: 6381-6388 (2004); the AAV-10 genome is provided in *Mol. Ther.,* 13(1): 67-76 (2006); and the AAV-11 genome is provided in *Virology,* 330(2): 375-383 (2004). Cloning of the AAVrh.74 serotype is described in Rodino-Klapac*., et al. Journal of translational medicine* **5**, 45 (2007). *Cis*-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the ITRs. Three AAV promoters (named p5, p19, and p40 for their relative map locations) drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p19), coupled with the differential splicing of the single AAV intron (*e.g.*, at AAV2 nucleotides 2107 and 2227), result in the production of four rep proteins (rep 78, rep 68, rep 52, and rep 40) from the rep gene. Rep proteins possess multiple enzymatic properties that are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology, 158*: 97-129 (1992).

[0064] AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells, for example, in gene therapy. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects many mammalian cells allowing the possibility of targeting many different tissues *in vivo*. Moreover, AAV transduces slowly dividing and non-dividing cells, and can persist essentially for the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of recombinant genomes feasible. Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, some or all of the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may be replaced with foreign DNA such as a gene cassette containing a promoter, a DNA of interest and a polyadenylation signal. The rep and cap proteins may be provided *in trans*. Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56ºC to 65ºC for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection.

[0065] Multiple studies have demonstrated long-term (>1.5 years) recombinant AAV-mediated protein expression in muscle. See, Clark *et al., Hum Gene Ther, 8*: 659-669

(1997); Kessler *et al., Proc Nat. Acad Sc. USA, 93*: 14082-14087 (1996); and Xiao *et al., J Virol, 70*: 8098-8108 (1996). See also, Chao *et al*., *Mol Ther*, *2*:619-623 (2000) and Chao *et al*., *Mol Ther*, *4*:217-222 (2001). Moreover, because muscle is highly vascularized, recombinant AAV transduction has resulted in the appearance of transgene products in the systemic circulation following intramuscular injection as described in Herzog *et al., Proc Natl Acad Sci USA, 94*: 5804-5809 (1997) and Murphy *et al., Proc Natl Acad Sci USA, 94*: 13921-13926 (1997). Moreover, Lewis *et al., J Virol, 76*: 8769-8775 (2002) demonstrated that skeletal myofibers possess the necessary cellular factors for correct antibody glycosylation, folding, and secretion, indicating that muscle is capable of stable expression of secreted protein therapeutics.

[0066] Recombinant AAV genomes of the disclosure comprise nucleic acid molecule of the disclosure and one or more AAV ITRs flanking a nucleic acid molecule. AAV DNA in the rAAV genomes may be from any AAV serotype for which a recombinant virus can be derived including, but not limited to, AAV serotypes (e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVRH10, AAVRH74, AAV11, AAV12, AAV13, or Anc80, AAV7m8 and their derivatives). Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. See, for example, Marsic *et al., Molecular Therapy*, 22(11): 1900-1909 (2014). As noted in the Background section above, the nucleotide sequences of the genomes of various AAV serotypes are known in the art.

[0067] The provided recombinant AAV (*i.e*., infectious encapsidated rAAV particles) comprise a rAAV genome. The term "rAAV genome" refers to a polynucleotide sequence that is derived from a native AAV genome that has been modified. In some embodiments, the rAAV genome has been modified to remove the native cap and rep genes. In some embodiments, the rAAV genome comprises the endogenous 5' and 3' inverted terminal repeats (ITRs). In some embodiments, the rAAV genome comprises ITRs from an AAV serotype that is different from the AAV serotype from which the AAV genome was derived. In some embodiments, the rAAV genome comprises a transgene of interest flanked on the 5' and 3' ends by inverted terminal repeat (ITR). In some embodiments, the rAAV genome comprises a "gene cassette." In exemplary embodiments, the genomes of both rAAV lack AAV rep and cap DNA, that is, there is no AAV rep or cap DNA between the ITRs of the genomes.

[0068] The rAAV genomes provided herein, in some embodiments, comprise one or more AAV ITRs flanking the transgene polynucleotide sequence. The transgene polynucleotide sequence is operatively linked to transcriptional control elements (including, but not limited to, promoters, enhancers and/or polyadenylation signal sequences) that are functional in

target cells to form a gene cassette. Examples of promoters are the miniCMV promoter having the nucleotide sequence of SEQ ID NO: 3. Additional promoters are contemplated herein including, but not limited to the chicken β actin promoter (CBA), the P546 promoter the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the elongation factor-1a promoter, the hemoglobin promoter, and the creatine kinase promoter.

[0069] Additionally provided herein are a miniCMV promoter sequence, and promoter sequences at least: 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the nucleotide sequence of the miniCMV (SEQ ID NO: 3) sequence which exhibit transcription promoting activity.

[0070] Other examples of transcription control elements are tissue specific control elements, for example, promoters that allow expression specifically within neurons or specifically within astrocytes. Examples include neuron specific enolase and glial fibrillary acidic protein promoters. Inducible promoters are also contemplated. Non-limiting examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline-regulated promoter. The gene cassette may also include intron sequences to facilitate processing of a transgene RNA transcript when expressed in mammalian cells. One example of such an intron is the SV40 intron.

[0071] rAAV genomes provided herein comprises a polynucleotide (SEQ ID NO: 1) encoding LIPA protein. In some embodiments, the rAAV genomes provided herein comprises a polynucleotide that encodes a polypeptide comprising an amino acid sequence that is at least: 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence encoded by the LIPA cDNA (SEQ ID NO 1).

[0072] rAAV genomes provided herein comprises a nucleotides 1853-3906 of SEQ ID NO: 4. In some embodiments, the rAAV genomes provided herein comprises a polynucleotide that at least: 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the nucleotide sequences of nucleotides 1853-3906 of SEQ ID NO: 4.

[0073] rAAV genomes provided herein, in some embodiments, a polynucleotide sequence that encodes an LAL protein and that hybridizes under stringent conditions to the polynucleotide sequence set forth in SEQ ID NO: 1 or the complement thereof.

[0074] DNA plasmids of the disclosure comprise rAAV genomes of the disclosure. The DNA plasmids are transferred to cells permissible for infection with a helper virus of AAV (e.g., adenovirus, E1-deleted adenovirus or herpesvirus) for assembly of the rAAV genome into infectious viral particles. Techniques to produce rAAV particles, in which an AAV genome to be packaged, rep and cap genes, and helper virus functions are provided to a cell are standard in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (*i.e.*, not in) the rAAV genome, and helper virus functions. The AAV rep and cap genes may be from any AAV serotype for which recombinant virus can be derived and may be from a different AAV serotype than the rAAV genome ITRs, including, but not limited to, AAV serotypes AAV-9, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAVrh.74, AAV-8, AAV-10, AAV-11, AAV-12 and AAV-13. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692 which is incorporated by reference herein in its entirety.

[0075] A method of generating a packaging cell is to create a cell line that stably expresses all the necessary components for AAV particle production. For example, a plasmid (or multiple plasmids) comprising a rAAV genome lacking AAV rep and cap genes, AAV rep and cap genes separate from the rAAV genome, and a selectable marker, such as a neomycin resistance gene, are integrated into the genome of a cell. AAV genomes have been introduced into bacterial plasmids by procedures such as GC tailing (Samulski *et al*., 1982, *Proc. Natl. Acad. S6. USA*, 79:2077-2081), addition of synthetic linkers containing restriction endonuclease cleavage sites (Laughlin *et al*., 1983, Gene, 23:65-73) or by direct, blunt-end ligation (Senapathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). The packaging cell line is then infected with a helper virus such as adenovirus. The advantages of this method are that the cells are selectable and are suitable for large-scale production of rAAV. Other examples of suitable methods employ adenovirus or baculovirus rather than plasmids to introduce rAAV genomes and/or rep and cap genes into packaging cells.

[0076] General principles of rAAV production are reviewed in, for example, Carter, 1992, Current Opinions in Biotechnology, 1533-539; and Muzyczka, 1992, Curr. Topics in Microbial. and Immunol., 158:97-129). Various approaches are described in Ratschin *et al*., *Mol. Cell. Biol*. 4:2072 (1984); Hermonat *et al., Proc. Natl. Acad. Sci. USA*, 81:6466 (1984); Tratschin et al., *Mo1. Cell. Biol*. 5:3251 (1985); McLaughlin *et al., J. Virol*., 62:1963 (1988); and Lebkowski *et al., Mol. Cell. Biol.,* 7:349 (1988). Samulski *et al.*, J. Virol., 63:3822-3828 (1989); U.S. Patent No. 5,173,414; WO 95/13365 and corresponding U.S. Patent No. 5,658.776 ; WO 95/13392; WO 96/17947; PCT/US98/18600; WO 97/09441 (PCT/US96/14423); WO 97/08298 (PCT/US96/13872); WO 97/21825 (PCT/US96/20777);

WO 97/06243 (PCT/FR96/01064); WO 99/11764; Perrin *et al. Vaccine* 13:1244-1250 (1995); Paul *et al. Human Gene Therapy* 4:609-615 (1993); Clark *et al. Gene Therapy* 3:1124-1132 (1996); U.S. Patent. No. 5,786,211; U.S. Patent No. 5,871,982; and U.S. Patent. No. 6,258,595. The foregoing documents are hereby incorporated by reference in their entirety herein, with particular emphasis on those sections of the documents relating to rAAV production.

[0077] The disclosure thus provides packaging cells that produce infectious rAAV. In one embodiment packaging cells may be stably transformed cancer cells such as HeLa cells, 293 cells and PerC.6 cells (a cognate 293 line). In another embodiment, packaging cells are cells that are not transformed cancer cells, such as low passage 293 cells (human fetal kidney cells transformed with E1 of adenovirus), MRC-5 cells (human fetal fibroblasts), WI-38 cells (human fetal fibroblasts), Vero cells (monkey kidney cells) and FRhL-2 cells (rhesus fetal lung cells).

[0078] The rAAV may be purified by methods standard in the art such as by column chromatography or cesium chloride gradients. Methods for purifying rAAV vectors from helper virus are known in the art and include methods disclosed in, for example, Clark *et al., Hum. Gene Ther., 10*(6): 1031-1039 (1999); Schenpp and Clark, *Methods Mol. Med., 69* 427-443 (2002); U.S. Patent No. 6,566,118 and WO 98/09657.

[0079] Compositions provided herein comprise rAAV and a pharmaceutically acceptable excipient or excipients. Acceptable excipients are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include, but are not limited to, buffers such as phosphate [e.g., phosphate-buffered saline (PBS)], citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, copolymers such as poloxamer 188, pluronics (e.g., Pluronic F68) or polyethylene glycol (PEG).

[0080] Dosages of rAAV to be administered in methods of the disclosure will vary depending, for example, on the particular rAAV, the mode of administration, the time of administration, the treatment goal, the individual, and the cell type(s) being targeted, and may be determined by methods standard in the art. Dosages may be expressed in units of viral genomes (vg). Dosages contemplated herein include about $1x10^7$, $1x10^8$, $1x10^9$,$5x10^9$,

6 $x10^9$, $7x10^9$, $8x10^9$, $9x10^9$, $1x10^{10}$, $2x10^{10}$, $3x10^{10}$, $4x10^{10}$, $5x10^{10}$, $1x10^{11}$, about $1x10^{12}$, about $1x10^{13}$, about $1.1x10^{13}$, about $1.2x10^{13}$, about $1.3x10^{13}$, about $1.5x10^{13}$, about 2 $x10^{13}$, about 2.5 $x10^{13}$, about 3 x 10^{13}, about 3.5 x 10^{13}, about 4x 10^{13}, about 4.5x 10^{13}, about 5 x 10^{13}, about $6x10^{13}$, about 7 $x10^{13}$, about $8x10^{13}$, about $9x10^{13}$, about $1x10^{14}$, about 2 $x10^{14}$, about 3 x 10^{14}, about 4x 10^{14}, about $5x10^{14}$, about $1x10^{15}$, to about $1x10^{16}$, or more total viral genomes.

[0081] Dosages of about $1x10^9$ to about 1 $x10^{10}$, about 5x 10^9 to about 5 $x10^{10}$, about $1x10_{10}$ to about 1x 10^{11}, about $1x10^{11}$ to about $1x10^{15}$ vg, about $1x10^{12}$ to about $1x10^{15}$ vg, about $1x10^{12}$ to about $1x10^{14}$ vg, about $1x10^{13}$ to about $6x10^{14}$ vg, about $1x10^{13}$ to about $1x10^{15}$ vg and about $6x10^{13}$ to about $1.0x10^{14}$ vg are also contemplated. One dose exemplified herein is $1x10^{13}$ vg administered via intravenous or intraperitoneal delivery.

[0082] Dosages are also may be expressed in units of vg/kg. Dosages contemplated herein include about $1x10^7$ vg/kg, $1x10^8$ vg/kg, $1x10^9$ vg/kg, $5x10^9$ vg/kg, 6 $x10^9$ vg/kg, $7x10^9$ vg/kg, $8x10^9$ vg/kg, $9x10^9$ vg/kg, $1x10^{10}$ vg/kg, $2x10^{10}$ vg/kg ^{10}, $3x10^{10}$ vg/kg, $4x10^{10}$ vg/kg, $5x10^{10}$ vg/kg, $1x10^{11}$ vg/kg, about $1x10^{12}$ vg/kg, about $1x10^{13}$ vg/kg, about $1.1x10^{13}$ vg/kg, about $1.2x10^{13}$ vg/kg, about $1.3x10^{13}$ vg/kg, about $1.5x10^{13}$ vg/kg, about 2 $x10^{13}$ vg/kg, about 2.5 $x10^{13}$ vg/kg, about 3 x 10^{13} vg/kg, about 3.5 x 10^{13} vg/kg, about 4x 10^{13} vg/kg, about 4.5x 10^{13} vg/kg, about 5 x 10^{13} vg/kg, about $6x10^{13}$ vg/kg, about 7 $x10^{13}$, about $8x10^{13}$, about $9x10^{13}$, about $1x10^{14}$ vg/kg, about 2 $x10^{14}$ vg/kg, about 3 x 10^{14} vg/kg, about 4x 10^{14} vg/kg about $5x10^{14}$ vg/kg, about $1x10^{15}$ vg/kg, to about $1x10^{16}$ vg/kg.

[0083] Dosages of about $1x10^9$ vg/kg to about 1 $x10^{10}$ vg/kg, about 5x 10^9 vg/kg to about 5 $x10^{10}$ vg/kg, about $1x10^{10}$ vg/kg to about 1x 10^{11} vg/kg, about $1x10^{11}$ vg/kg to about $1x10^{15}$ vg/kg, about $1x10^{12}$ vg/kg to about $1x10^{15}$ vg/kg, about $1x10^{12}$ vg/kg to about $1x10^{14}$ vg/kg, about $1x10^{13}$ vg/kg to about $2x10^{14}$ vg/kg, about $1x10^{13}$ vg/kg to about $1x10^{15}$ vg/kg and about $6x10^{13}$ vg/kg to about $1.0x10^{14}$ vg/kg are also contemplated. One dose exemplified herein is $1x10^{13}$ vg/g administered via intravenous or intraperitoneal delivery.

[0084] Methods of transducing a target cell with rAAV, *in vivo* or *in vitro*, are contemplated by the disclosure. The *in vivo* methods comprise the step of administering an effective dose, or effective multiple doses, of a composition comprising a rAAV of the disclosure to an animal (including a human being) in need thereof. If the dose is administered prior to development of a disorder/disease, the administration is prophylactic. If the dose is administered after the development of a disorder/disease, the administration is therapeutic. In embodiments of the disclosure, an effective dose is a dose that alleviates (eliminates or reduces) at least one symptom associated with the disorder/disease state being treated, that slows or prevents progression to a disorder/disease state, that slows or prevents

progression of a disorder/disease state, that diminishes the extent of disease, that results in remission (partial or total) of disease, and/or that prolongs survival. Example of a LAL-D contemplated for prevention or treatment with methods of the disclosure is Wolman Disease and Cholesterol Ester Storage Disease or a disorder related to lipid storage or accumulation such as coronary artery disease, atherosclerosis, type II diabetes, obesity, r non-alcoholic fatty liver disease, dyslipidemia or hypercholesterolemia.

[0085] Combination therapies are also contemplated by the disclosure. Combination as used herein includes both simultaneous treatment and sequential treatments. Combinations of methods of the disclosure with standard medical treatments are specifically contemplated, as are combinations with novel therapies. In some embodiments, the combination therapy comprises administering an immunosuppressing agent in combination with the gene therapy disclosed herein.

[0086] Administration of an effective dose of the compositions may be by routes standard in the art including, but not limited to, intramuscular, parenteral, intravenous, intraarterial, intraperitoneal, oral, buccal, nasal, pulmonary, intracranial, intraosseous, intraocular, rectal, or vaginal. Route(s) of administration and serotype(s) of AAV components of the rAAV (in particular, the AAV ITRs and capsid protein) of the disclosure may be chosen and/or matched by those skilled in the art taking into account the infection and/or disease state being treated and the target cells/tissue(s) that are to express the wild type LAL protein.

[0087] The disclosure provides for local administration and systemic administration of an effective dose of rAAV and compositions of the disclosure. For example, systemic administration is administration into the circulatory system so that the entire body is affected. Systemic administration includes enteral administration such as absorption through the gastrointestinal tract and parenteral administration through injection, infusion or implantation.

[0088] Transduction of cells with rAAV of the disclosure results in sustained expression of the LAL protein. The present disclosure thus provides methods of administering/delivering rAAV which express LAL protein to an animal, preferably a human being. These methods include transducing cells with one or more rAAV of the present disclosure.

[0089] The term “transduction” is used to refer to the administration/delivery of the coding region of the LIPA gene to a recipient cell either *in vivo* or *in vitro*, via a replication-deficient rAAV of the disclosure resulting in expression of LAL the recipient cell.

Immunosuppressing Agents

[0090] The immunosuppressing agent may be administered before or after the onset of an immune response to the rAAV in the subject after administration of the gene therapy. In

addition, the immunosuppressing agent may be administered simultaneously with the gene therapy or the protein replacement therapy. The immune response in a subject includes an adverse immune response or an inflammatory response following or caused by the administration of rAAV to the subject. The immune response may be the production of antibodies in the subject in response to the administered rAAV.

[0091] Exemplary immunosuppressing agents include glucocorticosteroids, janus kinase inhibitors, calcineurin inhibitors, mTOR inhibitors, cyctostatic agents such as purine analogs, methotrexate and cyclophosphamide, inosine monophosphate dehydrogenase (IMDH) inhibitors, biologics such as monoclonal antibodies or fusion proteins and polypeptides, and di peptide boronic acid molecules, such as Bortezomib.

[0092] The immunosuppressing agent may be an anti-inflammatory steroid, which is a steroid that decreases inflammation and suppresses or modulates the immune system of the subject. Exemplary anti-inflammatory steroid are glucocorticoids such as prednisolone, betamethasone, dexamethasone, methotrexate, hydrocortisone, methylprednisolone, deflazacort, budesonide or prednisone.

[0093] Janus kinase inhibitors are inhibitors of the JAK/STAT signaling pathway by targeting one or more of the Janus kinase family of enzymes. Exemplary janus kinase inhibitors include tofacitinib, baricitinib, upadacitinib, peficitinib, and oclacitinib.

[0094] Calcineurin inhibitors bind to cyclophilin and inhibits the activity of calcineurin Exemplary calcineurine inhibitors includes cyclosporine, tacrolimus and picecrolimus.

[0095] mTOR inhibitors reduce or inhibit the serine/threonine-specific protein kinase mTOR. Exemplary mTOR inhibitors include rapamycin (also known as sirolimus), everolimus, and temsirolimus.

[0096] The immunosuppressing agents include immune suppressing macrolides. The term “immune suppressing macrolides” refer to macrolide agents that suppresses or modulates the immune system of the subject. A macrolide is a class of agents that comprise a large macrocyclic lactone ring to which one or more deoxy sugars, such as cladinose or desoamine, are attached. The lactone rings are usually 14-, 15-, or 16-membered. Macrolides belong to the polyketide class of agents and may be natural products. Examples of immunosuppressing macrolides include tacrolimus, pimecrolimus, and rapamycin (also known as sirolimus).

[0097] Purine analogs block nucleotide synthesis and include IMDH inhibitors. Exemplary purine analogs include azathioprine, mycophenolate such as mycophenolate acid or mycophenolate mofetil and lefunomide.

[0098] Exemplary immunosuppressing biologics include abatacept, adalimumab, anakinra, certolizumab, etanercept, golimumab, infliximab, ixekizumab, natalizumab, rituximab, secukinumab, tocilizumab, ustekinenumab, vedolizumab, basiliximab, belatacep, and daclizumab.

[0099] In particular, the immunosuppressing agent is an anti-CD20 antibody. The term anti-CD20 specific antibody refers to an antibody that specifically binds to or inhibits or reduces the expression or activity of CD20. Exemplary anti-CD20 antibodies include rituximab, ocrelizumab or ofatumumab.

[00100] Additional examples of immuosuppressing antibodies include anti-CD25 antibodies (or anti-IL2 antibodies or anti-TAC antibodies) such as basiliximab and daclizumab, and anti-CD3 antibodies such as muromonab-CD3, otelixizumab, teplizumab and visilizumab, anti-CD52 antibodies such as alemtuzumab.

[00101] The following EXAMPLES are provided by way of illustration and not limitation. Described numerical ranges are inclusive of each integer value within each range and inclusive of the lowest and highest stated integer.

EXAMPLES

Example 1
Gene Therapy Constructs Encoding LIPA

[00102] AAV genome constructs encoding LIPA were generated as set forth in Figure 1, which depicts the AAVrh74 vector design with the full-length transcript of LIPA cDNA under the control of a miniCMV promoter (SEQ ID NO: 3).

[00103] A human GFP cDNA clone was obtained from Origene, Rockville, MD. The LIPA cDNA alone was further subcloned into a self-complementary AAVrh74 genome under the control of a miniCMV promoter. The plasmid construct also included an intron such as the simian virus 40 (SV40) chimeric intron, and a polyadenylation signal (PolyA). The constructs were packaged into either AAVrh74 genome.

[00104] The LIPA cDNA expression cassette had a Kanamycin resistance gene, and an optimized Kozak sequence, which allows for more robust transcription. rAAV vectors were produced by a modified cross-packaging approach whereby the AAVrh74 vector genome can be packaged into multiple AAV capsid serotypes [Rabinowitz et al., J Virol. 76 (2):791-801 (2002)]. Production was accomplished using a standard three plasmid DNA/CaPO4 precipitation method using HEK293 cells. HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin. The production plasmids were: (i) plasmids encoding the therapeutic proteins, (ii) rep2-capX

modified AAV helper plasmids encoding cap serotype AAVrh74 isolate, and (iii) an adenovirus type 5 helper plasmid (pAdhelper) expressing adenovirus E2A, E4 ORF6, and VA I/II RNA genes. A quantitative PCR-based titration method was used to determine an encapsidated vector genome (vg) titer utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems). [Clark et al., Hum Gene Ther. 10 (6): 1031-1039 (1999)]. A final titer (vg ml−1) was determined by quantitative reverse transcriptase PCR using the specific primers and probes utilizing a Prism 7500 Real-time detector system (PE Applied Biosystems, Grand Island, NY, USA). Aliquoted viruses were kept at −80 °C.

[00105] All plasmids used to make AAV genomes to be packaged also contain a Kanamycin resistance gene (KanR) outside of the ITR sequences used for packaging of the genome. This allows for the DNA encoding the AAV genome to be transformed into bacteria to produce large amounts of DNA in the presence of Kanamycin, which will kill all non-transformed bacteria. KanR is not packaged into the AAV capsid in the AAV genome used to treat patients, but its presence allows for DNA production in bacteria.

[00106] The map for plasmid r(sc) AAVrh74.miniCMV.LIPA is set out in Figure 2 and the sequence of the entire plasmid is provided in SEQ ID NO: 4. The vector scAAVrh74.miniCMV.LIPA comprises the nucleotide sequence within and inclusive of the ITR's of SEQ ID NO: 4. The rAAV vector comprises the 5' AAV2 ITR, miniCMV promoter, the coding sequence for the LIPA gene, SV40 late polyA, and 3' AAV2 ITR. The plasmid set forth in SEQ ID NO: 4 further comprises kanamycin resistance with pUC origin of replication.

[00107] **Table 2** shows the molecular features of the plasmid (SEQ ID NO: 4), in which range refers to the nucleotides in SEQ ID NO: 4 and ► indicates the kanamycin gene is in the forward orientation.

Name	Range	Strand	Length
F1 ori	1158 - 1465	►	308
LacZ alpha	1783 - 1851	►	69
Wt 5' ITR	1853 - 1982	►	130
Mini CMV	1992 - 2219	►	228
LIPA transcript variant 1	2254 - 3453	►	1200
SV40 late polyA	3480 - 3671	►	192
Wt 3' ITR (with d TR)	3679 - 3906	►	228
ori	4025 – 4613	►	589
KanR	4735 - 5550	►	816

Example 2

LIPA Gene Replacement in LIPA-Deficient Mice

[00108] The following study tested AAV-based *LIPA* (human) gene replacement therapy in pure-bred *Lipa*-deficient (*Lipa*$^{-/-}$) mice. *Lipa*$^{-/-}$ mice, like WD and CESD patients, develop severe liver dysfunction and damage as the result of the massive loading of cholesterol esters and triglycerides into this organ (Du et al. *Hum Mol Genet* **7**: 1347-1354, 1998; Du et al. *J Lipid Res* **42**: 489-500, 2001). For example, the mice develop hepatosplenomegaly, elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and elevated liver and spleen cholesterol and triglycerides. Disease is evident within 3 weeks of birth, and *Lipa*$^{-/-}$ mice have profound disease by 4 months, showing a 3- to 6-fold increase in the size of the liver and the spleen. Mice succumb to disease several months thereafter, beginning at 6 months of age.

[00109] Mice: The *lal*$^{-/-}$ mice were first generated by Du et al in 1998. The mouse model has been widely used to study the role of Lal in multiple organ systems. The *lal*$^{-/-}$ mice on a mixed genetic background of 129Sv and CF-1 survive into adulthood, and are fertile, but die at ages of 7 to 8 months. The *lal*$^{-/-}$ mice show massive accumulation of TGs and CEs in liver, spleen, and small intestine. These mice resemble hepatosplenomegaly, the major clinical manifestation of WD and CESD in human. The *lal*$^{-/-}$ mice provide a model to study human WD and CESD, but more importantly, serve as a powerful tool to investigate the systemic impacts of lysosomal lipolysis on metabolic and immune homeostasis.

[00110] One day-old (P1) *Lipa*$^{-/-}$ mice were intravenously administered a single injection rscAAVrh74.mCMV.*LIPA.* 8x10^{13}vg/kg rscAAVrh74.mCMV.*LIPA* was intravenously administered via the retro-orbital vein. The *Lipa*$^{-/-}$ mice used were bred on a pure FvB/NJ background, which almost doubled the litter size relative to C57Bl/6J-bred mice but did not significantly change disease phenotypes. Without treatment by 4 months of age, livers in *Lipa*$^{-/-}$ mice have become severely enlarged and damaged.

[00111] In the mock-treated *Lipa*$^{-/-}$ mice at 4 months of age, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels mice were elevated 10-fold compared to wild type mice, and these elevations were reduced 90% by AAV treatment (Fig. 2). For both the AST and ALT measures, this was a highly significant change ($p < 0.0001$ for both comparisons). AST and ALT are markers for liver damage, and they are the primary clinical biomarker used to follow LAL-D disease in patients.

[00112] Treatment with rscAAVrh74.mCMV.*LIPA* also resulted in a complete normalization of serum LDL-cholesterol, which was increased in *Lipa*$^{-/-}$ mice due to increased export of elevated cholesterol from the liver (Fig. 3). Other serum measures that are impacted by disease, for example reduced serum HDL-cholesterol (which mediates cholesterol influx to the liver) and reduced serum glucose (which likely reflects reduced

nutrient uptake into the intestine), both of which are reduced by more than half in 4mo *Lipa*$^{-/-}$ mice, were significantly increased towards baseline wild type levels by AAV treatment (not shown). Other measures of abnormal liver function, for example elevated serum total bilirubin and total protein, were also completely corrected by treatment with rscAAVrh74.mCMV.*LIPA*, though these changes were only statistically significant for total protein (e.g., Tot Bilirubin: WT 0.38±0.02mg/dL, *Lipa*$^{-/-}$ 0.54±.013mg/dL, *Lipa*$^{-/-}$ Treated 0.31±0.04mg/dL; Tot protein: WT 5.55±0.07g/dL, *Lipa*$^{-/-}$ 6.82±0.10g/dL ($p<0.00001$ vs. WT), *Lipa*$^{-/-}$ Treated 5.78±0.18 ($p<0.001$ vs. untreated *Lipa*$^{-/-}$).

[00113] By 4 months of age, *Lipa*$^{-/-}$ mice are easily recognizable by sight, as they have enlarged and distended abdomens due to the dramatic increase in the size of the liver and spleen, a symptom of the human disease as well (Burton et al. *J Pediatr Gastroenterol Nutr* **61**: 619-625, 2015). By the 6-month endpoint, both the spleen and the liver had increased more than 5-fold in total size (Fig. 4). This is largely due to the profound loading of triglycerides into these organs, but other organs were also affected; intestine, heart, and kidney, for example, show increased size due to fat loading (Fig. 4). Brain size, by contrast, is not increased, while muscle size decreased, most likely due to impaired nutrient absorption (Fig. 4). Treatment with the $8x10^{13}$vg/kg dose of rscAAVrh74.mCVM.*LIPA* reduced the increase in organ size by at least 70% at 6 months of age in all tested organs, with all such organs showing a significant improvement toward their baseline wild type measures (Fig. 4).

[00114] These improvements in organ size were matched by significant reductions in staining using Oil Red O (with hematoxylin counterstain in blue, Fig. 5). Oil Red O staining, to identify lipid burden in intestine, spleen, liver, and kidney, showed very significant decreases in staining in AAV-treated *Lipa*$^{-/-}$ mice compared to mock-treated *Lipa*$^{-/-}$ controls at 6 months of age. Such decreased staining was highly suggestive of reduced fat content. This was confirmed with biochemical measures of triglyceride content in liver and spleen. Here, the more than 5-fold increase in triglyceride content in untreated *Lipa*$^{-/-}$ mice was significantly reduced by AAV treatment, approaching baseline wild type levels in both instances (Fig. 6). AAV treatment also led to improvements in the overall activity levels of mice on open field tests, where mice showed as much or more ambulation activity than wild type mice. For example, at 6 months, average total ambulatory events per five-minute interval were decreased by 35% in mock-treated *Lipa*$^{-/-}$ mice compared to wild type FVB/NJ controls but this measure was increased to 1.35 times wild type activity in AAV-treated *Lipa*$^{-/-}$ mice (WT: 4477±381 events/5min, *Lipa*$^{-/-}$*:* 2925±771 events/5min, *Lipa*$^{-/-}$ Treated: 6069±72 events/5min, $p<0.05$ for treated vs. untreated *Lipa*$^{-/-}$ comparison).

[00115] Surprisingly, while it was expected that the liver would be easily transduced with AAV in *Lipa*$^{-/-}$ mice, the liver was only transduced with 1.7±0.4vg/nucleus at the 8x10^{13}vg/kg IV dose (n=4), a mere 2-fold elevation in transduction relative to skeletal muscle in the same mice (0.9±0.1vg/nucleus, n=4). While this level of transduction suggests saturation of cells in the liver (>1vg/nucleus), it is not uncommon with other AAV vectors at this dose to generate 10-100vg/nucleus [22]. This lower level of transduction is likely due to the fact that hepatocytes in the liver divide in the first month of life in mice as the liver grows, making it impossible to transduce such cells with treatment at postnatal day 2, a time prior to the genesis of such liver cells.

Example 3
Gene Therapy of Young Adult Mice

[00116] Because hepatocytes divide in the liver as it grows until about one month of age, treatment of young adult (2-month-old) mice was investigated. The 2-month *Lipa*$^{-/-}$ mice were intravenously administered a single injection rscAAVrh74.mCMV.*LIPA*. 8x10^{13}vg/kg rscAAVrh74.mCMV.*LIPA* via the retro-orbital vein. Treatment of adult *Lipa*$^{-/-}$ mice with rscAAVrh74.mCMV.*LIPA* led to a logarithmic increase in transduction of viral genomes into the liver, increasing from 2 to 50 vg/nucleus (Fig. 7).

[00117] This level of transduction led to an increased human LIPA transgene expression exceeded normal mouse LIPA gene expression found in wild type tissues, as measured by qRT-PCR (Fig. 8). The overall appearance of the liver from a 4mo *Lipa*$^{-/-}$ mice treated at 2 months of age was no different than wild type (FVBn), showing no fat whatsoever, while a 4mo *Lipa*$^{-/-}$ from a mouse treated at P1 still contained some fat, though not nearly as much as an untreated *Lipa*$^{-/-}$ mouse, which was also greatly increased in size (Fig. 9).

[00118] The reduced fat content seen visually was confirmed by the measured reduction in cholesterol and triglycerides in treated Lipa$^{-/-}$ (LIPA KO) mice in both liver and spleen, which in the 2mo condition were not significantly different from wild type (Fig. 10).

[00119] The weights of liver, spleen and intestine were all reduced to near normal levels with treatment at 2 months, despite the fact that these organs had already doubled in size due to fat burden by this point in untreated Lipa$^{-/-}$ mice (Fig. 11). Thus, rscAAVrh74.mCMV.LIPA can both prevent and reverse disease symptoms in a LAL-D model. Blood cells in lymph nodes showed greater prevention of fat burden with treatment at 2mo compared to 2 days (Fig. 11). As mouse blood cells turn over in the course of months, this suggests a trans effect of rscAAVrh74.mCMV.LIPA treatment in adult mice, as blood cells would have lost AAV expression due to turnover by 4 months of age. This finding correlated with the fact that LIPA enzyme activity was actually elevated in the serum of Lipa$^{-/-}$

mice treated with rscAAVrh74.mCMV.LIPA as adults (Fig. 12), along with its elevation in liver tissue (Fig. 13).

[00120] This data suggest that LIPA enzyme is released from the liver and is expressed systemically in the blood as the result of gene therapy treatment in the adult animal, providing therapeutic protein in trans to tissues where cells turn over constantly, such as the blood. This allows rscAAVrh74.mCMV.LIPA gene therapy to also provide enzyme replacement therapy to transduced cells and tissues when the proper dose and timing of therapy is given, but also to cells not transduced by the gene therapy. Such a therapy would allow for constant prevention of disease throughout the whole body on an ongoing basis during the patient's life.

Example 3

rscAAVrh74.miniCMV.LIPA treatment can inhibit and reverse hepatosplenomegaly and elevated serum liver enzymes in *Lipa*$^{-/-}$ mice

[00121] As an extension to the experiments described in Example 2, rscAAVrh74.miniCMV.LIPA was administered at various ages. These experiments tested an AAV dose that, when taking into account differences in titering methods, was equivalent to the highest doses currently being used in gene therapy clinical trials (36, 37). A dose of 8.4 x 10^{13} vector genomes (vg) per kilogram (vg/kg) of rscAAVrh74.miniCMV.LIPA AAV gene therapy was intravenously injected via the facial vein at early (P2, postnatal day 2) or via tail vein at middle (P60, postnatal day 60) or advanced (P120, postnatal day 120) disease stages in *Lipa*$^{-/-}$ mice. Mice were followed to endpoints of P60 (2 months of age), P120 (4 months of age) and P180 (6 months of age) (Figure 14A). At these time points, mice were necropsied, organs (liver, spleen, kidneys, intestine, mesenteric lymph node, heart, lung, thymus, brain) and muscles (left and right gastrocnemius and quadriceps) were harvested for biodistribution and gene expression. Harvested non-muscle organs were weighed and then immersed in OCT before being frozen in dry ice-cooled isopentane. Muscles were weighed and then snap-frozen in liquid nitrogen-cooled isopentane.

[00122] Hepatosplenomegaly, or the enlargement of the liver and spleen, and yellowing of organs due to increased fat deposition are both defining features of LAL-D and of disease in *Lipa*$^{-/-}$ mice. Both phenotypes were present and progressed with age in *Lipa*$^{-/-}$ mice (Figure 14B-D). Liver weight increased over time to comprise as much as 25% of total body weight by 6 months of age, in contrast to being only 5% of body weight at all 3 ages in wild type (WT) mice (Figure 14C). Similarly, spleen weight increased, on average, to 2% of total body weight at 6 months of age in *Lipa*$^{-/-}$ mice compared to 0.3% in WT (Figure 14D,). Treatment of *Lipa*$^{-/-}$ mice at P2, P60, and P120 resulted in reduced liver weight and normalized liver

color, but early treatment (P2) was far less effective than treatment at P60 or P120. P2 injection reduced liver size to wild-type levels at 2 and 4 months, suggesting disease inhibition, but only partially reduced liver weight, by about 50%, at 6 months. By contrast, P60 and P120 injections were more effective despite already present evidence of hepatosplenomegaly at these ages, reducing liver weight to 8% and 10% of total body weight, respectively, at 6 months, suggesting disease reversal. P2 injection was more effective at reducing spleen size than it was for liver at the 6-month endpoint (Figure 14D), and treatment at all 3 time points reduced spleen weight to near wild type levels. Intestines and mesenteric lymph nodes also showed increased weight in *Lipa*$^{-/-}$ mice (Figure 14E, F). Weight in intestine was increased to 4.5% of total body weight at 6 months of age compared to 3% in WT (Figure 14E), while weight of mesenteric lymph node was increased to 0.6% of body weight compared to 0.1% in WT (Figure 14F). Here again, AAV treatment reduced intestine and mesenteric lymph node weight in a manner similar to responses seen with liver, with P2 injection showing improvement to near wild type levels at 2 or 4 months that was lost by 6 months, while injection at P60 or P120 showed reductions in weight at the 6-month endpoint. Unlike liver, spleen, and intestine, there were no data that showed a complete normalization of lymph node weight at any time point with any of the treatment times tested. Instead, at best, only a 50% average reduction in weight was achieved by 6 months (Figure 14F).

[00123] Serum ALT and AST activity were also measured, and these enzyme when elevated indicate liver damage (Figure 14G, H). Both serum ALT and AST levels were elevated 20-fold in *Lipa*$^{-/-}$ mice compared to WT by 6 months. Here, treatment at all time points resulted in decreased serum ALT/AST levels, with a more pronounced effect with injection at P60 and P120 than at P2. As with liver weights, injection at P2 reduced serum ALT and AST levels at the 2- and 4-month time points to near wild-type levels, but showed only about a 50% reduction at 6 months . Injection of this dose of rscAAVrh74.miniCMV.LIPA in WT mice did not significantly elevate serum transaminase levels at any of the time points tested. Despite the increased weight and serum liver enzyme levels at 6 months for P2 injection, the several P2-treated *Lipa*$^{-/-}$ mice that lived beyond the 6-month endpoint showed a significant increase in overall lifespan relative to untreated *Lipa*$^{-/-}$ controls (Figure 15). Untreated *Lipa*$^{-/-}$ mice showed a median survival of 224 days, whereas the P2-treated *Lipa*$^{-/-}$ mice had a median survival of 506 days, more than double the median life span of untreated *Lipa*$^{-/-}$ mice.

[00124] The body weight of *Lipa*$^{-/-}$ mice did not differ from that of WT mice at any study time point (Figure 16A). Additionally, a significant reduction in muscle mass was observed in the *Lipa*$^{-/-}$ mice, about 25% (Figure 15B, C). Due to the enlarged liver, *Lipa*$^{-/-}$ mice presented

with a distended abdomen. Open field studies which determined fine, ambulatory, center, peripheral, and rearing movement events were performed as previously described (49) were carried out to determine if mobility was affected (Figure 16D-H). Fine movement (such as sniffing and grooming) and peripheral movement (movement at the periphery of the open field area) were not affected in *Lipa*$^{-/-}$ mice relative to WT, but center-cage based ambulation and rearing were significantly decreased. All such measures were improved with treatment at all time points. Such muscle atrophy may also contribute to ambulation differences in additional to abdominal distension in *Lipa*$^{-/-}$ mice, and these muscle weights were increased back to WT levels with P60 and P120 injection, while they only were marginally increased for P2 (Supplemental Figure 16B, C).

Example 4

AAV transduction in organs results in LIPA expression and restored lysosomal acid lipase enzyme activity

[00125] Biodistribution of AAV vgs was assessed in various organs and tissues from all injection time points using quantitative real-time PCR (Figure 17A) as described in (50). At the 6-month endpoint, there was a difference in organ biodistribution between mice injected at P2 versus mice injected at P60 or P120; injections at P2 showed greater AAV transduction in the heart and lungs (21.82±8.41 vg/nucleus, and 7.87±1.16 vg/nucleus, respectively; Table 3 while injection at P60 or P120 showed greater transduction in the liver (406.13±117.59 vg/nucleus and 90.69±21.48 vg/nucleus, respectively), with higher levels (20-50 vg/nucleus) also in kidney, lung, spleen, and thymus (Figure 17A, Table 4). Comparison of kidneys and muscles from the left and right side of the body plan suggested even AAV distribution. With the injections at P2, transduction levels were relatively stable over the course of the study in most organs, though there was a sharp decline in transduction between 4 and 6 months in the heart and liver (Tables 3 and 4, below). With injections at P60, transduction levels were also stable when measured at 2- and 4-months post-injection.

Table 3 - **.** Biodistribution of AAV at 6-month endpoint. Values are represented as mean vg/nucleus ± SD.

	Injected at P2 (n=8)	**Injected at P60 (n=8)**	**Injected at P120 (n=5)**
Brain	1.20±0.14	2.55±0.81	1.64±0.21
Heart	21.82±8.41	20.52±8.79	12.9412.11
Intestines	0.14±0.05	17.93±9.08	12.58±3.73
Liver	1.62±0.39	406.13±117.59	90.69±21.48
Lung	7.87±1.16	23.91±7.15	45.47±14.73
Lymph node	0.03±0.01	8.14±2.59	4.99±1.94
L kidney	0.48±0.12	34.95±11.97	33.00±6.93

R kidney	0.55±0.09	30.66±10.44	38.83±8.58
Spleen	0.07±0.02	35.66±13.19	28.31±9.92
Thymus	2.52±1.28	49.44±14.81	19.25±8.36
L gastrocnemius	1.10±0.15	10.75±2.66	6.19±3.48
R gastrocnemius	1.86±0.73	10.79±4.61	11.89±5.07
L quadricep	1.65±0.33	7.11±1.48	4.55±0.65
R quadricep	1.38±0.25	7.50±3.29	6.81±1.53

Table 4 - Biodistribution of AAV at the 2- and 4- month endpoints. Values are represented as mean vg/nucleus ± SD

	Injected at P2, 2 month endpoint (n=3)	**Injected at P2, 4 month endpoint (n=3)**	**Injected at P60, 4 month endpoint (n=3)**
Brain	0.28±0.06	1.70±1.09	1.13±0.48
Heart	47.27±4.76	74.34±18.01	0.97±0.61
Intestines	0.05±0.01	0.11±0.02	8.97±1.33
Liver	10.73±7.29	9.21±4.09	340.12±37.33
Lung	6.85±1.01	18.42±3.32	18.61±1.25
Lymph node	0.46±0.45	0.02±0.00	18.55±5.58
L kidney	0.45±0.09	0.66±0.18	23.74±4.53
R kidney	0.51±0.05	0.84±0.45	17.78±3.59
Spleen	0.23±0.07	0.08±0.01	19.26±1.79
Thymus	1.15±0.95	1.63±1.44	7.09±4.36
L gastrocnemius	2.92±0.60	0.54±0.06	4.09±2.35
R gastrocnemius	2.23±0.95	0.95±0.10	5.08±3.09
L quadricep	0.78±0.15	0.74±0.29	0.91±0.13
R quadricep	0.67±0.10	0.68±0.07	0.98±0.14

[00126] Next, quantitative reverse transcription (RT)-PCR was used to measure mRNA levels of the human LIPA transgene in treated mice compared to normal expression of endogenous mouse Lipa gene in wild-type tissues. At the 6-month study endpoint, there was 3-fold and 15-fold LIPA expression in the lung and heart, respectively with injection at P2 (Figure 17B), consistent with the finding of 7 and 20 vg/nucleus of AAV vgs in these organs at this time point. P2 injection achieved a near normal level of gene expression in liver, with human LIPA/mouse WT Lipa equal to 0.8. Interestingly there was only a 1.5-fold increase in liver in the same ratio for P60 or P120 injection at 6 months, despite the fact that there was, on average, 90 or 400 vgs/nucleus in liver at this time point, respectively, suggesting feedback inhibition on AAV-mediated LIPA transgene expression. Similarly, AAV transduction in the kidneys and lymph node did not result in increased expression LIPA/Lipa ratios despite there being 5-30 vg/nucleus in those tissues at the P60 and P120 time points.

Also of interest was the fact that P2-injected skeletal muscles (gastrocnemius and quadriceps femoris) showed a 20-fold LIPA/Lipa ratio at the 6-month time point for P2, while P60 and P120 injected muscles showed a lower ratio despite having higher AAV vgs. rscAAVrh74.miniCMV.LIPA treatment at any time point did not alter endogenous mouse Lipa expression (not shown), but tissues in *Lipa*$^{-/-}$ mice did show some reductions in endogenous Lipa gene expression relative to wild type (not shown).

[00127] Lysosomal acid lipase (LAL) enzyme activity in liver, spleen, and serum from treated and control mice was also measured (Figure 17C-E). Overall LAL enzyme activity was reduced by 90% in *Lipa*$^{-/-}$ liver relative to WT (Figure 17C). At the 6-month endpoint, liver enzyme activity did not significantly differ between the untreated *Lipa*$^{-/-}$ mice and those treated at P2 at all examined time points (Figure 17C). When injected at P60 and P120, however, AAV treatment led to a significant increase in enzyme activity that exceeded WT activity by 4-fold and 2.5-fold, respectively. In spleen, overall LAL activity was reduced about 80% relative to WT at 6 months. Similar to liver, P2-injected spleens showed no improvement in activity at 6 months. Unlike liver, however, the P60 treatment time point in spleen also showed no improvement, while P120 did show an increase, though one that did not reach WT levels (Figure 17D).

[00128] Serum LAL enzyme activity was also assayed to determine whether exogenous LAL was being secreted from cells as a result of treatment in a manner that might be utilized in trans by other tissues. Frozen liver and spleen samples were homogenized in LAL tissue extraction buffer (0.1M sodium phosphate pH 6.8, 1mM EDTA, 0.02% sodium azide, 10mM DTT, 0.5% NP-40). Protein concentrations were determined with the bicinchoninic acid assay (Pierce), using BSA as the standard. LAL activity was determined using 4-methylumbelliferyl palmitate (4-MUP; Gold Biotechnology) as the substrate, as previously described (51, 52). Briefly, 1µg of protein was added to 0.345 mM substrate solution (0.345mM 4-MUP, 90.9mM sodium acetate, pH 4.0, 1% (v/v) Triton X-100 and 0.0325% (w/v) cardiolipin), and enzymatic reactions were performed in triplicate in the presence or absence of the LAL inhibitor Lalistat2 (Sigma Aldrich). Reactions were incubated at 37°C for 3 hours in the dark. Reactions were terminated by adding 200 µl of 150 mM EDTA, pH 11.5. A standard curve was prepared ranging from 0-33.3 µM 4-methylumbelliferone (4-MU; Gold Biotechnology). Fluorescence was measured on a SpectraMax M2 plate reader (Molecular Devices) using a 355-nm excitation filter and a 460-nm emission filter. LAL activity (pmol/min/µg) was calculated by subtracting the enzymatic activity of the inhibited reaction from that of the -uninhibited reaction.

[00129] Serum enzyme activity was only performed on mice treated at P60 and P120, as there was not enough serum remaining after blood chemistry analysis from P2-treated mice.

Overall LAL activity in untreated LIPA$^{-/-}$ mice was about one third that of the WT (11.80±2.89 vs 29.43±2.91 pmol/min/μl serum). With treatment at P60 and P120, serum LAL enzyme activity was restored to WT levels (Figure 17E).

Example 5

Triglyceride and cholesterol levels are reduced in rscAAVrh74.miniCMV.LIPA-treated *Lipa*$^{-/-}$ mice

[00130] Total lipids were extracted from snap-frozen tissues using the Lipid Extraction Kit (Chloroform Free) (abcam) per manufacturer's protocol. Triglycerides were measured using the Infinity Triglycerides Reagent (Thermo Fisher Scientific), and total cholesterol was measured using the Infinity Cholesterol Reagent (Thermo Fisher Scientific). Lipid concentrations were determined against a standard curve of triglycerides or cholesterol standards (Pointe Scientific). Measurements were performed in triplicate, and absorbance values at 500 nm were measured on a Synergy 2 plate reader (BioTek Instruments).

[00131] When assayed at the 2-, 4-, and 6-month time points, total cholesterol in the liver of untreated *Lipa*$^{-/-}$ mice was constantly elevated, on average 19.96±3.97 μg/mg liver, a value approximately 12-fold higher than the WT (Figure 18). Cholesterol content in the spleen increased more slowly, from 2.65±0.66 μg/mg at 2 months, up to 5.51±0.91 μg/mg at 6 months. Cholesterol content also increased with age in WT spleen (from 1.44±0.70 μg/mg at 2 months to 2.23±0.36 μg/mg at 6 months). Treatment at P2 significantly decreased cholesterol levels in the liver and spleen at 2 months and 4 months post-injection. At 6 months, total cholesterol levels for P2 treatment reverted to untreated *Lipa*$^{-/-}$ levels in both the liver and the spleen (Figure 18B), while cholesterol content remained reduced to near WT levels for P60 and P120 injection.

[00132] Triglyceride levels in the liver of untreated *Lipa*$^{-/-}$ mice doubled between 2 months and 4 months of age (14.04±4.15 μg/mg to 29.32±3.70 μg/mg) and then remained constant at 6 months (30.66±10.45 μg/mg) (Figure 18C). Thus, at 6 months, these values, on average, were elevated 6-fold compared to WT (average 4.70±0.34 μg/mg). At all time points, treatment reduced liver triglycerides significantly, approaching or reaching WT-levels (Figure 18C). In the spleen, triglyceride content in *Lipa*$^{-/-}$ mice increased more gradually between 2 months and 6 months of age (2.27±1.32 μg/mg to 4.31±2.82 μg/mg), and it was not until 6 months of age that *Lipa*$^{-/-}$ spleen triglyceride content was significantly greater than WT. Unlike liver, treatment at P2 and P60 did not significantly alter triglyceride content in the spleen at the 2 month, 4 month, or 6 month endpoint (Figure 18D). Instead, only treatment at P120 showed a significant decrease.

[00133] Serum changes in *Lipa*$^{-/-}$ mice included reduced total cholesterol, triglycerides, HDL cholesterol, and free fatty acids, along with elevated LDL cholesterol (Figure 18E-I). Much as seen with lipid levels in the liver and spleen, P2 treatment did not significantly ameliorate any of these changed serum lipid levels at the 2-, 4- or 6-month endpoint, but there were often trends toward improvement. By contrast, treatment at P60 and P120 offset reductions in total cholesterol, HDL cholesterol and free fatty acids in *Lipa*$^{-/-}$ mice, generating levels near those found in WT mice at the 6-month endpoint. Similarly, treatment partially corrected triglycerides and LDL cholesterol.

[00134] Oil Red O (ORO) staining of tissue sections was used to visualize neutral lipids in the liver, spleen, and intestine (Figure 18J). Compared to the WT, there were large accumulations of lipids stained with ORO in untreated *Lipa*$^{-/-}$ liver, spleen, and intestine, with almost ubiquitous strong staining being present in the liver. With treatment at all time points, there was a decrease in overall ORO staining, but this was most pronounced for treatment at P60 and P120. After treatment, ORO staining appeared primarily as lipid islands within the tissue, with the more diffuse staining seen in untreated *Lipa*$^{-/-}$ mice being absent from the remainder of the section.

Example 6
LIPA expression reduces macrophages in the liver

[00135] To determine the extent of liver hepatocyte expression in treated livers and the extent of macrophage occupancy by performing LIPA and CD68 immunostaining. 10 μm frozen tissue sections were prepared from liver samples. Slides were fixed in acetone for 10 min at -20°C, allowed to air dry to evaporate excess acetone and washed 2x in PBS. Slides were incubated in BLOXALL Endogenous Blocking Solution (Vector Laboratories) for 10 minutes, washed 2x in PBS, then blocked in 2.5% normal serum for 30 minutes. Slides were incubated overnight at 4°C with antibodies to LIPA (1:1000; HPA057052, Sigma Aldrich), or CD68 (1:500; MCA1957GA, Bio-Rad). Following incubation, slides were washed 2x in PBS and incubated with ImmPRESS HRP Reagent (Vector Laboratories) for 30 minutes. Slides were then washed 2x in PBS and stained with ImmPACT DAB staining solution (Vector Laboratories) for 5 minutes. They were then counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, differentiated, and mounted in Cytoseal XYL (Thermo Fisher Scientific) with a glass cover slip.

[00136] As shown in Figure 19, at the 6-month endpoint, P2-treated *Lipa*$^{-/-}$ mice showed only very diffuse and weak staining in liver cells, with little to no punctate staining that would be expected for localization of protein to lysosomes. By contrast, mice injected at P60 and

P120 showed increased punctate immunostaining that was evident throughout liver sections. Anti- CD68 staining, a marker used to detect macrophages and Kupffer cells, showed highly elevated staining in untreated *Lipa*$^{-/-}$ mice. With treatment at all time points, there was a decrease in CD68-positive macrophages, though staining remained within lipid islands present in the liver. Thus, LIPA and CD68 immunostaining supported the notion that P60 and P120 treatment led to high levels of LIPA expression in the liver and to a lowered macrophage occupancy.

Example 7

Lower doses of rscAAVrh74.miniCMV.LIPA still show therapeutic benefits

[00137] A dose of 8.4 x 10^{13} vg/kg was administered to ensure saturation to determine if gene therapy was feasible for LAL-D. Given the promising data from this dosage, an experiment was designed to determine if lower doses of the gene therapy vector would still prove efficacious. Since injection at P60 (mid-stage) disease provided the most positive results with the high dose, this injection protocol was repeated with 4.2 x 10^{13}, 2.1 x 10^{13}, and 1.05 x 10^{13} vg/kg of rscAAVrh74.miniCMV.LIPA, ultimately lowering dose 8-fold relative to our starting dose. These mice were followed until 6 months of age (4 months post-injection).

[00138] The gross pathology of the liver and spleen was first examined. With decreasing dose, the liver progressively appeared larger and more yellowed, indicating increased fat deposition (Figure 20A). Nevertheless, treatment at all four doses showed more normal liver appearance when compared to untreated *Lipa*$^{-/-}$ mice (Figure 20A), with the two higher doses (8.4 x 10^{13} and 4.2 x 10^{13} vg/kg) having slightly more effect than the two lower doses. The spleen also appeared longer and thicker as dose decreased, but again it remained far smaller and less fattened than would occur without treatment (Figure 20A). Hepatosplenomegaly was reduced at all four doses; the percent of body weight for the liver and the spleen with all 4 doses was significantly reduced relative to untreated *Lipa*$^{-/-}$ mice (Figure 20B, C). There was, however, an apparent increase in relative weight as at the two lowest dosages used (1.05 x 10^{13} vg/kg and 2.1 x 10^{13} vg/kg) relative to 4.2 x 10^{13} vg/kg. Likewise, all four doses showed a reduction in the weight of the intestine (Figure 20D), while only the two highest doses showed a reduction in the weight of the mesenteric lymph node (Figure 20E). Serum ALT and AST values were significantly decreased at all four doses, again with a slight trend toward higher levels at the lower doses (Figure 20F and G).

[00139] Biodistribution of AAV was examined in all organs and tissues for the dosing experiment (Figure 21A, Table 5). There was a 2-fold decrease in AAV between doses 8.4 x

10^{13} vg/kg vs 4.2 x 10^{13} vg/kg, and 2.1 x 10^{13} vg/kg vs 1.05 x 10^{13} vg/kg, while there was a steeper than 2-fold decrease in AAV vgs/nucleus between doses of 4.2 x 10^{13} vg/kg and 2.4 x 10^{13} vg/kg in all organs. Most importantly, at the lowest dose of 1.05 x 10^{13} vg/kg, there was still approximately 20 vg/nucleus in the liver and 2 vg/nucleus in spleen. Interestingly, this translated to less expected human LIPA transcript expression (Figure 21B); in the liver, at the highest dose (8.4 x 10^{13} vg/kg), there was only about a 2-fold increase in LIPA expression, normalized to endogenous mouse wild type Lipa expression, despite there being more than 200 vg/nucleus. Using the lowest dose (1.05 x 10^{13} vg/kg), LIPA transcript levels were still restored to WT levels (Figure21B), resulting in WT-levels of enzyme activity in the liver (Figure 21C). Low expression of AAV and LIPA transcript at all doses in the spleen did not lead to significantly increased enzyme activity relative to untreated *Lipa*$^{-/-}$ mice (Figure 21D). LAL enzyme activity in serum was elevated relative to untreated *Lipa*$^{-/-}$ at all four doses used (Figure 21E).

Table 5 - Biodistribution of AAV at the various doses, treated at P60. Values represent mean vg/nucleus ± SD.

	1.05x10^{13} vg/kg (n=3)	**2.1x10^{13} vg/kg (n=3)**	**4.2x10^{13} vg/kg (n=3)**	**8.4x10^{13} vg/kg (n=8)**
Brain	0.23±0.11	0.77±0.17	1.46±0.62	2.55±0.81
Heart	2.69±1.13	3.70±2.38	12.51±1.55	20.52±8.79
Intestines	2.50±1.03	4.29±2.21	10.72±3.26	17.93±9.08
Liver	20.05±9.32	39.99±12.80	316.38±95.55	406.13±117.59
Lung	2.95±1.68	3.30±0.15	23.89±8.48	23.91±7.15
Lymph node	0.40±0.08	0.75±0.37	5.32±4.45	8.14±2.59
L kidney	5.91±3.28	10.94±4.56	24.38±3.99	34.95±11.97
R kidney	5.99±3.81	9.23±2.65	44.94±10.31	30.66±10.44
Spleen	2.10±0.47	3.24±1.32	33.41±12.28	35.66±13.19
Thymus	2.41±1.53	6.73±1.84	74.99±2.58	49.44±14.81
L gastrocnemius	1.11±0.37	2.66±1.21	6.97±2.76	10.75±2.66
R gastrocnemius	1.89±0.53	2.54±0.73	12.25±4.62	10.79±4.61
L quadricep	0.70±0.25	1.54±0.61	9.26±3.41	7.11±1.48
R quadricep	0.48±0.10	1.37±0.67	6.18±4.14	7.50±3.29

[00140] All four doses also significantly reduced cholesterol (Figure 22A, B) and triglyceride (Figure 22C, D) content in liver relative to untreated *Lipa*$^{-/-}$ mice. Both liver and spleen cholesterol and triglyceride content were reduced to be equivalent to or nearly equivalent to WT levels at all four doses used. Spleen triglyceride levels were reduced less than triglyceride levels in the liver, though all measures were significantly different. Thus, reductions in liver and spleen lipid content can be achieved in *Lipa*$^{-/-}$ mice with a dose of rscAAVrh74.miniCMV.LIPA as low as 1.05 x 10^{13} vg/kg.

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SEQ ID NO: 1 LIPA gene variant 1

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ptrs.miniCMV.LIPA AAV vector plasmid sequence (SEQ ID NO: 4)

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CCCACTCGTGC

What is claimed is:

1. A polynucleotide comprising
(a) a regulatory control element comprising the nucleotide sequence of SEQ ID NO: 3; and

(b) a lipase A (LIPA) cDNA sequence comprising the nucleotide sequence set forth in SEQ ID NO: 1.

2. The polynucleotide of claim 1 comprising nucleotides 1853-3906 of SEQ ID NO: 4.

3. The polynucleotide of claim 1 or 2 comprising (a) a nucleotide sequence that is at least 95% identical to SEQ ID NO: 4 or (b) the nucleotide sequence of SEQ ID NO: 4.

4. A recombinant adeno-associated virus (rAAV) having a genome comprising a polynucleotide sequence of claim 1 or 2.

5. The rAAV of claim 4, wherein the rAAV is of the serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVRH10, AAVrh74, AAV11, AAV12, AAV13 or Anc80, AAV7m8 and their derivatives.

6. An rAAV particle comprising the rAAV of claim 4 or 5.

7. A composition comprising the rAAV of claim 4 or 5 or the viral particle of claim 6.

8. The composition of claim 7, wherein the composition is formulated for intravenous delivery.

9. Use of a polynucleotide of any one of claims 1-3, an rAAV of claim 4 or 5, a rAAV particle of claim 6 or a composition of claim 7 or 8 for the preparation of a medicament for the treatment of a Lysosomal Acid Lipase Deficiency (LAL-D) disorder in a subject in need thereof, wherein the LAL-D disorder is Wolman Disease or cholesterol ester storage disease (CESD).

10. Use of a polynucleotide of any one of claims 1-3, an rAAV of claim 4 or 5, a rAAV particle of claim 6 or a composition of claim 7 or 8 for the preparation of a medicament for the treatment of a disorder related to lipid storage or accumulation in a subject in need thereof, wherein the disorder is coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

11. Use of a polynucleotide of any one of claims 1-3, an rAAV of claim 4 or 5, a rAAV particle of claim 6 or a composition of claim 7 or 8 for the preparation of a medicament for treating dyslipidemia or hypercholesterolemia in a subject in need thereof.

12. The use of any one of claims 9-11, wherein the medicament is to be administered with an immunosuppressing agent.

13. The use of any one of claims 9-12, wherein the medicament is formulated for intravenous or intraperitoneal delivery.

14. A composition for use in treating a lysosomal acid lipase deficiency (LAL-D) or a disorder related to lipid storage or accumulation in a subject in need thereof, wherein the composition comprises a polynucleotide of any one of claims 1-3, an rAAV of claim 4 or 5, a rAAV particle of claim 6 or a composition of claim 7 or 8.

15. The composition for use of claim 14 wherein the LAL-D is Wolman disease or cholesterol ester storage disease.

16. The composition for use of claim 14 or 15 wherein the subject has a mutation in the LAL-D gene.

17. The composition for use of claim 14 wherein the disorder is coronary artery disease, atherosclerosis, type II diabetes, obesity or non-alcoholic fatty liver disease.

18. A composition for use in treating dyslipidemia or hypercholesterolemia in a subject in need thereof or for decreasing triglycerides, cholesterol, and/or fatty acids in a subject, wherein the composition comprises a polynucleotide of any one of claims 1-3, an rAAV of claim 4 or 5, a rAAV particle of claim 6 or a composition of claim 7 or 8.

19. The composition for use of any one of claims 14-18, wherein the composition is to be administered with an immunosuppressing agent.

20. The composition for use of any one of claims 14-18, wherein the composition is formulated for intravenous delivery.