Crotamine as a vehicle for non-viral gene delivery for Pompe disease

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Abstract

Genetic deficiency of lysosomal acid alpha glucosidase or acid maltase (GAA) results in Pompe disease (PD), encompassing at least five clinical subtypes of varying severity. The current approved enzyme replacement therapy (ERT) for PD is via IV infusion every 2 weeks of a recombinant human GAA (rhGAA) secreted by Chinese hamster ovary (CHO) cells (alglucosidase alfa/Myozyme, Sanofi/Genzyme). Although alglucosidase alfa has proven to be efficient in rescuing cardiac abnormalities and extending the life span of the infantile form, the response in skeletal muscle is variable. ERT usually begins when the patients are symptomatic and secondary problems are already present which are compounded by low alglucosidase alfa uptake, transient nature (every 2 weeks with a rapid return to defect levels), variable glycogen reduction, autophagic accumulation, immune response and high cost. A consensus at a recent US Acid Maltase Deficiency (AMD) conference suggested that a multi-pronged approach including gene therapy, diet, exercise, etc. must be evaluated for a successful treatment of PD. Compared to replication defective viruses, non-viral gene transfer offers fewer safety concerns and, if recent studies are validated, has a wider range of cells. In order for gene therapy (GT) to succeed, the gene of interest must be delivered into the affected cell and expressed to overcome the inherited deficiency. Cell penetrating peptides (CPPs) enter eukaryotic cells through an energy-independent mechanism and efficiently carry biologically active and therapeutic molecules into cells and localize in the cytoplasm or nucleus. CPPs are usually covalently linked to the cargo, including peptides and DNA. Crotonamine (Cro) from the South American rattlesnake-*Crotalus durissus terrificus* venom, can bind electrostatically to plasmid DNA to deliver into cells, including muscle. We have assembled a bacterial expression vector for Cro and purified the recombinant Cro (rCro). Transient transfection in AMD fibroblasts and *ex vivo* in whole blood from an adult Pompe patient with rCro complexed with the pcDNA3 x *hGAA* cDNA demonstrated increased GAA activity. In GAA knockout (KO) mice receiving a single injection of rCro complexed to pcDNA3 x *hGAA* cDNA intraperitoneally (IP), we found increased GAA activity in tissues after 48 hr. After 8 treatments-IP over 55 days, we found increased vertical hang-time activity, reduced glycogen deposition, increased GAA activity/*hGAA* plasmid in tissues and minimal immune-reaction to rCro. A subsequent study of 5 administrations every 2 to 3 weeks showed reverse of the clinical phenotypes by running wheel activity, Rotarod, grip-strength meter, open field mobility and T-maze. Tissue culture experiments in PD fibroblast, lymphoid and skeletal muscle cell lines showed increased GAA activity after rCro transient gene delivery.

Introduction

Lysosomal acid maltase or GAA hydrolyzes linear α -1,4 glucosidic linkages in substrates ranging from large polymers (glycogen) to maltose and the artificial substrate 4-methylumbelliferyl- α -D-glucoside (1-18). Genetic deficiency of GAA results in Pompe disease (PD) encompassing at least 5 subtypes of varying severity (1,19) presenting as intracellular accumulation of glycogen in multiple tissues with skeletal muscle being the primary target, manifesting as myopathy and cardiomyopathy. The infantile PD is characterized by no GAA activity, muscle hypotonia, feeding difficulties and hypertrophic cardiomyopathy leading to death within the first year of life (20). The juvenile and adult onset forms are caused by partial enzyme deficiency, manifesting with progressive muscle weakness leading to wheelchair/ventilator dependence and premature death from respiratory insufficiency (21-24). The current approved enzyme replacement therapy (ERT) for PD is via IV infusion every 2 weeks of a recombinant human GAA (rhGAA) secreted by CHO cells (alglucosidase alfa/Myozyme, Sanofi/Genzyme). Although alglucosidase alfa has proven to be efficient in rescuing cardiac abnormalities and extending the life span of the infantile form, the response in skeletal muscle is variable. In juvenile and adult onset patients, only mild improvements in motor and respiratory functions have been achieved and is unsatisfactory in the reversal of skeletal muscle pathology. In infants, muscle pathology and degree of glycogen deposition correlates with the severity of symptoms and the earlier ERT is introduced, the better chance of response (24). In infants, glycogen is found mostly within large neurons of hindbrain, spinal cord and sensory ganglia with severe degeneration in axon terminals of primary sensory neurons (25-27). Treatments must address the neuropathology and hearing loss in infants (28-35). In juvenile and adult onset patients, mild improvements in motor and respiratory functions have been achieved, but are unsatisfactory in the reversal of skeletal muscle pathology (1,20-24, 36-40). Lysosomal glycogen accumulation leads to multiple secondary abnormalities (autophagy, lipofuscin, mitochondria, trafficking and signaling) that may be amenable to long-term therapy. ERT usually begins when the patients are symptomatic and secondary problems present (41). Gutschmidt et al. (42) found that ERT in late onset patients' clinical outcomes, particularly lung function, muscle strength, and walking capability tend to deteriorate over time, indicating that additional efforts must be made to improve alglucosidase alfa's effectiveness and supplement therapies developed. Some patients experienced infusion-associated reactions due to bronchial spasm with flushing and pulmonary artery blockage during infusion. Although alglucosidase alfa has been a wonderful first step in treating PD, it has revealed subtle aspects that must be dealt with for successful treatment (2,43-49). A consensus at a recent US Acid Maltase Deficiency (AMD)

conference in San Antonio, Texas, suggested that a multi-pronged approach including gene therapy, diet, exercise, etc. must be evaluated for a successful treatment of PD.

Compared to replication defective viruses, non-viral GT offers fewer safety concerns and, if recent studies are validated, has a wider range of cells. Dunbar et al. (50) reviewed the current status of gene therapies with viral vectors, adoptive transfer of genetically engineered T-cells, hematopoietic stem cells and gene editing after 30 years of promise and setbacks. GT is rapidly becoming a critical component for inherited immune disorders, hemophilia, eye/neurodegenerative disorders and lymphoid cancers. The CRISPR/Cas9 gene editing approach would provide ways to correct or alter an individual's genome with precision. Non-viral GT and their products should reduce cost and minimize immune responses and toxicity.

In order for GT to succeed the gene of interest must be delivered into the affected cell and expressed to overcome the inherited deficiency. Cell penetrating peptides (CPPs) have the ability to enter eukaryotic cells through an energy-independent mechanism and can carry biologically active and therapeutic compounds. CPPs are usually multiple sequences of short and positively charged peptides, rich in arginine and lysine residues, which penetrate cellular membranes and localize in the cytoplasm or the nucleus (51-53). CPPs are usually covalently linked to the cargo or synthesized as a fusion protein (54,55) including peptides/proteins, DNA, siRNA and liposomes (51,53,55,56). Studies suggest CPPs translocate cells via endocytosis (57), non-endocytotic pathways (58,59), pore formation (57,60) and membrane-associated heparan sulfate (61-72). Crotonamine (vCro) is a myonecrotic polypeptide in the venom of the S. American rattlesnake, *Crotalus durissus terrificus*. Radis-Baptista et al. (73) isolated the 340 bp *crotonamine* cDNA from venom glands. It contained an open reading frame of 198 bp with 5'-50 and 3'-30 bp untranslated regions, a signal peptide sequence and a poly (A⁺) signal. vCro is a 42 amino acid cationic polypeptide with 11-basic residues and 6-Cys forming 3 disulfide bonds in -YKQCHKKGGHCFPEKEICLPSSDFGKMDCRWRWKCKKGS-G. vCro contains an antiparallel β -sheet (residues 9–13 and 34–38) and an α -helix (residues 1–7) stabilized by 3 disulfide bonds between Cys4-Cys36, Cys11-Cys30 and Cys18-Cys37 (74). Kerkis et al. (75) reported vCro can penetrate into different cell types, specifically associates with centrosomes/chromatin in the nucleus, binds to the chromosomes at the S/G2 phase and is stable over a wide pH range and temperatures. Nascimento et al. (76) showed that vCro is unique by binding electrostatically to plasmid DNA. Two to 4 μ g of DNA complexed with 10 μ g of vCro was effective in transfecting cultured cells. Mice treated-IP with 2 μ g of DNA complexed with 10 μ g of vCro for 24 hr or 30 days showed bone marrow, liver, bronchioles/lung, spleen, muscle, heart and brain were transduced (77-87). vCro does not demonstrate any

immune-toxicity after long-term treatment (daily-IP of 1 µg for 21 days) in mice (78,88,89). Proteomics showed that small snake venom proteins of <5kD as vCro do not produce antibody response (90-92). Yamane et al. (93) showed specificity for proliferating cells, low cytotoxic effects and no hemolytic activity of vCro against non-tumor mammal cell lines. Chen et al. (94) demonstrated in a 3D model of vCro that residues 31-Arg-Trp-Arg-Trp-Lys-35 are potential DNA binding sites. Porta et al. (95) found that chemically synthesized full-length Cro compared favorably with its native counterpart including the ability to transfect and transport DNA into eukaryotic cells.

In this study, we have assembled a bacterial expression vector for Cro and purified the rCro. Transient transfection in PD transformed fibroblast cells and *ex vivo* in whole blood from an adult patient with rCro complexed with the pcDNA3 x *hGAA* cDNA demonstrated increased GAA activity. In GAA KO mice receiving a single injection of rCro x pcDNA3 x *hGAA* cDNA IP, we found increased GAA activity in tissues after 48 hr. After 8 treatments-IP over 55 days, we found increased vertical hang-time activity, reduced glycogen deposition, increased GAA activity/*hGAA* plasmid in tissues and minimal immune-reaction to rCro. A subsequent study of 5 administrations showed reverse of the clinical phenotypes by running wheel activity, Rotarod, grip-strength meter, open field mobility and T-maze. Experiments in PD fibroblast, lymphoid lines and skeletal muscle cell lines showed increased GAA activity after rCro transient gene delivery.

Materials and Methods

Construction of a *Cro* prokaryotic expression vector and expression/purification in *E. coli* We constructed a prokaryotic expression mini-gene with the *Cro* cDNA in pUC19 under the control of the *LacZ* promoter/operator with a 5'-His tag and a Xa protease site (96,97). rCro was induced with IPTG for ~2 hr and purified by nickel affinity chromatography via the His-tag, followed by Xa protease digestion (Novagen, Inc.) and analyzed by SDS-PAGE.

Cell culture and transfection of PD human skeletal muscle, fibroblast and lymphoid cell lines Human lymphoid (GM6314, GM13793, GM14450) or fibroblast (GM4912, GM1935, GM3329) or transformed TR4912 cell lines from infantile or adult PD were maintained in 15% fetal bovine serum, RPMI 1640 or DMEM supplemented with glutamine, penicillin and streptomycin at 37°C-5% CO₂. A human PD skeletal muscle cell line (homozygous for the IVS1 c.-32-13t>g)(98) and normal skeletal muscle cells were grown in PromoCell skeletal muscle growth media. Cells were plated at 0.3-0.4 x 10⁶ in 1.5 ml media in 6-well plates-24 hours before transfection with either rCro or lipofectamine 3000 (ThermoFisher) or Lipofectin (99-102) with the mammalian expression plasmid-pcDNA3 x human *GAA* cDNA (bp 1 to 3,150, 2,856 bp of coding cDNA (10,99-104). The rCro-*GAA* plasmid complexes were formed by mixing 1, 2 or 4 µg of plasmid DNA with 10 µg of rCro in 150 mM NaCl or PBS at RT for 10 min. Cells were incubated for 48 hr and assayed for GAA with 4-methylumbelliferyl-α-D-glucoside (4-MU-Glyc) at pH 4 and as an internal control, neutral α-glucosidase (NAG)(see below). Cells were harvested after 48 hr, washed with PBS, lysed by addition of 0.5 ml of 0.01 M sodium phosphate pH 7.5, frozen and thawed 2-3x, spun for 5 minutes in a microfuge to clarify and assayed for human GAA and NAG as described below. Cells were also exposed to a rhGAA (R and D Systems #8329-GH-025).

Ex vivo studies We transfected 3 x 3ml heparinized whole blood from an adult PD patient with 4 µg of the *hGAA* cDNA plasmid complexed with 10 µg of rCro or Lipofectin, incubated at 37°C for 24 hrs and WBCs isolated by hypaque-ficol density centrifugation. Cells were assayed for GAA/NAG as below.

Enzyme Assay. The lysate (50 µl) was assayed for GAA in 100 µl of the artificial substrate 4-methylumbelliferyl-α-D-glucoside (4-MU-Glyc) (one mg per ml) in 0.5 M sodium acetate pH 4.0 for 6 to 18 hours at 37°C and fluorescence determined in a fluorometer (excitation-360_{nm} and emission-460_{nm}) (Sequoia-Turner, Mountainview, CA). For assay of neutral α-glucosidase (NAG) as an internal control, 4-MU-Glyc in 0.5 M sodium phosphate at pH 7.5 was used (10,105). All assays were done in triplicate. Bradford protein assay used 150 µl reagent (BioRad #500-0006) diluted 1->5 with water in a 96 well flat-bottomed microtiter plate. Standards were serial dilutions of BSA from 1000 µg/ml to 2 µg/ml for 1 to 25 µl sample volume. Samples were read at A_{595nm} before 60 min.

In vivo studies GAA KO mice We utilized the GAA KO mouse developed by Raben et al. (106) with the exon 6^{neo} disruption (Jackson Labs; B6,129-Gaa^{tm1Rabn/J}, exon 6^{neo}), wild-type BALB/c or 129/J or GAA KO mice mock-treated with PBS. We IP infused GAA KO mice (~4-6 months old) with a single or multiple doses of 4 or 20 µg of *hGAA* plasmid complexed to 10 µg rCro. At 7 days, mice were sacrificed and tissues were assayed for GAA and NAG and compared to wild-type mice and mock treated GAA KO mice. Also, a weekly treatment for 55 days and 8 treatments with 10 µg rCro complexed with 0 or 4 µg or 20 µg *hGAA* plasmid and measured vertical hang-time (forelimb strength)(106), GAA activity/plasmid content in tissues, immune-reaction to Cro and glycogen deposition.

Phenotypic evaluation of GAA KO mice after rCro-hGAA plasmid treatment GAA KO mice (106) were treated IP with either 4 µg or 10 µg of *hGAA* plasmid complexed to 10 µg of rCro in 100 µl PBS every 2 to 3 weeks for 5 administrations. Tissues, urine, blood slides, weight and serum were collected. Mice were tested every ~3 weeks between 8:00-11:00AM to avoid circadian variation for: motor activity by running wheel (RW-Mini-Mitter Co., Inc., OR); fore-limb muscle strength by grip-strength meter (GSM)(Columbus Inst. OH), motor coordination/balance with a Rotarod (AJATPA Expert Industries, India), open-field mobility by 5 min video/distance traveled and spatial preference/spontaneous learning with a T-maze (Stoelting, IL) (100-102).

ELISA for antibodies to rCro In Falcon plates #3912, rCro was added at 1 µl/well in 100 µl PBS at 4°C overnight, washed 3x with PBS and blocked with 200 µl PBS-1g% BSA-0.05% Tween-20 for 18 hr at RT. Then, add 1 µl serum in 100 µl PBS-overnight at 4°C, wash 3x with PBS, 1x-PBS-0.05% Tween-20 and 2X PBS. Add 100 µl goat anti-mouse IgG-HRP (BioRad #172-1101) at 1:500 in PBS/BSA/Tween-20 at 4°C overnight. Wash 3x PBS, 1x PBS-0.05% Tween-20, 2x PBS and stain with 100 µl of 3,3',5,5'-tetramethylbenzidine (Sigma #T5525) in 9 ml of 0.05 M phosphate-citrate buffer, pH 5.0 (Sigma #P4922) with 20 µl of 30% H₂O₂ for 30-60 minutes at room temperature. The reaction is stopped with 50 µl 1 N HCl and read at A_{450nm}

Assay for glycogen content In a 96 well microtiter plate, add 25 µl sample, 25 µl 0.05 M sodium phosphate pH 6.5, +/- 0.5 µl amyloglucosidase (Sigma #A-1602), placed overnight at 37°C (107). Glycogen standards (rabbit liver, 2 mg/ml) and D-Glucose standards were at 400 µM, 200 µM and 100 µM. Add 20 µl of Eton Bioscience glucose assay solution (#1200031002) and incubate for 30 minutes at 37°C. The reaction was stopped with 25 µl of 0.5 M acetic acid and read at A_{490nm}.

PCR for *hGAA* plasmid in tissues PCR utilized Bulldog Fastmix Frenchie according to instructions with 1 µg genomic DNA from tissues after 8 treatments or 55 days or 1 ng of pcDNA3x*hGAA* cDNA. DNA was extracted using standard phenol-chloroform methods. The human *GAA* cDNA primers are: GAAFM106 5'-CCATTTGTGATCTCCCGCTCGACCTTTG-3' (bp1733-1810) and GAAFM94 5'-GGTGGTCCACAGTCCAGGTGCTAGAG-3' (actual primer sequence; bp2201-2227). The cycling conditions are: hot-start 94°C for 2 min, disassociation-94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min for 40 cycles with a final extension for 10 min. The PCR amplicons (20 µl) are electrophoresed in 1.5% agarose and visualized under UV light with ethidium bromide staining.

Statistical analysis Student t-test for probability associated with a population and standard deviation based upon the population were determined using Microsoft Excel software and considered significant at p value ≤ 0.05 .

Results

Construction of an rCro expression vector and expression/purification in *E. coli* We constructed a prokaryotic expression mini-gene with the *Cro* cDNA in pUC19 under the control of the *LacZ* promoter/operator with a 5'-His tag and a Xa protease site (96,97). The rCro was induction with IPTG for ~2hr and purified by nickel affinity chromatography via the His-tag, followed by Xa protease digestion and analyzed by SDS-PAGE (76,78). **Figure 1** shows in lane 1-lysate pre-Ni column; lane 2-lysate post Ni column; lane-3 first wash fraction of Ni column and lane 4-eluted fraction with the arrow showing the correct size of rCro at ~5 kD. The rCro was also characterized for binding time to plasmid DNA and plasmid binding capacity. The rCro showed maximum plasmid binding of 4 µg to 10 µg rCro and a minimum time of one minute similar to the published data.

rCro transient transfection of an PD transformed fibroblast cell line TR4912, an immortalized infantile PD fibroblast cells (no GAA activity or mRNA)(108) were transfected with various amounts (1, 2 or 4 µg) of *hGAA* plasmid complexed to 10 µg of rCro. After 48 hr, cells were assayed for GAA and NAG (**Graph 1**). As a positive control for transfection, cells were transfected with Lipofectin and *hGAA* plasmid. Transfected TR4912 cells increased from 0.0001 to 5, 16 and 48 GAA/NAG ratio for 1, 2 or 4 µg plasmid respectively with 10 µg rCro. Cells transfected with Lipofectin (99-102) had a ratio of 12, while all mock treated cells were undetectable. GAA/mg protein did not change results (not shown). Thus, rCro mediated gene delivery was greater than lipid mediated.

Ex vivo studies We transfected 3 x 3 ml heparinized whole blood from an adult PD patient with 4 µg of the *hGAA* cDNA plasmid complexed with 10 µg of rCro or Lipofectin that was incubated at 37°C for 24 hr and WBCs isolated by hypaque-ficol density centrifugation. Cells were assayed for GAA/NAG ratio. Mocked treated WBCs had a relative GAA/NAG ratio of 5; blood treated with the *hGAA* plasmid x rCro complexes had a GAA/NAG ratio of 45, while lipid had a GAA/NAG ratio of 25.

Short-term in vivo rCro gene delivery in GAA KO mice 4 µg of *hGAA* plasmid was complexed to 10 µg rCro that was administered IP to three GAA KO mice in 100 µl-PBS. After 48 hr, tissues were assayed. Tissues-heart, skeletal muscle, liver, kidney and diaphragm showed significant increases (**Table 1**)(mean±SD). GAA/mg protein did not change results (not shown).

Long-term in vivo rCro gene delivery and vertical hang-time measurement We are utilizing non-viral *in vivo* gene delivery with rCro that is transient in nature as an episome (maximum expression for 3-14 days)(109-126), thus we decided upon a weekly treatment for 55 days or 8 treatments schedule to maintain and monitor sustained GAA levels. We

treated four groups (age matched) of GAA KO mice-IP (n=2-3) weekly with 10 µg rCro complexed with 0 or 4 µg or 20 µg *hGAA* plasmid and measured vertical hang-time (forelimb strength)(106). The 8 times treated animals were well tolerated as mice gained weight over 55 days (data not shown). Both treated groups showed a steady and significant improvement in vertical hang-time (**Figure 2**). Interestingly, mice in the 4 µg plasmid group gradually increased to the 20 µg plasmid group, suggesting that a lower dose may be effective long-term. Both treated groups showed ~67% of wild-type mice hang-time. All treatments were significant at $p \leq 0.05$.

Biodistribution (BD) in tissues and glycogen reduction after 8 treatments over 55 days We found increased GAA activity in heart, hind-limb skeletal muscle, kidney, liver and diaphragm from treated GAA KO mice (**Table 2**) expressed as GAA/NAG. The % of wild type ranged from 11-32% (in red). Similar increases were found when expressed as GAA/mg protein (not shown). After 8 treatments over 55 days, we found tissue glycogen levels reduced from 11 to 96% (**Table 3**)(106). GAA/mg protein did not change results (not shown).

Immune-antibody response Serum antibody titer to rCro at 55 days/8 treatments for rCro alone was 35ng/ml, with rCro-4 µg plasmid = 70 ng/ml and for 20 µg plasmid = 60 ng/ml, thus demonstrating low immune response (78,87-92).

PCR for *hGAA* plasmid in tissues Agarose gel electrophoresis shows PCR amplicons from 1 µg genomic DNA from tissues (liver, heart, skeletal muscle, kidney and diaphragm) after 8 treatments over 55 days or positive control-1 ng of pcDNA3 x *hGAA* cDNA (**Figure 3**). We found the *hGAA* amplicon present in all treated tissues to varying degree, however the liver required nested PCR to detect. DNA from WT tissues (not shown) and mock treated GAA KO mice (lane 2) were negative for the *hGAA* amplicon.

Assessment of spontaneous alternation in WT and GAA KO mice Spontaneous alternation is used to assess the cognitive ability of rodents to choose one of the 2 goal arms of the T-maze. The advantage of a free choice procedure is that hippocampal or lesioned animals often develop a side preference and scores below 50%. Controls generally achieve at least 60-80% correct alternation. We assessed spontaneous alternative learning for cognitive ability in the T-maze in both male and female GAA KO mice and WT-129/C57 mice from 2-9 months of age. We found that deficiency in spontaneous learning appeared by 2-3 months in male and 3-4 months in female GAA KO mice (**Graph 2**). All conditions were significant ($p \leq 0.05$).

Phenotypic evaluation of GAA KO mice after rCro-*hGAA* plasmid treatment GAA KO mice (106) were treated IP every 2 to 3 weeks with either 4 µg or 10 µg of *hGAA* plasmid complexed to 10 µg of rCro for 5 administrations (**Table 4**). Mice were tested every ~3 weeks for: motor activity by running wheel (RW); fore-limb muscle strength by grip-

strength meter (GSM), motor coordination/balance with a Rotarod, open-field mobility by 5 min video/distance traveled and spontaneous learning with a T-maze. All measurements improved significantly after 2 treatments for both doses of *hGAA* plasmid compared to pre-treatment measurements. All measurements were significant at $p \leq 0.05$ compared to pre-treated mice. All treatments were significant at $p \leq 0.05$.

rCro transient transfection in a human PD skeletal muscle, lymphoid and fibroblast cell lines A human PD skeletal muscle cell line, three PD lymphoid and three PD fibroblast cell lines were transfected with 4 μ g of *hGAA* plasmid complexed to 10 μ g of rCro. After 48 hr, cells were assayed for GAA and NAG. Cells were also transfected with Lipofectamine 3000-*hGAA* plasmid or exposed to rhGAA (skeletal muscle only) for 48 hr and assayed. Mock treated PD and normal cell lines were controls. We found that lipo-*hGAA* plasmid increased GAA to 23% of normal while rCro-*hGAA* plasmid increased GAA to 28% of normal and rhGAA increased GAA to 20% of normal in PD skeletal muscle cells (mean $\pm$ SD)(**Graph 3**). In transfected PD fibroblast and lymphoid cell lines transfected with rCro-*hGAA* plasmid, increased GAA/NAG ratios was observed to various degrees as compared to normal cells (**Graphs 4 and 5**). All treatments were significant at $p \leq 0.05$. GAA/mg protein did not change results (not shown).

Discussion

Genetic deficiency of lysosomal GAA results in Pompe's disease (PD), encompassing at least five clinical subtypes of varying severity (19). The current approved ERT for PD is via IV infusion every 2 weeks of a rhGAA secreted by CHO cells (alglucosidase alfa). Although alglucosidase alfa has proven to be efficient in rescuing cardiac abnormalities and extending the life span of the infantile form, the response in skeletal muscle is variable. In juvenile and adult patients, only mild improvements in motor and respiratory functions have been achieved and is unsatisfactory in the reversal of skeletal muscle pathology. ERT usually begins when the patients are symptomatic and secondary problems are already present which are compounded by low (about 1 %) alglucosidase alfa uptake in muscle (41), transient nature of administered (every 2 weeks with a rapid return to defect levels), variable glycogen reduction, autophagic accumulation, immune response and high cost. Although alglucosidase alfa has been a wonderful first step in treating PD, it has revealed subtle aspects that must be dealt with for successful treatment (2,43-49).

Compared to replication defective viruses, non-viral gene transfer offers fewer safety concerns and, if recent studies are validated, has a wider range of cells. Dunbar et al. (50) reviewed the current status of GTs with viral vectors, adoptive transfer of genetically engineered T-cells, hematopoietic stem cells and gene editing after 30 years of promise and setbacks. The CRISPR gene-editing approach would provide ways to correct or alter an individual's genome with precision. Recent findings showed that there was low level of integration with AAV GT in dogs sometimes near genes affecting cell growth and must be monitored for development of cancers (127). There have been many studies using viral mediated GT in PD cells and animal models using adenovirus, adeno-associated virus, lentivirus and retroviruses (128-151). Barry Byrne and his colleagues at the Univ. of Florida and investigators at Duke University are developing GT using AAV viral systems (152-177). Over the next 10 years, viral delivered GTs are expected to come into their own as a treatment option for a variety of diseases with two therapies have regulatory approval (Novartis' Zolgensma for spinal muscular atrophy and Spark's Luxturna for a rare form of genetic blindness). GTs offer great rewards to treat many diseases, but there are also significant risks. Over the past year, several clinical studies have been placed on hold or stopped entirely due to safety concerns. Audentes Therapeutics had a temporary hold placed on the GT for X-linked myotubular myopathy following several patient deaths that has recently lifted by the FDA. Uniqure was placed on hold for its hemophilia B trial after a patient in the study developed liver cancer. Other studies found some patients developed acute myeloid leukemia (AML). A sickle cell disease clinical trial using CRISPR gene-editing to turn on a fetal form of

hemoglobin reported promising results last year. However, CRISPR gene-editing can have off-target effects and rearrange chromosomes and if it can trigger cancer may not be known for several years. In December 2020, uniQure reported that a patient in a GT trial for hemophilia had developed a liver tumor and planned to evaluate whether its vector, an adeno-associated virus (AAV), had a role. Even though AAVs are supposed to be safer than lentiviruses because they are not designed to insert their gene or cargo into the genome, animal studies have found they can (178). While the precise mechanisms that led to these toxicities remain unknown, some hypotheses have focused on the role of antibodies to AAV that either preexist or rapidly accumulate following vector infusion. The formation of vector–antibody complexes could activate complement through the classic pathway or activate innate immunity through Fc-dependent uptake in antigen-presenting cells. The complexity of host–vector interactions in GT and the species-specific differences in those interactions have long limited the ability to use preclinical data to predict clinical outcomes. This is especially true with regard to immune-mediated vector toxicities. However, these limitations should not be seen as a reason to abandon animal modeling. Instead, the early clinical development of GT vectors may require an iterative process, especially if unexpected toxicities are observed (179-181).

Promising non-viral delivery systems are currently undergoing clinical trials (109-117,124-126). GT is the modulation of gene expression in specific cells to treat pathological conditions by introducing exogenous nucleic acids by carriers or vectors as DNA, mRNA, small interfering RNA (siRNA) or microRNAs. Conventional non-viral delivery methods are hydrodynamic pressure techniques, electroporation, ballistic bombardment and microinjection. Cationic polymer methods include lipofection, cationic peptides, PEI and receptor-mediated. We have previously investigated various delivery systems for non-viral GT with several mammalian expression plasmids containing the *hGAA* cDNA *in vitro* and *in vivo* were Helios gene gun (99,100), liposome (cationic lipid GL-67)(unpublished)(99-101) and PEI (unpublished) that included CMV, muscle specific and modified CMV regulatory promoters (101,102). In Australia with Peter Healy-DVM, two PD shorthorn cows were infused 4x over 4 months with a lipid-plasmid complex (150mg/100kg). Treated animals did not progress with muscle weakness and serum CPK dropped from 1000U pre-Tx to 500U by 4 months and were "healthy". Skeletal muscle, diaphragm and intercostal muscle showed GAA increased from ~1% to 10-20% of normal (unpublished).

In order for GT to succeed, the gene of interest must be delivered into the affected cell and expressed to overcome the inherited deficiency. CPPs enter eukaryotic cells through an energy-independent mechanism and efficiently carry biologically active and therapeutic molecules into cells and localize in the cytoplasm or nucleus. CPPs are usually covalently linked to the cargo, including peptides and DNA. vCro from the South American rattlesnake *Crotalus*

durissus terrificus venom can bind electrostatically to plasmid DNA to deliver into cells, including muscle. We decided to use a weekly administration regimen to obtain maximum and sustained GAA levels based upon the transient nature of non-viral GT and short-term episomal longevity. Similar to *in vitro* transfection, maximum expression and maintenance may be only 3-14 days before the plasmid is lost however long term detection has been observed (109-124). We have assembled a bacterial expression vector for Cro and purified the rCro. Transient transfection in PD fibroblasts and *ex vivo* in whole blood from an adult PD patient with rCro complexed with the pcDNA3 x *hGAA* cDNA, demonstrated increased GAA activity. In GAA KO mice receiving a single injection of rCro x *hGAA* cDNA IP, we found increased GAA activity in tissues after 48 hr. After 8 treatments-IP over 55 days, increased vertical hang-time activity, reduced glycogen deposition, increased GAA activity/*hGAA* plasmid in tissues and minimal immune-reaction to rCro. A subsequent study of 5 administrations showed reverse of the clinical phenotypes by running wheel activity, Rotarod, grip-strength meter, open field mobility and T-maze. Experiments in PD fibroblast, lymphoid lines and skeletal muscle cell lines showed increased GAA activity after rCro transient gene delivery.

From DNA vaccine (182-185), the production of the recombinant protein may be released into the circulation and taken up by distant tissues, functioning as an "enzyme-factory or depot." With future experiments, if we achieve 10% of normal, we will consider the treatment a success since adult PD patients have 2-10% and survive to the 5/6th decade. If over-expression is observed, we will add a Tet-off doxycycline (TRE) to regulate expression dose-dependently. If plasmid related cytotoxicity is found, we will remove CpG dimer sequences by minicircle synthesis (182,185). We also hypothesize there will be positive selective pressure to maintain the plasmid in tissues to be "healthier" by being able to hydrolyze glycogen to glucose (109-126,149-151). In some studies, plasmid integration into chromosomes was observed, however integration has the potential to cause tumorigenic events (109-126,149-151). Additionally, to further minimized immune-reaction to rCro, we will evaluate a smaller rCro (94).

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Legends

Figure 1 Composite diagram of a rCro prokaryotic expression vector and expression/purification in *E. coli* SDS-PAGE shows standards in the left lane, lane 1-lysate pre-Ni column; 2-lysate post Ni column; 3-first wash fraction of Ni column and 4-eluted fraction with the arrow showing the correct size of rCro at ~5kD.

Figure 2 Long-term *in vivo* treatment and vertical hang-time measurement We treated four groups (age matched) of GAA KO mice-IP (exon 6^{neo})(n=2-3) weekly with 10 µg rCro complexed with 0 or 4 µg or 20 µg *hGAA* plasmid and measured vertical hang-time (forelimb strength). Both treated groups showed a steady and significant improvement in vertical hang-time. Interestingly, mice in the 4 µg plasmid group gradually increased to the 20 µg plasmid group. Both Tx groups showed ~67% of WT mice hang-time. Mean±SD not shown to not complicate the graph.

Figure 3 PCR for *hGAA* plasmid in tissues PCR was performed on 1 µg genomic DNA from tissues after 8 treatments (Tx) or 1 ng of pcDNA3 x *hGAA* cDNA. Agarose gel electrophoresis of the amplicons-on left lane = STDs-ThermoFisher GeneRuler 1 kb ladder, lane 1=plasmid, lane 2=mock treated liver, lane 3=treated liver, lane 4=treated heart, lane 5=treated skeletal muscle, lane 6=treated kidney and lane 7=treated diaphragm.

Graph 1 rCro transient transfection of a PD transformed fibroblast cell line TR4912, an immortalized infantile PD fibroblast cells (no GAA activity or mRNA) were transiently transfected with rCro-*hGAA* plasmid complexes formed by mixing 1, 2 or 4 µg of plasmid DNA with 10 µg of rCro. Cells were incubated for 48 hr and assayed for GAA with 4-MU-Glyc at pH4 and as an internal control, neutral α-glucosidase (NAG). As a positive control for transfection, cells were transfected with Lipofectin and plasmid. Transfected TR4912 cells increased from 0.0001 to 5, 16 and 48 GAA/NAG for 1, 2 or 4 µg plasmid with 10 µg rCro. Cells transfected with Lipofectin had a ratio of 12, while all mock treated cells were undetectable.

Graph 2 Mean % correct spontaneous alternation by T-maze and age Spontaneous alternation is used to assess the cognitive ability of rodents to choose one of the 2 goal arms of the T-maze. The advantage of a free choice procedure is that hippocampal or lesioned animals often develop a side preference and scores below 50%. Controls generally achieve at least 60-80% correct alternation. We assessed spontaneous alternative learning for cognitive ability in the T-maze in both male and female GAA KO mice and WT-129/C57 mice from 2-9 months of age. All conditions were significant ($p \leq 0.05$).

Graph 3 Human normal and PD myoblast cells by rCro-*hGAA* plasmid delivery A human PD skeletal muscle cell line cell line was transfected with 4 µg of *hGAA* plasmid complexed to 10 µg of rCro or with Lipofectamine 3000-*hGAA*

plasmid or exposed to rhGAA. After 48 hr, cells were assayed for GAA and NAG. Mock treated AMD and normal cell lines were controls. We found that lipo-*hGAA* plasmid increased GAA to 23% of normal while rCro-*hGAA* plasmid increased GAA to 28% of normal and rhGAA increased GAA to 20% of normal in PD myoblast cells (mean+/-SD). All treatments were significant at $p \leq 0.05$.

Graph 4 PD fibroblast cells treated with either liposome or rCro-*hGAA* plasmid delivery Human three PD fibroblast cell lines were transfected with 4 µg of *hGAA* plasmid complexed to 10 µg of rCro or with Lipofectamine 3000-*hGAA* plasmid. After 48 hr, cells were assayed for GAA and NAG. Mock treated PD and normal cell lines were controls. In transfected PD fibroblast cell lines transfected with rCro-*hGAA* plasmid, increased GAA/NAG ratios was observed to various degrees as compared to normal cells. All treatments were significant at $p \leq 0.05$.

Graph 5 PD LL treated with either liposome or rCro-*hGAA* plasmid delivery Human PD three lymphoid cell lines were transfected with 4 µg of *hGAA* plasmid complexed to 10 µg of rCro or with Lipofectamine 3000-*hGAA* plasmid. After 48 hr, cells were assayed for GAA and NAG. Mock treated AMD and normal cell lines were controls. In transfected PD lymphoid cell lines transfected with rCro-*hGAA* plasmid, increased GAA/NAG ratios was observed to various degrees as compared to normal cells. All treatments were significant at $p \leq 0.05$.

Figures, Graphs and Tables

Graph 3 Human normal and PD skeletal muscle cells by rCro gene delivery with a plasmid containing the *hGAA* cDNA

Graph 4 PD fibroblasts treated with either liposome or rCro plasmid gene delivery

Graph 5 PD LL treated with either liposome or rCro x *hGAA* plasmid gene delivery

Table 1	<i>rCRO Gene Delivery</i>					
	Sk. Muscle	Heart	Diaphragm	Kidney	Liver	Lung
Mock Treated						
GAA ^{-/-} Mice	0.03±0.004	0.052±0.007	0.061±0.009	0.09±0.008	0.07±0.006	0.08±0.005
1X-Treated GAA ^{-/-} Mice-post-48 hrs.	0.19±0.07	0.11±0.08	0.16±0.09	0.10±0.07	0.54±0.1	0.63±0.2
Normal 129	1.8±0.3	0.57±0.2	0.91±0.15	1.3±0.5	0.88±0.13	1.23±0.23

TABLE 2. <u>Mouse tissue GAA/NAG (mean ± SD) after 8 Tx over 55 days.</u>						
	Hind-limb Skeletal Muscle	Heart	Diaphragm	Liver	Kidney	
Treated GAA KO-4ug plasmid + CRO	0.22 ± 0.02	0.14 ± 0.02	0.18 ± 0.03	0.14 ± 0.04	0.23 ± 0.04	
% of WT	28%	18%	28%	11%	21%	
Treated GAA KO-20ug plasmid + CRO	0.22 ± 0.01	0.11 ± 0.01	0.21 ± 0.04	0.21 ± 0.05	0.23 ± 0.01	
% of WT	28%	15%	32%	16%	21%	
Treated GAA KO-CRO	0.054 ± 0.01	0.07 ± 0.01	0.077 ± 0.1	0.12 ± 0.08	0.29 ± 0.02	
Mock GAA KO-PBS	0.07 ± 0.044	0.065 ± 0.044	0.08 ± 0.14	0.05 ± 0.009	0.15 ± 0.02	
Wild-type	0.8 ± 0.11	0.76 ± 0.23	0.65 ± 0.38	1.33 ± 0.36	1.12 ± 0.08	

TABLE 3.	% glycogen reduction (mean) compared to mock treated in GAA KO mouse tissues.					
	Hind-limb Skeletal Muscle	Heart	Diaphragm	Liver	Kidney	
Treated GAA KO -4ug plasmid + CRO	96	11	47	29	48	
Treated GAA KO -20ug plasmid + CRO	28	17	49	32	47	

Table 4	RW	GSM	Rotarod	T-maze	Open-field mobility
Mean±SD	rev/12 hrs	lbs at release	Time (sec)	% correct alternation	cm/min ±SD
GAA KO M/F					
Pre-Tx	404±128	0.130±60	112±120	23±12	182±80
4ug plasmid/10ug Cro					
2x injections	2047±677*	0.253±99*	296±131*	50±19*	318±174*
4x injections	3156±647*	ND	ND	ND	ND
5 x injections	2177±647*	0.232±87*	287±145*	51±7*	229±73*
10ug plasmid/10ug Cro					
2x injections	1730±366*	0.293±117*	131±59*	58±13*	388±86*
4x injections	3536±1687*	ND	ND	ND	ND
5 x injections	3508±2007*	0.249±97*	288±129*	53±18*	334±56*
WT-129/C57 M/F n=8	4912±596	0.627±265	245±98	61±10	334±96

*p<0.05 when compared to pre-treatment data by T-test (1-tail, 2-equal variance)

References

1. Martiniuk, F., Chen, A., Mack, A., Codd, W., Hanna, B., Arvanitopoulos, E. and Slonin, A. Carrier frequency for glycogen storage disease type II in New York and estimates of affected individuals born with the disease. Am. J. Med. Genet. 79:69-72, 1998.
2. Kikuchi, T., Yang, H.W., Pennybacker, M., Ichihara, N., Mizutani, M., Van Hove, J.L. and Chen, Y.T. Clinical and metabolic correction of Pompe disease by enzyme therapy in acid maltase-deficient quail. J. Clin. Invest. 101:827-833, 1998.
3. DeBarsy, T., Jacquemin, P., Devos, P. and Hers, H.G. Rodent and human acid alpha glucosidase. Purification, properties and inhibition by antibodies. Investigation in type II glycogenosis. Eur. J. Biochem. 31:156-165, 1965.
4. Brown, B.I. and Brown, D.H. The subcellular distribution of enzymes in type II glycogenosis and the occurrence of oligo alpha-1,4-glucosidase in human tissues. Biochim. Biophys. Acta. 110:124-133, 1965.
5. Koster, J.F. and Slee, R.G. Some properties of human acid α -glucosidase. Biochim. Biophys. Acta. 482:89-97, 1977.
6. Jeffrey, P.L., Brown, D.H. and Brown, B.I. Studies of lysosomal α -glucosidase. Biochem. 9:1416-1422, 1970.
7. Palmer, T.N. The substrate specificity of acid α -glucosidase from rabbit muscle. Biochem. J. 124:701-711, 1971.
8. Fujimori, K., Hizukuri, S. and Nikuni, Z. Studies on acid α -1,4-glucosidase from bovine spleen. B.B.R.C. 32:811-816, 1968.
9. Oude Elferink, R.P.J. Biosynthesis, transport and processing of lysosomal alpha glucosidase. PhD Thesis, University of Amsterdam, 1985.
10. Martiniuk, F., Bodkin, M., Tzall, S. and Hirschhorn, R. Identification of the base pair responsible for a human acid α -glucosidase allele with lower affinity for glycogen (GAA 2) and transient gene expression in deficient cells. Am. J. Human Genetics 47:440-445, 1990.
11. Solomon, E., Swallow, D., Burgess, S. and Evans, L. Assignment of the human acid alpha glucosidase gene to chromosome 17 using somatic cell hybrids. Ann. Hum. Genet. 42:273-281, 1981.
12. Nickel, B.E., Chudley, A.E., Pabello, P.D. and McAlpine, P.J. Exclusion mapping of the *GAA* locus to chromosome 17q21-25. Cytogenet. Cell Genet. 32:303-308, 1982.
13. Haines J.L., Ozelius L.J., McFarlane H., Menon A., Tzall, S., Martiniuk, F., Hirschhorn, R., Breakefield, X.O. and Gusella, J.F. A genetic linkage map of chromosome 17. Genomics 8:1-6, 1989.
14. Martiniuk, F., Ellenbogen, A., Hirschhorn, K. and Hirschhorn, R. Further regional localization of the genes for human acid alpha glucosidase (GAA), peptidase D (PEPD) and alpha mannosidase (MANB) by somatic cell hybridization. Hum. Genet. 69:109-111, 1985.
15. Swallow, D.M., Corney, G., Harris, H. and Hirschhorn, R. Acid α -glucosidase: a new polymorphism in man demonstratable by affinity electrophoresis. Ann. Hum. Genet. 38:391-406, 1975.
16. Nickel, B.E. and McAlpine, P.J. Extension of human acid α -glucosidase polymorphism by isoelectric focusing on polyacrylamide gel. Ann. Hum. Genet. 46:97-103, 1982.
17. Beratis, N.G., LaBadie, G.U. and Hirschhorn, K. An isozyme of acid α -glucosidase with reduced catalytic activity for glycogen. Am. J. Hum. Genet. 32:137-149, 1980.
18. La Badie, G. U. Biochemical and immunologic studies of acid alpha glucosidase deficiency, a genetically heterogeneous, inherited neuromuscular disease. (Ph.D. Thesis, City University of New York, Mt. Sinai Hospital), 1986.
19. Slonim, A.E., Bulone, L., Goldberg, T., Minikes, J., Slonim, E., Galanko, J. and Martiniuk, F. Modification of the natural history of adult-onset acid maltase deficiency by nutrition and exercise therapy. Muscle Nerve. 35:70-7, 2007.
20. van den Hout, H.M., Hop, W., van Diggelen, O.P., Smeitink, J.A., Smit, G.P., Poll-The, B.T., Bakker, H.D., Loonen, M.C., de Klerk, J.B., Reuser, A.J. and van der Ploeg, A.T. The natural course of infantile Pompe's disease: 20 original cases compared with 133 cases from the literature. Pediatrics 112:332-340, 2003.
21. Winkel, L.P., Hagemans, M.L., van Doorn, P.A., Loonen, M.C., Hop, W.J., Reuser, A.J. and van der Ploeg, A.T. The natural course of non-classic Pompe's disease; a review of 225 published cases. J. Neurol. 252:875-884, 2005.
22. Engel, A., Gomez, M.R., Seybold, M.E. and Lambert, E.H. The spectrum and diagnosis of acid maltase deficiency. Neurology 23:95-106, 1973.
23. Mehler, M. and DiMauro, S. Residual GAA activity in late-onset acid maltase deficiency. Neurol. 27:178-184, 1997.
24. Slonim, A., Bulone, L., Ritz, S., Goldberg, T., Chen, A. and Martiniuk, F. Identification of two subtypes of infantile acid maltase deficiency: Evaluation of twenty-two patients and review of the literature. J. Pediatrics 137:283-285, 2000.
25. DeRuisseau, L.R., Fuller, D.D., Qiu, K., DeRuisseau, K.C., Donnelly, W.H., Mah, C., Reier, P.J. and Byrne, B.J. Neural deficits contribute to respiratory insufficiency in Pompe disease. PNAS 106:9419-9424, 2009.
26. Mancal, E.L., Aponte, G.E. and Berry, R.G. Pompe's disease (diffuse glycogenosis) with neuronal storage. J. Neuropathol. Exp. Neurol. 24:85-96, 1965.

27. Sidman, R.L., Taksir, T., Fidler, J., Zhao, M., Dodge, J.C., Passini, M.A., Raben, N., Thurberg, B.L., Cheng, S.H. and Shabuddin, I.S. Temporal neuropathological and behavioral phenotype of 6Neo/6Neo Pompe disease mice. *J. Neuropathol. Exp. Neurol.* 67:803–818, 2008.
28. Richard, E., Douillard-Guilloux, G. and Caillaud, C. New insights into therapeutic options for Pompe disease. *IUBMB. Life* 63:979-986, 2011.
29. Fuller, D.D., ElMallah, M.K., Smith, B.K., Corti, M., Lawson, L.A., Falk, D.J. and Byrne B.J. The respiratory neuromuscular system in Pompe disease. *Respiratory Physiology & Neurobiology* 189:241-249, 2013.
30. Vilchez, D., Ros, S., Cifuentes, D., Pujadas, L., Vallès, J., García-Fojeda, B., Criado-García, O., Fernández-Sánchez, E., Medraño-Fernández, I., Domínguez, J., García-Rocha, M., Soriano, E., Rodríguez de Córdoba, S. and Guinovart, J.J. Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. *Nat. Neurosci.* 10:1407–1413, 2007.
31. Chien, Y.H., Lee, N.C., Peng, S.F. and Hwu, W.L. Brain development in infantile-onset Pompe disease treated by enzyme replacement therapy. *Pediatr. Res.* 60:349–352, 2006.
32. Turner, S., Hoyt, A.K., ElMallah, M.K., Falk, D.J., Byrne, B.J. and Fuller, D.D. Neuropathology in respiratory-related motoneurons in young Pompe (Gaa^{-/-}) mice. *Respiratory Physiology & Neurobiology* 227:48–55, 2016.
33. Kamphoven, J.H.J., de Ruiter, M.M., Winkel, L.P.F., Van den Hout, H.M.P., Bijman, J., De Zeeuw, C.I., Hoeve, H.L., Van Zanten, B.A., Van der Ploeg, A.T. and Reuser, A.J.J. Hearing loss in infantile Pompe's disease and determination of underlying pathology in the knockout mouse. *Neurobiology of Disease* 16:14–20, 2004.
34. van Capelle, C.I., Goedegebure, A., Homans, N.C., Hoeve, H.L.J., Reuser, A.J.J. and van der Ploeg, A.T. Hearing loss in Pompe disease revisited: results from a study of 24 children. *J. Inherit. Metab. Dis.* 33:597–602, 2010.
35. van der Beek, N.A.M.E., Verschuure, H., Reuser, A.J.J., van der Ploeg, A.T., van Doorn, P.A. and Poublon, R.M.L. Hearing in adults with Pompe disease. *J. Inherit. Metab. Dis.* 35:335–341, 2012.
36. Tagers, J.M., Oude Elferink, R.P.J., Reuser, A.J.J., Kroos, M., Ginsel, L.A., Franzen, J.A.M. and Klumperman, J. Alpha glucosidase deficiency; Pompe's disease. *Enzyme* 38:280-285, 1987.
37. Raben, N., Ralston, E., Chien, Y.H., Baum, R., Schreiner, C., Hwu, W.L., Zaal, K.J. and Plotz, P.H. Differences in the predominance of lysosomal and autophagic pathologies between infants and adults with Pompe disease: implications for therapy. *Mol. Genet. Metab.* 101:324-331, 2010.
38. Reuser, A.J.J., Kroos, M., Elferink, R.P.J.O. and Tager, J.M. Defects in synthesis, phosphorylation, and maturation of acid alpha glucosidase. *J. Biol. Chem.* 260:8336-8341, 1985.
39. Strothotte, S., Strigl-Pill, N., Grunert, B., Kornblum, C., Eger, K., Wessig, C., Deschauer, M., Breunig, F., Glocker, F.X., Vielhaber, S., Brejova, A., Hilz, M., Reiners, K., Müller-Felber, W., Mengel, E., Spranger, M. and Schoser, B. Enzyme replacement therapy with alglucosidase alfa in 44 patients with late-onset glycogen storage disease type 2: 12-month results of an observational clinical trial. *J. Neurol.* 257:91-97, 2010.
40. van der Ploeg, A.T., Clemens, P.R., Corzo, D., Escolar, D.M., Florence, J., Groeneveld, G.J., Herson, S., Kishnani, P., Laforet, P., Lake, S.L., Lange, D.J., Leshner, R.T., Mayhew, J.E., Morgan, C., Nozaki, K., Park, D.J., Pestronk, A., Rosenbloom, B., Skrinar, A., van Capelle, C.I., van der Beek, N.A., Wasserstein, M. and Zivkovicet, S.A. A randomized study of alglucosidase alfa in late-onset Pompe's disease. *N. Engl. J. Med.* 362:1396-1406, 2010.
41. Lim, J., Li, L. and Raben, N. Pompe disease: from pathophysiology to therapy and back again. *Frontiers in Aging Neuroscience* 6:1-14, 2014.
42. Gutschmidt, K., Musumeci, O., Díaz-Manera, J., Chien, Y-H., Knop, K.C., Wenninger, S., Montagnese, F., Pugliese, A., Tavilla, G., Alonso-Pérez, J., Wuh-Liang Hwu, P., Toscano, A. and Schoser, B. STIG study: real-world data of long-term outcomes of adults with Pompe disease under enzyme replacement therapy with alglucosidase alfa. *J. Neurol.* Feb 5: 1–11, 2021, doi: 10.1007/s00415-021-10409-9, PMCID: PMC7862044 PMID: 33543425.
43. Raben, N., Jatkar, T., Lee A., Lu, N., Dwivedi, S., Nagaraju, K. and Plotz, P.H. Glycogen stored in skeletal but not in cardiac muscle in acid α -glucosidase mutant (Pompe) mice is highly resistant to transgene-encoded human enzyme. *Molecular Therapy* 6:601-608, 2002.
44. Neufeld, E. F. Lysosomal storage diseases. *Annu. Rev. Biochem.* 60:257–280, 1991.
45. Reuser, A.J., Kroos M.A., Hermans, M.M., Bijvoet, A.G., Verbeet, M.P., Van Diggelen, O.P., Kleijer, W.J. and Van der Ploeg, A.T. Glycogenosis type II (acid maltase deficiency). *Muscle Nerve* 3S:61–69, 1995.
46. Van den Hout, J.M., Reuser, A.J., de Klerk, J.B., Arts, W.F., Smeitink, J.A. and Van der Ploeg, A.T. Enzyme therapy for Pompe disease with recombinant human α -glucosidase from rabbit milk. *J. Inherit. Metab. Dis.* 24:266–274, 2001.
47. Van den Hout, H., Reuser, A.J., Vulto, A.G., Loonen, M.C., Cromme-Dijkhuis, A. and Van der Ploeg, A.T. Recombinant human α -glucosidase from rabbit milk in Pompe patients. *Lancet* 356:397–398, 2000.
48. Amalfitano, A., Bengur, A.R., Morse, R.P., Majure, J.M., Case, L.E., Veerling, D.L., Mackey, J., Kishnani, P., Smith, W., McVie-Wylie, A., Sullivan, J.A., Hoganson, G.E., Phillips, J.A. 3rd, Schaefer, G.B., Charrow, J., Ware, R.E., Bossen, E.H. and Chen, Y.T. Recombinant human acid α -glucosidase enzyme therapy for infantile glycogen storage disease type

II: results of a phase I/II clinical trial. *Genet. Med.* 3:132–138, 2001.

49. Bijvoet, A.G., Van Hirtum, H., Kroos, M.A., Van de Kamp, E.H., Schoneveld, O., Visser, P., Brakenhoff, J.P., Weggeman, M., van Corven, E.J., Van der Ploeg, A.T. and Reuser, A.J. Human acid α -glucosidase from rabbit milk has therapeutic effect in mice with glycogen storage disease type II. *Hum. Mol. Genet.* 8:2145–2153, 1999.
50. Dunbar, C.E., High, K.A., Joung, J.K., Kohn, D.B., Ozawa, K. and Sadelain, M. Gene therapy comes of age. *Science* 359:1-10, 2018.
51. Gupta, B., Levchenko, T.S. and Torchilin, V.P. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv. Drug Deliv. Rev.* 57:637-651, 2005.
52. Zorko, M. and Langel, U. Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv. Drug Deliv. Rev.* 57:529-545, 2005.
53. Kerkis, A., Hayashi, M.A.F., Yamane, Y. and Kerkis, I. Properties of cell penetrating peptides (CPPs). *IUBMB. Life* 58:1-7, 2006.
53. Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R. and Hawiger, J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.* 270:14255-14258, 1995.
54. Rojas, M., Donahue, J.P., Tan, Z. and Lin, Y.Z. Genetic engineering of proteins with cell membrane permeability. *Nat. Biotechnol.* 16:370-375, 1998.
55. Schwarze, S.R. and Dowdy, S.F. *In vivo* protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends in Pharm. Sci.* 21:45-48, 2000.
56. Jarver, P. and Langel, U. The use of cell-penetrating peptides as a tool for gene regulation. *Drug Discov. Today* 9:395-402, 2004.
57. Richard, J.P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M.J., Chernomordik, L.V. and Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 278:585-590, 2003.
58. Derossi, D., Chassaing, G. and Prochiantz, A. Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol.* 8:84-87, 1998.
59. Futaki, S. Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. *Int. J. Pharm.* 245:1-7, 2002.
60. Drin, G., Cottin, S., Blanc, E., Rees, A.R. and Temsamani, J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.* 278:31192-31201, 2003.
61. Ziegler, A., Nervi, P., Dürrenberger, M. and Seelig, J. The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. *Biochemistry* 44:138-148, 2005.
62. Derossi, D., Joliot, A.H., Chassaing, G. and Prochiantz, A. The third helix of the *Antennapedia* homeodomain translocates through biological membranes. *J. Biol. Chem.* 269:10444–10450, 1994.
63. Green, M. and Loewenstein, P. M. Autonomous functional domain of chemically synthesized human immunodeficiency virus tat *trans*-activator protein. *Cell* 55:1179–1188, 1988.
64. Frankel, A. and Pabo, C. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 55:1189–1193, 1988.
65. Elliott, G. and O'Hare, P. Intercellular trafficking and protein delivery by a herpes virus structural protein. *Cell* 88:223–233, 1997.
66. Ford, K. G., Darling, D., Souberbielle, B. and Farzaneh, F. Protein transduction: a new tool for the study of cellular ageing and senescence. *Mech. Ageing Dev.* 121:113–121, 2000.
67. Martin, A., O'Hare, P., McLauchlan, J. and Elliott, G. Herpes simplex's virus tegument protein VP22 contains overlapping domains for cytoplasmic localization, microtubule interaction, and chromatin binding. *J. Virol.* 76:4961–4970, 2002.
68. Schwarze, S. R., Ho, A., Vocero-Akbani, A. and Dowdy, S. F. *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* 285:1569–1572, 1999.
69. Dilber, M., Phelan, A., Aints, A., Mohamed, A., Elliott, G., Edvard Smith, C. and O'Hare, P. Intercellular delivery of thymidine kinase prodrug activating enzyme by the Herpes simplex's virus protein, VP22. *Gene Ther.* 6:12–21, 1999.
70. Cashman, S.M., Sadowski, S.L., Morris, D.J., Frederick, J. and Kumar-Singh, R. Intercellular trafficking of adenovirus-delivered HSV VP22 from the retinal pigment epithelium to the photoreceptor - Implication for gene therapy. *Mol. Ther.* 6:813–823, 2002.
71. Nagahara, H., Vocero-Akbani, A., Snyder, E., Ho, A., Latham, D., Lissy, N., Becker Hapak, M., Ezhevsky, S. and Dowdy, S. Transduction of full length TAT fusion proteins into mammalian cells: TAT-p27kip1 induces cell migration. *Nat. Med.* 4:1449–1452, 1998.

72. Phelan, A., Elliott, G. and O'Hare, P. Intercellular delivery of functional p53 by the herpes virus protein VP22. Nat. Biotechnol. 16:440–443, 1998.
73. Radis-Baptista, G., Oguiura, N., Hayashi, M., Camargoc, M., Gregod, K., Oliveira, E. and Yamane, T. Nucleotide sequence of crotoamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37:973–984, 1999.
74. Fadela, V., Bettendorff, P., Herrmann, T., de Azevedo Jr, W.F., Oliveira, E.B., Yamane, T. and Wuthrich, K. Automated NMR structure determination and disulfide bond identification of the myotoxin crotoamine from *Crotalus durissus terrificus*. Toxicon 46:759–767, 2005.
75. Kerkis, A., Kerkis, I., Radis-Baptista, G., Oliveira, E., Vianna-Morgante, A., Pereira, L. and Yamane, T. Crotoamine is a novel cell-penetrating protein from the venom of rattlesnake *Crotalus durissus terrificus*. FASEB J. 18:1407–1409, 2004.
76. Nascimento, D., Hayashi, M., Kerkis, A., Oliveira, V., Oliveira, E.B., Radis-Baptista, G., Bonciani Nader, H., Yamane, T., dos Santos Tersariol, I. and Kerkis, I. Crotoamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. J.B.C. 282:21349–60, 2007.
77. Costa, B.A., Sanches, L., Gomide, A.B., Bizerra, F., Dal Mas, C., Oliveira, E.B., Perez, K.R., Itri, R., Oguiura, N. and Hayashi, M.A. Interaction of the rattlesnake toxin crotoamine with model membranes. J. Phys. Chem. B. 118:5471–9, 2014.
78. Kerkis, I., Hayashi, M.A., Prieto da Silva, A.R., Pereira, A., De Sá Júnior, P.L., Zaharenko, A.J., Radis-Baptista, G., Kerkis, A. and Yamane, T. State of the art in the studies on crotoamine, a cell penetrating peptide from South American rattlesnake. Biomed. Res. Int. 2014:1–9, 2014.
79. Sieber, M., Bosch, B., Hanke, W. and Fernandes de Lima, V.M. Membrane-modifying properties of crotoamine, a small peptide-toxin from *Crotalus durissus terrificus* venom. Biochim. Biophys. Acta. 1840:945–50, 2014.
80. Coronado, M.A., Gabdulkhakov, A., Georgieva, D., Sankaran, B., Murakami, M.T., Arni, R.K. and Betzel, C. Structure of the polypeptide crotoamine from the Brazilian rattlesnake *Crotalus durissus terrificus*. Acta Crystallogr. D. Biol. Crystallogr. 69:1958–64, 2013.
81. Rodrigues, M., de la Torre, B.G., Andreu, D. and Santos, N.C. Kinetic uptake profiles of cell penetrating peptides in lymphocytes and monocytes. Biochim. Biophys. Acta. 1830:4554–63, 2013.
82. Chen, P.C., Hayashi, M.A., Oliveira, E.B. and Karpel, R.L. DNA-interactive properties of crotoamine, a cell-penetrating polypeptide and a potential drug carrier. PLoS One 7:e48913, 2012.
83. Coronado, M.A., Georgieva, D., Buck, F., Gabdoulkhakov, A.H., Ullah, A., Spencer, P.J., Arni, R.K. and Betzel, C. Purification, crystallization and preliminary X-ray diffraction analysis of crotoamine, a myotoxic polypeptide from the Brazilian snake *Crotalus durissus terrificus*. Acta Crystallogr. Sect F Struct. Biol. Cryst. Commun. 68:1052–4, 2012.
84. Hayashi, M.A., Oliveira, E.B., Kerkis, I. and Karpel R.L. Crotoamine: a novel cell-penetrating polypeptide nanocarrier with potential anti-cancer and biotechnological applications. Methods Mol. Biol. 906:337–52, 2012.
85. Radis-Baptista, G. and Kerkis, I. Crotoamine, a small basic polypeptide myotoxin from rattlesnake venom with cell-penetrating properties. Curr. Pharm. Des. 17:4351–61, 2011.
86. Kerkis, I., de Sa Silva, F., Pereira, A., Kerkis, A. and Radis-Baptista, G. Biological versatility of crotoamine—a cationic peptide from the venom of a South American rattlesnake. Expert Opin. Investig. Drugs 19:1515–25, 2010.
87. Nascimento, F.D., Sancey, L., Pereira, A., Rome, C., Oliveira, V., Oliveira, E.B., Nader, H.B., Yamane, T., Kerkis, I., Tersariol, I.L., Coll, J.L. and Hayashi, M.A. The natural cell penetrating peptide crotoamine targets tumor tissue *in vivo* and triggers a lethal calcium-dependent pathway in cultured cells. Molecular Pharmaceutics 9:211–221, 2012.
88. Pereira, A., Kerkis, A., Hayashi, M. A. Pereira, A.S., Silva, F.S., Oliveira, E.B., Prieto da Silva, A.R., Yamane, T., Radis-Baptista, G. and Kerkis I. Crotoamine toxicity and efficacy in mouse models of melanoma. Expert Opinion on Investigational Drugs 20:1189–1200, 2011.
89. Leon, G. Sanchez, L., Hernandez, A., Villalta, M., Herrera, M., Segura, A., Estrada, R. and Gutiérrez, J.M. Immune response towards snake venoms. Inflammation and Allergy-Drug Targets 10:381–398, 2011.
90. Lomonte, B., Escolano, J., Fernández, J., Sanz, L., Angulo, Y., Gutiérrez, J.M. and Calvete, J.J. Snake venomomics and antivenomics of the arboreal neotropical pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii*. J. Proteome Res. 7:2445–2457, 2008.
91. Gutiérrez, J.M., Sanz, L., Escolano, J., Fernández, J., Lomonte, B., Angulo, Y., Rucavado, A., Warrell, D.A. and Calvete, J.J. Snake venomomics of the lesser Antillean pit vipers *Bothrops caribbaeus* and *Bothrops lanceolatus*: correlation with toxicological activities and immunoreactivity of a heterologous antivenom. J. Proteome Res. 7:4396–4408, 2008.
92. Calvete, J.J., Sanz, L., Angulo, Y., Lomonte, B. and Gutiérrez, J.M. Venoms, venomomics and antivenomics. FEBS Lett. 583:1736–1743, 2009.
93. Yamane, E.S., Bizerra, F.C., Oliveira, E.B., Moreira, J.T., Rajabi, M., Nunes, G.L.C., de Souza, A.O., da Silva, I.D.C.G., Yamane, T., Karpel, R.L., Silva Jr, P.I. and Mirian, A.F. Unraveling the antifungal activity of a South American rattlesnake toxin crotoamine. Biochimie 95:231–240, 2013.

- 94.Chen, P.C., Hayashi, M.A., Oliveira, E.B., and Karpel, R.L. DNA-interactive properties of crotamine, a cell-penetrating polypeptide and a potential drug carrier. PLoS One 7:e48913, 2012.
- 95.Porta, L., Fadel, V., Campeiro, J., Oliveira, E., Godinho, R. and Hayashi, M. Biophysical and pharmacological characterization of a full-length synthetic analog of the antitumor polypeptide crotamine. Journal of Molecular Medicine 98:1561–1571, 2020.
- 96.Hoffmann, A. and Roeder, R.G. Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Research 19:6337, 1991.
- 97.Vu, T.T.T., Jeong, B., Yu, J., Koo, B.K., Jo, S.H., Robinson, R.C. and Choe, H. Soluble prokaryotic expression and purification of crotamine using an N-terminal maltose-binding protein tag. Toxicon 92:157-165, 2014.
- 98.Hintze, S., Limmer, S., Dabrowska-Schlepp, P., Berg, B., Kriehoff, N., Busch, A., Schaaf, A., Meinke, P. and Schoser, B. Moss-derived human recombinant GAA provides an optimized enzyme uptake in differentiated human muscle cells of Pompe disease. Int. J. Mol. Sci. 21:2642-2657, 2020.
- 99.Martiniuk, F., Chen, A., Mack, A., Donnabella, V., Slonim, A., Bulone, L., Arvanitopoulos, E., Raben, N., Plotz, P. and Rom, W.N. Helios gene gun particle delivery for therapy of acid maltase deficiency. DNA and Cell Biology 21:717-725, 2002.
- 100.Martiniuk, F. Gene therapy for Pompe's disease. Current Medical Literature's Journal on Lysosomal Storage Disease 29-37, 2002.
- 101.Martiniuk, F. Liposome mediated gene therapy for treatment of Pompe's disease. Oral presentation. Prospect of treatment of rare diseases. Trieste, Italy 2003.
- 102.Martiniuk, F., Donnabella, V., Arvanitopoulos, E., Raben, N., Plotz, P., Cheng, S. and Rom, W.N. Correction of disease phenotype in acid maltase deficient knockout mice following cationic lipid mediated gene therapy. submitted, 2021.
- 103.Martiniuk, F., Chen, A., Donnabella, V., Arvanitopoulos, E., Slonim, A.E., Raben, N., Plotz, P. and Rom W.N. Correction of glycogen storage disease type II by enzyme replacement with a recombinant human acid maltase produced by over-expression in a CHO-DHFR (neg) cell line. Biochem. Biophys. Res. Commun. 276:917-923, 2000.
- 104.Martiniuk, F., Mehler, M., Tzall, S., Meredith, G. and Hirschhorn, R. Sequence of the cDNA and 5' flanking region for human acid alpha glucosidase, detection of an intron in the 5' untranslated leader sequence, definition of 18-bp polymorphisms, and differences with previous cDNA and amino acid sequences. DNA 9:85-94, 1990.
- 105.Martiniuk, F., Mehler, M., Tzall, S., Meredith, G. and Hirschhorn R. Extensive genetic heterogeneity in patients with acid a-glucosidase deficiency. Am. J. Human Genetics 47:73-78, 1990.
- 106.Raben, N., Nagaraju, K., Lee, E., Kessler, P., Byrne, B., Lee, L., LaMaurex, M., King, J., Sauer, B. and Plotz, P. Targeted disruption of the acid alpha glucosidase gene in mice causes an illness with critical features of both infantile and adult human glycogen storage disease type II. J.B.C. 273:19086-92, 1998.
- 107.Gomez-Lechon, M.J., Ponsoda, X., and Castell, J.V. A microassay for measuring glycogen in 96-well-cultured cells. Analytical Biochemistry 236: 296–301, 1996.
- 108.Martiniuk, F., Mehler, M., Bodkin, M., Tzall, S., Hirschhorn, K., Zhong, N. and Hirschhorn, R. Identification of the mutation from an adult onset patient with glycogenosis type II expressing only one allele. DNA and Cell Biology 10:681-687, 1991.
- 109.Leamy, V.L., Martin, T., Mahajan, R., Vilalta, A., Rusalov, D., Hartikka, J., Bozoukova, V., Hall, K.D., Morrow, J., Rolland, A.P., Kaslow, D.C. and Lalor, P.A. Comparison of rabbit and mouse models for persistence analysis of plasmid based vaccines. Hum. Vaccin. 2:113-8, 2006.
- 110.Vilalta, A., Mahajan, R.K., Hartikka, J., Leamy, V., Martin, T., Rusalov, D., Bozoukova, V., Lalor, P., Hall, K., Kaslow, D.C. and Rolland, A. Cationic lipid-formulated plasmid DNA-based *Bacillus anthracis* vaccine: evaluation of plasmid DNA persistence and integration potential. Human Gene Therapy 16:1151–1156, 2005.
- 111.Vilalta, A., Mahajan, R.K., Hartikka, J., Rusalov, D., Martin, T., Bozoukova, V., Leamy, V., Hall, K., Lalor, P., Rolland, A. and Kaslow, D.C. I. Poloxamer-formulated plasmid DNA-based human cytomegalovirus vaccine: evaluation of plasmid DNA biodistribution/persistence and integration. Human Gene Therapy 16:1143–1150, 2005.
- 112.Bertoni, C., Jarrahan, S., Wheeler, T.M., Li, Y., Olivares, E.C., Calos, M.P. and Rando, T.A. Enhancement of plasmid-mediated gene therapy for muscular dystrophy by directed plasmid integration. PNAS 103:419–424, 2006.
- 113.Ledwith, B.J., Manam, S., Troilo, P.J., Barnum, A.B., Pauley, C.J., Griffiths, T.G. 2nd, Harper, L.B., Schock, H.B., Zhang, H., Faris, J.E., Way, P.A., Beare, C.M., Bagdon, W.J. and Nichols, W.W. Plasmid DNA vaccines: assay for integration into host genomic DNA. Dev. Biol. (Basel) 104:33-43, 2000.
- 114.Glover, D.J., Lipps, H.J. and Jans, D.A. Towards safe, nonviral therapeutic gene expression in humans. Nature Reviews Genetics 6:299-311, 2005.
- 115.Wang, X., Sarkar, D.P., Mani, P., Steer, C.J., Chen, Y., Guha, C., Chandrasekhar, V., Chaudhuri, A., Roy-Chowdhury, N., Kren, B.T. and Roy-Chowdhury, J. Long-term reduction of jaundice in Gunn rats by nonviral liver-

targeted delivery of sleeping beauty transposon. *Hepatology* 50:815–824, 2009.

- 116.Miao, C.H., Thompson, A.R, Loeb, K. and Ye, X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer *in vivo*. *Molecular Therapy* 3:947-957, 2001.
- 117.Wang, Z., Troilo, P.J., Wang, X., Griffiths II, T.G., Pacchione, S.J., Barnum, A.B., Harper, L.B., Pauley, C.J., Niu, Z., Denisova, L., Follmer, T.T., Rizzuto, G., Ciliberto, G., Fattori, E., Monica, N.L., Manam, S. and Ledwith, H.J. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Therapy* 1:711–721, 2004.
- 118.Puttni, S., van Zwieten, R.W., Saugy, D., Lekka, M., Hogger, F., Ley, D., Kulik, A.J. and Mermod, N. MAR-mediated integration of plasmid vectors for *in vivo* gene transfer and regulation. *BMC Molecular Biology* 14:26-37, 2013.
- 119.Martin, T., Parker, S., Hedstrom, R., Le, T, Hoffman, J., Hobart, P. and Lew, D. Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. *Human Gene Therapy* 10:759-768, 1999.
- 120.Tolmachov, O.E. Building mosaics of therapeutic plasmid gene vectors. *Curr. Gene Ther.* 11:466-78, 2011.
121. Liu, R., Li, Y., Hu, R., Jin, T., Deng, S., Liang, W., Zhang, N., Chen, J., Prudhomme, G.J., Jia, W.W., Ma, D. and Wang, Q. A site-specific genomic integration strategy for sustained expression of glucagon-like peptide-1 in mouse muscle for controlling energy homeostasis. *Biochem. Biophys. Res. Commun.* 403:172-7, 2010.
- 122.Aneja, M.K., Imker, R. and Rudolph, C. Phage phiC31 integrase-mediated genomic integration and long-term gene expression in the lung after nonviral gene delivery. *J. Gene Med.* 9:967-75, 2007.
- 123.Fernando, S. and Fletcher, B.S. Sleeping beauty transposon-mediated nonviral gene therapy. *BioDrugs* 20:219-29, 2006.
- 124.Coelho-Castelo, A.A., Trombone, A.P., Rosada, R.S., Santos, R.R., Jr, Bonato, V.L., Sartori, A. and Silva, C.L. Tissue distribution of a plasmid DNA encoding *Hsp65* gene is dependent on the dose administered through intramuscular delivery. *Genet. Vaccines Ther.* 4:1-10, 2006.
- 125.Ohlfest, J.R., Frandsen, J.L., Fritz, S., Lobitz, P.D., Perkinson, S.G., Clark, K.J., Nelsestuen, G., Key, N.S., McIvor, R.S., Hackett, P.B. and Largaespada, D.A. Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the Sleeping Beauty transposon system. *Blood* 105:2691-8, 2005.
- 126.Williams, J.A. Carnesa, A.E. and Clague, P. Hodgson, C.P. Plasmid DNA vaccine vector design: impact on efficacy, safety and upstream production. *Biotechnol. Adv.* 27: 353–370, 2009.
- 127.Nguyen, G.N., Everett, J.K., Kafle, S., Roche, A.M., Raymond, H.E., Leiby, J., Wood, C., Assenmacher, C.A., Merricks, E.P., Long, C.T., Kazazian, H.H., Nichols, T.C., Bushman, F.D. and Sabatino, D.E. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nature Biotechnology* 39:47–55, 2021.
- 128.Lin, C.Y., Ho, C.H., Hsieh, C.H. and Kikuchi, T. Adeno-associated virus-mediated transfer of human acid maltase gene results in a transient reduction of glycogen accumulation in muscle of Japanese quail with acid maltase deficiency. *Gene Therapy* 9:554-63, 2002.
- 129.The Journal of Gene Medicine Clinical Trial website.
- 130.Fraites, T.J., Schleissig, M.R., Shanely, R.A. Walter, G.A., Cloutier, D.A., Zolotukhin, I., Pauly, D.F., Raben, N., Plotz, P.H., Powers, S.K., Kessler, P.D. and Byrne, B.J. Correction of the enzymatic and functional deficits in a model of Pompe disease using adeno-associated virus vectors. *Molecular Therapy* 5:561-578, 2002.
- 131.Ding, E.Y., Hodges, B.L., Hu, H., McVie-Wylie, A.J., Serra, D., Migone, F.K., Pressley, D., Chen, Y.T. and Amalfitano, A. Adenovirus-mediated transfer of human acid- α -glucosidase gene into glycogen storage disease type II knockout mice. *Human Gene Therapy* 12:955–965, 2001.
- 132.Amalfitano, A., McVie-Wylie, H., Hu, T.L., Dawson, T.L., Raben, N., Plotz, P. and Chen, Y.T. Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid- α -glucosidase (Pompe gene therapy). *PNAS* 96:8861–8866, 1999.
- 133.Ding, E., Hu, H., Hodges, B.L., Migone, F. Serra, D., Xu, F., Chen, Y.T. and Amalfitano, A. Efficacy of gene therapy for a prototypical lysosomal storage disease (GSD-II) is critically dependent on vector dose, transgene promoter, and the tissues targeted for vector transduction. *Molecular Therapy* 5:436-446, 2002.
- 134.Zaretzky, J.Z., Candotti, F., Boerkoel, C., Adams, E.M., Yewdell, J.W., Blaese, R.M. and Plotz, P.H. Retroviral transfer of acid α -glucosidase to enzyme deficient myoblasts results in phenotype spread of the genotypic correction by both secretion and fusion. *Hum. Gene Therapy* 8:1555-63, 1997.
- 135.Rucker, M., Fraites, T.J, Porvasnik, S. L., Lewis, M., A., Zolotukhin, I., Cloutier, D. A. and Byrne, B.J. Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease. *Development* 131:3007-3019, 2004.

- 136.** Cresawn, K. O., Fraites, T. J., Wasserfall, C., Atkinson, M., Lewis, M., Porvasnik, S., Liu, C., Mah, C. and Byrne, B. J. Impact of humoral immune response on distribution and efficacy of recombinant Adeno-associated virus-derived acid α -glucosidase in a model of glycogen storage disease type II. *Hum. Gene Ther.* 16:68-80, 2005.
- 137.** Kiang, A., Hartman, Z.C., Liao, S., Xu, F., Serra, D., Palmer, D.J., Ng, P. and Amalfitano, A. Fully deleted adenovirus persistently expressing GAA accomplishes long-term skeletal muscle glycogen correction in tolerant and nontolerant GSD-II mice. *Mol. Ther.* 13:127-34, 2006.
- 138.** Franco, L. M., Sun, B., Yang, X., Bird, A., Zhang, H., Schneider, A., Brown, T., Young, S.P., Clay, T.M., Amalfitano, A., Chen, Y.T. and Koeberl, D.D. Evasion of immune responses to introduced human acid α -glucosidase by liver-restricted expression in glycogen storage disease type II. *Mol. Ther.* 12:876-84, 2005.
- 139.** An, Y., Young, S.P., Kishnani, P.S., Millington, D.S., Amalfitano, A., Corz, D. and Chen, Y.T. Glucose tetrasaccharide as a biomarker for monitoring the therapeutic response to enzyme replacement therapy for Pompe disease. *Mol. Genet. Metab.* 85:247-54, 2005.
- 140.** Sun, B., Zhang, H., Franco, L.M., Young, S.P., Schneider, A., Bird, A., Amalfitano, A., Chen, Y.T. and Koeberl, D.D. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. *Mol. Ther.* 11:57-65, 2005.
- 141.** Xu, F., Ding, E., Migone, F., Serra, D., Schneider, A., Chen, Y.T. and Amalfitano, A. Glycogen storage in multiple muscles of old GSD-II mice can be rapidly cleared after a single intravenous injection with a modified adenoviral vector expressing hGAA. *J. Gene Med.* 7:171-8, 2005.
- 142.** Xu, F., Ding, E., Liao, S.X., Migone, F., Dai, J., Schneider, A., Serra, D., Chen, Y.T. and Amalfitano, A. Improved efficacy of gene therapy approaches for Pompe disease using a new, immune-deficient GSD-II mouse model. *Gene Ther.* 11:1590-8, 2004.
- 143.** McVie-Wylie, A.J., Ding, E.Y., Lawson, T., Serra, D., Migone, F.K., Pressley, D., Mizutani M., Kikuchi, T., Chen, Y.T. and Amalfitano, A. Multiple muscles in the AMD quail can be "cross-corrected" of pathologic glycogen accumulation after intravenous injection of an [E1-, polymerase-] adenovirus vector encoding human acid- α -glucosidase. *J. Gene Med.* 5:399-406, 2003.
- 144.** Sun, B.D., Chen, Y.T., Bird, A., Amalfitano, A. and Koeberl, D.D. Long-term correction of glycogen storage disease type II with a hybrid Ad-AAV vector. *Mol. Ther.* 7:193-201, 2003.
- 145.** Sun, B., Chen, Y.T., Bird, A., Xu, F., Hou, Y., Amalfitano, A. and Koeberl, D. Packaging of an AAV vector encoding human acid α -glucosidase for gene therapy in glycogen storage disease type II with a modified hybrid adenovirus-AAV vector. *Molecular Therapy* 7:467-477, 2003.
- 146.** Sun, B., Zhang, H., Bird, A., Li, S., Young, S.P. and Koeberl, D.D. Impaired clearance of accumulated lysosomal glycogen in advanced Pompe disease despite high-level vector-mediated transgene expression. *J. Gene Med.* 11:913-920, 2009.
- 147.** Maga, J.A., Zhou, J., Kambampati, R., Peng, S., Wang, X., Bohnsack, R.N., Thomm, A., Golata, S., Tom, P., Dahms, N.M., Byrne, B.J. and LeBowitz, A.H. Glycosylation-independent lysosomal targeting of acid α -glucosidase enhances muscle glycogen clearance in Pompe. *J. Biol. Chem.* 288:1428-1438, 2013.
- 148.** Nicolino, M.P., Puech, J.P., Kremer, E.J., Reuser, A.J., Mbebi, C., Verdière-Sahuqué, M., Kahn, A. and Poenaru, L. Adenovirus-mediated transfer of the acid α -glucosidase gene into fibroblasts, myoblasts and myotubes from patients with glycogen storage disease type II leads to high level expression of enzyme and corrects glycogen accumulation. *Human Molecular Genetics* 20:R61-R68, 2011.
- 149.** Ziegler, R.J., Bercury, S.G., Fidler, J., Zhao, M.A., Foley, J., Taksir, T.V., Ryan, S., Hodges, B.J., Scheule, R.K., Shihabuddin, L.S. and Seng, H. Cheng ability of adeno-associated virus serotype 8-mediated septic expression of acid α -glucosidase to correct the biochemical and motor function deficits of presymptomatic and symptomatic Pompe mice. *Human Gene Therapy* 19:609-621, 2008.
- 150.** Kyosen, S.O., Iizuka, S., Kobayashi, H., Kimura, T., Fukuda, T., Shen, J., Shimada, Y., Ida, H., Et, Y. and Ohashi, T. Neonatal gene transfer using lentiviral vector for murine Pompe disease: long-term expression and glycogen reduction. *Gene Therapy* 17:521-530, 2010.
- 151.** van Til, N.P., Stok, M., Aerts Kaya, F.S., de Waard, M.C., Farahbakhshian, E., Visser, T.P., Kroos, M.A., Jacobs, E.H., Willart, M.A., van der Wegen, P., Scholte, B.J., Lambrecht, B.N., Duncker, D.J., van der Ploeg, A.T., Reuser, A.J., Verstegen, M.M. and Wagemaker, G. Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood* 115:5329-5337, 2010.
- 152.** Pauly, D.F., Fraites, T.J., Toma, C., Bayes, H.S., Huie, M.L., Hirschhorn, R., Plotz, P.H., Raben, N., Kessler, P.D. and Byrne, B.J. Intercellular transfer of the virally derived precursor form of acid α -glucosidase corrects the enzyme deficiency in inherited cardioskeletal myopathy Pompe disease. *Human Gene Therapy* 12:527-538, 2001.
- 153.** Conlon, T.J., Erger, K., Porvasnik, S., Cossette, T., Roberts, C., Combee, L., Islam, S., Kelley, J., Cloutier, D., Clement, N., Abernathy, C.R. and Byrne, B.J. Preclinical toxicology and biodistribution studies of recombinant adeno-

associated virus 1 human acid α -glucosidase. Human Gene Therapy Clinical Development 24:127–133, 2013.

154.Falk, D.J., Soustek, M.S., Todd, A.G., Mah, C.S., Cloutier, D.A., Kelley, J.S., Clement, N., Fuller, D.D. and Byrne, B.J. Comparative impact of AAV and enzyme replacement therapy on respiratory and cardiac function in adult Pompe mice. Molecular Therapy — Methods & Clinical Development 2:15007-15, 2015.

155.Corti, M., Cleaver, B., Clement, N., Conlon, T.J., Kaitlyn J. Faris, K.J., Wang, G., Benson, J., Tarantal, A.F., Fuller, D., Herzog, R.W. and Byrne, B.J. Evaluation of readministration of a recombinant adeno-associated virus vector expressing acid alpha-glucosidase in Pompe disease: Preclinical to clinical planning. Human Gene Therapy Clinical Development 26:185-193, 2015.

156.Doerfler, P.A., Todd, A.G., Clement, N., Falk, D.J., Nayak, S., Herzog, R.W. and Byrne, B.J. Copackaged AAV9 vectors promote simultaneous immune tolerance and phenotypic correction of Pompe disease. Human Gene Therapy 27:43-53, 2015.

157.Smith, B.K., Martin, D., Lawson, L.A., Vernot, V., Marcus, J., Islam, S., Shafi, N., Corti, M., Collins, S.W. and Byrne, B.J. Inspiratory muscle conditioning exercise and diaphragm gene therapy in Pompe disease: Clinical evidence of respiratory plasticity. Experimental Neurology 287:216–224, 2017.

158.Byrne, B.J., Falk, D.J., Pacak, C.A., Nayak, S., Herzog, R., Elder, M.E., Collins, S.W., Conlon, T.J., Clement, N., Cleaver, B.D., Cloutier, D.A., Porvasnik, S.L., Islam, S., Elmallah, M.K., Martin, A., Smith, B.K., Fuller, D.D., Lawson, L.A. and Mah, C. Pompe disease gene therapy. Human Molecular Genetics 20:R61–R68, 2011.

159.Smith, B.K., Collins, S.W., Conlon, T.J., Mah, C., Lawson, L.A., Martin, A.D., Fuller, D.D., Cleaver, B.D., Clement, N., Phillips, D., Islam, S., Dobjia, N. and Byrne, B.J. Phase I/II trial of adeno-associated virus-mediated alpha-glucosidase gene therapy to the diaphragm for chronic respiratory failure in Pompe disease: initial safety and ventilatory outcomes. Human Gene Therapy 24:630–640, 2013.

160.Mah, C., Cresawn, K.O., Fraites, T.J., Pacak, C.A., Lewis, M.A., Zolotukhin, I. and Byrne, B.J. Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors. Gene Therapy 12:1405–1409, 2005.

161.Mah, C.S., Falk, D.J., Germain, S.A., Kelley, J.S., Lewis, M.A., Cloutier, D.A., DeRuisseau, L.R., Conlon, T.J., Cresawn, K.O., Fraites, T.J., Campbell-Thompson, M., Fuller, D.D. and Byrne, B.J. Gel-mediated delivery of AAV1 vectors corrects ventilatory function in Pompe mice with established disease. Molecular Therapy 18:502–510, 2010.

162.Mah, C., Pacak, C.A., Cresawn, K.O., DeRuisseau, L.R., Germain, S., Lewis, M.A., Cloutier, D.A., Fuller, D.D. and Byrne, B.J. Physiological correction of Pompe disease by systemic delivery of adeno-associated virus serotype 1 vectors. Molecular Therapy 15:501–507, 2007.

163.ElMallah, M.K., Falk, D.J., Nayak, S., Federico, R.A., Sandhu, M.S., Poirier, A., Byrne, B.J. and Fuller, D.D. Sustained correction of motoneuron histopathology following intramuscular delivery of AAV in Pompe mice. Molecular Therapy 22:702–712, 2014.

164.Barry J Byrne, Shelley Collins, University of Florida, Safety study of recombinant adeno-associated virus acid alpha-glucosidase to treat Pompe disease. ClinicalTrials.gov Identifier: NCT00976352 Phase I/II Trial of Diaphragm Delivery of Recombinant Adeno-Associated Virus Acid Alpha-Glucosidase (rAAV1-CMV-GAA) Gene Vector in Patients With Pompe Disease.

165.Byrne, B.J., Corti, M. and Elder, E. University of Florida, Evaluation of Re-administration of Recombinant Adeno-Associated Virus Acid Alpha-Glucosidase (rAAV9-DES-hGAA) in Patients With Late-Onset Pompe Disease (LOPD) Re-administration of Intramuscular AAV9 in Patients With Late-Onset Pompe Disease (AAV9-GAA IM) ClinicalTrials.gov Identifier: NCT02240407.

166.Bond, J.E., Kishnani, P.S. and Koeberl, D.D. Immunomodulatory, liver depot gene therapy for Pompe disease. Cell Immunol. S0008-8749L30238-1, 2017.

167.Puzzo, F., Colella, P., Biferi, M.G., Bali, D., Paulk, N.K., Vidal, P., Collaud, F., Simon-Sola, M., Charles, S., Harget, R., Leborgne, C., Meliani, A., Cohen-Tannoudji, M., Astord, S., Gjata, B., Sellier, P., van Wittenberghe, L., Vignaud, A., Boisgerault, F., Barkats, M., Laforet, P., Kay, M.A., Koeberl, D.D., Ronzitti, G. and Mingozzi, F. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secreted acid α -glucosidase. Sci. Transl. Med. 9:1-27, 2017.

168.Han, S.O., Ronzitti, G., Arnson, B., Leborgne, C., Li, S., Mingozzi, F. and Koeberl, D. Low-dose liver-targeted gene therapy for Pompe disease enhances therapeutic efficacy of ERT via immune tolerance induction. Mol. Ther. Methods Clin. Dev. 4:126-136, 2017.

169.Han, S.O., Li, S. and Koeberl, D.D. Salmeterol enhances the cardiac response to gene therapy in Pompe disease. Mol. Genet. Metab. 118:35-40, 2016.

170.Wang, G., Young, S.P., Bali, D., HuttM J., Li, S., Benson, J. and Koeberl, D.D. Assessment of toxicity and biodistribution of recombinant AAV8 vector-mediated immunomodulatory gene therapy in mice with Pompe disease. Mol. Ther. Methods Clin. Dev. 1:14018, 2014.

171.Zhang, P., Luo, X., Bird, A., Li, S. and Koeberl, D.D. Deficiency in MyD88 signaling results in decreased antibody responses to an adeno-associated virus vector in murine Pompe disease. Biores. Open Access 1:109-14, 2012.

172. Zhang, P., Sun, B., Osada, T., Rodriguiz, R., Yang, X.Y., Luo, X., Kemper, A.R., Clay, T.M. and Koeberl, D.D. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. Hum. Gene Ther. 23:460-72, 2012.
173. Sun, B., Li, S., Bird, A. and Koeberl, D.D. Hydrostatic isolated limb perfusion with adeno-associated virus vectors enhances correction of skeletal muscle in Pompe disease. Gene Ther. 2010 17:1500-5, 2010.
174. Koeberl, D.D. and Kishnani, P.S. Immunomodulatory gene therapy in lysosomal storage disorders. Curr. Gene Ther. 9:503-10, 2009.
175. Sun, B., Kulis, M.D., Young, S.P., Hobeika, A.C., Li, S., Bird, A., Zhang, H., Li, Y., Clay, T.M., Burks, W., Kishnani, P.S. and Koeberl, D.D. Immunomodulatory gene therapy prevents antibody formation and lethal hypersensitivity reactions in murine pompe disease. Mol. Ther. 18:353-60, 2010.
176. Sun, B., Zhang, H., Bird, A., Li, S., Young, S.P. and Koeberl, D.D. Impaired clearance of accumulated lysosomal glycogen in advanced Pompe disease despite high-level vector-mediated transgene expression. J. Gene Med. 11:913-20, 2009.
177. Sun, B., Young, S.P., Li, P., Di, C., Brown, T., Salva, M.Z., Li, S., Bird, A., Yan, Z., Auten, R., Hauschka, S.D. and Koeberl, D.D. Correction of multiple striated muscles in murine Pompe disease through adeno-associated virus-mediated gene therapy. Mol. Ther. 16:1366-71, 2008.
178. Kaiser, J. Liver tumor in gene therapy recipient raises concerns about virus widely used in treatment. Science Dec. 22, 2020.
179. www.biospace.com/article/despite-recent-setbacks-gene-therapy-offers-promise-of-treating-devastating-diseases.
180. Audentes Therapeutics. Letter to the MTM disease community. <https://myotubulartrust.org/audentes-therapeutics-letter-23-june-2020/> (last accessed June 28, 2020).
181. Wilson, J.M. and Flotte, T.R. Moving forward after two deaths in a gene therapy trial of myotubular myopathy. Human Gene Therapy 31:695-696, 2020.
182. Farris, E., Brown, D.M., Ramer-Tait, A.E. and Pannier, A.K. Micro- and nanoparticulates for DNA vaccine delivery. Exp. Biol. Med. 241:919-29, 2016.
183. Shah, M.A., Ali, Z., Ahmad, R., Qadri, I., Fatima, K. and He, N. DNA mediated vaccines delivery through nanoparticles. J. Nanosci. Nanotechnol. 15:41-53, 2015.
184. Tregoning, J.S. and Kinnear, E. Using plasmids as DNA vaccines for infectious diseases. Microbiol. Spectr. 2:doi: 10.1128/microbiolspec. PLAS-0028-2014. Review. PMID: 26104452, 2014.
185. cms.plasmidfactory.com/en/