Evaluation of Readministration of a Recombinant Adeno-Associated Virus Vector Expressing Acid Alpha-Glucosidase in Pompe Disease: Preclinical to Clinical Planning

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A recombinant serotype 9 adeno-associated virus (rAAV9) vector carrying a transgene that expresses codon-optimized human acid alpha-glucosidase (*hGAA*, or *GAA*) driven by a human desmin (*DES*) promoter (i.e., rAAV9-DES-*hGAA*) has been generated as a clinical candidate vector for Pompe disease. The rAAV9-DES-*hGAA* vector is being developed as a treatment for both early- and late-onset Pompe disease, in which patients lack sufficient lysosomal alpha-glucosidase leading to glycogen accumulation. In young patients, the therapy may need to be readministered after a period of time to maintain therapeutic levels of *GAA*. Administration of AAV-based gene therapies is commonly associated with the production of neutralizing antibodies that may reduce the effectiveness of the vector, especially if readministration is required. Previous studies have demonstrated the ability of rAAV9-DES-*hGAA* to correct cardiac and skeletal muscle pathology in *Gaa*^{-/-} mice, an animal model of Pompe disease. This article describes the IND-enabling preclinical studies supporting the program for a phase I/II clinical trial in adult patients with Pompe. These studies were designed to evaluate the toxicology, biodistribution, and potential for readministration of rAAV9-DES-*hGAA* injected intramuscularly into the tibialis anterior muscle using an immune modulation strategy developed for this study. In the proposed clinical study, six adult participants with late-onset Pompe disease will be enrolled. The goal of the immune modulation strategy is to ablate B-cells before the initial exposure of the study agent in one leg and the subsequent exposure of the same vector to the contralateral leg four months after initial dosing. The dosing of the active agent is accompanied by a control injection of excipient dosing in the contralateral leg to allow for blinding and randomization of dosing, which may also strengthen the evidence generated from gene therapy studies in the future. Patients will act as their own controls. Repeated measures, at baseline and during the three months following each dosing will assess the safety, biochemical, and functional impact of the vector.

BACKGROUND

POMPE DISEASE IS A PROGRESSIVE and often fatal neuromuscular disorder resulting from a mutation in the gene for acid alpha-glucosidase (*GAA*), an enzyme necessary to degrade lysosomal glycogen. Accumulation of glycogen in multiple tissues results in cardiac, respiratory, and skeletal muscle dysfunction. Patients with complete deficiency of *GAA* are the most severely affected and suffer early mortality

because of cardiopulmonary failure, usually in the first year of life (i.e., early onset or infantile Pompe disease). Patients with some residual activity will present at a later age, but still demonstrate progressive respiratory insufficiency and generalized weakness (i.e., late-onset Pompe disease, LOPD). Respiratory muscle weakness leads to symptoms of respiratory insufficiency, and death eventually occurs because of respiratory failure.^{1,2} Skeletal

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muscle weakness affects the lower limbs and results in loss of walking function and wheelchair dependency.³

The current approach to treatment of Pompe disease is to supplement GAA activity in muscle by enzyme replacement therapy (ERT), a bi-weekly infusion of human GAA (Myozyme or Lumizyme), which was approved by the EMEA and FDA in 2006. However, ERT does not effectively target GAA deficiency and glycogen accumulation in the nervous system, since it does not cross the brain–blood barrier. These findings underlie the unmet need for an alternative therapeutic approach that efficiently targets the neural aspects of Pompe disease.

Glycogen accumulation has been extensively evaluated in Pompe muscle tissue and linked to overall muscle dysfunction. In particular, it is generally accepted that diaphragmatic dysfunction is the primary reason for ventilatory deficits in Pompe disease. However, we have recently shown that GAA enzyme deficiency in the central nervous system (CNS) significantly contributes to respiratory insufficiency in Pompe patients and animal models. The mechanism of the neural contribution to weakness in Pompe appears to be related to maladaptation of neuromuscular junctions (NMJ)⁴ and motor neuron dysfunction.^{5–7} Falk et al.⁴ showed loss of NMJ integrity in *Gaa*^{−/−} mice compared with controls. In addition, these studies demonstrated that reconstitution of the NMJ in Pompe animals following AAV9-DES-GAA administration. Following longitudinal sectioning of a *Gaa*^{−/−}+AAV9-GAA nerve, an increased signal in growth associated protein 43 (Gap43) labeling was observed. Gap43 has been associated with axonal regeneration and long-term potentiation, and is a crucial component of the presynaptic terminal.^{8,9} These observations support the requirement of an approach that will address the CNS deficits in Pompe and restore proper muscular function.⁵

In support of this hypothesis, recent reports reveal that glycogen accumulation in neurons is associated with apoptosis in cell culture¹⁰ and that spinal neurons seem to be particularly susceptible to excessive glycogen content. Furthermore, several case reports have shown glycogen accumulation in the CNS of Pompe patients.^{11–17} Postulating that GAA deficiency in motor neurons would contribute to respiratory insufficiency, we tested the hypothesis that *Gaa*^{−/−} mice would exhibit reduced ventilation and this would be reflected by attenuated efferent phrenic motor discharge. We showed that *Gaa*^{−/−} mice exhibit high glycogen content in the spinal cord and phrenic motoneurons, and

these animals exhibited reduced ventilation during quiet breathing. Neurophysiological data indicated that efferent phrenic motor output was substantially lower in *Gaa*^{−/−} mice compared with controls.^{4,7,11} In human subjects, we observed a similar motoneuron pathology in the cervical spinal cord, and glycogen accumulation was greater in spinal cord compared to the brain.¹¹ These novel observations raise important considerations for the approach to Pompe disease therapy, since the only currently available strategy using ERT does not effectively target GAA deficiency and glycogen accumulation in the CNS. AAV-mediated gene delivery to the respiratory musculature and associated motor neurons is the basis for the future therapeutic approach in Pompe disease. Clinical approaches to the delivery of vectors to the brain and spinal cord are currently being explored in several related neurological disorders.^{18,19}

Recombinant adeno-associated viral vectors (rAAV) are widely used gene therapy agents for the treatment of genetic diseases. rAAV has been used in several clinical trials for the treatment of different conditions, including Leber's congenital amaurosis,^{20,21} hemophilia B,^{22,23} Pompe disease,²⁴ Sanfilippo syndrome,²⁵ lipoprotein lipase deficiency,^{26,27} alpha-1 antitrypsin deficiency,²⁸ and limb-girdle muscular dystrophy.^{29,30} However, a critical challenge remains for the success of gene therapy: managing the host's immune response to both the vector capsid and transgene product. These immune responses raise concerns regarding the safety and longevity of gene expression. The development of antibodies through natural exposure to AAV is frequent early in life and may influence the use of AAV as a gene therapy vector.^{31,32} This may be critical in developing effective therapeutic strategies for congenital myopathies that may require repeat administration of AAV vectors. Addressing this issue will ensure that subjects who have received a nontherapeutic vector dose in early phase studies will not be precluded from receiving an effective dose in the future. Furthermore, many subjects may require re-dosing later in life, since increasing muscle mass or loss of copy number with age may reduce transgene expression. However, potent humoral and cellular memory responses to AAV may compromise the subsequent use of the same vector.^{31–33} For these reasons, we are developing a clinically applicable strategy to manage these immune responses, in order to achieve safe and long-term expression of a therapeutic gene by AAV-mediated gene therapy.

One of the strategies to overcome or prevent the development of neutralizing antibodies (NAbs) in

rAAV-mediated gene therapy is pharmacological modulation of the humoral immune response. In a recent study,³⁴ we evaluated the immune response of a Pompe patient dosed with an AAV1-hGAA vector after receiving rituximab and sirolimus to modulate reactions against ERT. A key finding of this single-subject case report is that B-cell ablation with rituximab before AAV vector exposure results in nonresponsiveness to both the capsid and transgene, therefore allowing for the possibility of repeat administration in the future. Based on this observation, we proposed to test this clinical strategy in a prospective trial to evaluate AAV vector readministration. To that end, we have begun IND-enabling toxicology studies to evaluate the parameters for both primary and subsequent dosing with AAV9 vectors, including dose, route of administration, and timing of immune modulation. These IND-enabling toxicology and biodistribution studies are ongoing. These studies have been proposed to the FDA in support of a human study to directly test the safety and utility of this approach by intramuscular (IM) or systemic administration routes, and the rationale for these studies as well as nonclinical supporting studies is presented in this article.

ASSESSMENT OF THE SAFETY, BIODISTRIBUTION, AND IMMUNOGENICITY OF rAAV9-DES-hGAA ADMINISTERED ONCE AND TWICE IN MALE AND FEMALE 129SVE *Gaa*^{-/-} KNOCKOUT MICE

The goals of this mouse study were to (1) assess the safety and biodistribution of the gene therapy vector following single and repeated IM administration; (2) confirm the dose–response of the test vector for efficacy in *Gaa*^{-/-} mice; (3) assess the dynamics of the NAb response to the vector following single and repeated administration; and (4) compare the safety, biodistribution, and immunogenicity of the IM injection to a systemic administration of the vector.

Experimental design

In total, 280 mice (140 male and 140 female) were included in this study and randomly assigned by gender and body weight into different dose subgroups (labeled as groups 1–6 in Table 1). Group 1 animals received vehicle solution and the animals in groups 2–4 received the rAAV9-DES-hGAA vector at three different doses (1.25×10^9 , 1.25×10^{10} , and 1.25×10^{11} vector genome (vg)/animal, respectively) via IM injection into the tibialis anterior (TA) muscle of the right hind leg. To fully

Table 1. Dose calculation

Mouse study				
Dose group	vg/animal/dose	vg/animal (total)	vg/mg	vg/kg
2 (IM) ^a	1.25×10^9	1.25×10^9	5×10^7	
3 (IM) ^a	1.25×10^{10}	1.25×10^{10}	5×10^8	
4 (IM) ^a	1.25×10^{11}	1.25×10^{11}	5×10^9	
5 (IM) ^a	1.25×10^{10}	2.5×10^{10}	5×10^8	
8 (IV) ^b	7.5×10^{12}	7.5×10^{12}	NA	3×10^{14}
Nonhuman primate study				
2–5 (IM) ^c	2.5×10^{13}	2.5×10^{13}	1.25×10^9	
6–7 (IM) ^c	2.5×10^{13}	5×10^{13}	1.25×10^9	
8 (IV) ^d	4×10^{14}	8×10^{14}	not determined	2 times 1.0×10^{14}

IM, intramuscular; IV, intravenous; TA, tibialis anterior.

^aAssuming mouse TA weight = 20 mg average.

^bAssuming mouse weight = 25 g average.

^cAssuming nonhuman primate TA weight = 20 g average.

^dAssuming nonhuman primate weight = 4 kg average.

consider the exposure of the vector *in vivo* we have provided information on vector dose per animal, per kg, and per mg of the exposed tissue following direct delivery (Table 1). Group 5 animals were administered the rAAV9-DES-hGAA vector at 1.25×10^{10} vg/animal in the right hind leg and after 4 weeks the second dose of the vector (1.25×10^{10} vg/animal) in the left hind leg, both via IM injection to the TA muscle. Group 6 mice received the rAAV9-DES-hGAA vector via intravenous (IV) (tail vein injection) at a dose of 3.0×10^{14} vg/kg once. The day of initial vehicle or vector administration was designated study day (SD) 1.

In-life endpoints were evaluated for all study animals and included detailed clinical observations and body weights. After initial vector/vehicle dose, subgroups of group 1–4 animals were euthanized at SD 29 ± 1 , SD 57 ± 1 , and SD 113 ± 2 ; group 5 animals at SD 113 ± 2 ; and group 6 animals at SD 29 ± 1 (Table 2). Study endpoints were chosen to assess mice early and three months postdosing. Animals in each subgroup were allocated equally to two cohorts (i.e., 5M/5F “biodistribution mice,” referred to as “BD mice,” and 5M/5F “histopathology mice,” referred to as “HP mice”) for different endpoint analyses. After euthanasia, blood was collected via cardiac puncture. Blood from the 5M/5F BD mice was used for assessment of hematology and vector genome concentration, and blood from the 5M/5F HP mice for assessment of serum chemistry, neutralizing anti-AAV9 antibodies, anti-GAA antibodies, and GAA activity. All animals received a complete necropsy and gross pathology assessment. Weights of selected organs were recorded. Selected tissues were harvested from the 5M/5F BD mice for assessment of vector

Table 2. Experimental design

Group	Dose ^a		Subgroup (euthanasia at SD ^c)	N (M/F)	Number of animals ^b	
	Vector (vg/animal; right hind leg for IM and tail vein for IV)	Re-dose vector (vg/animal; left hind leg for IM)			Biodistribution mice	Histopathology mice
1	Vehicle, IM	NA	1a (29 ± 1) 1b (57 ± 1) 1c (113 ± 2)	10/10 10/10 10/10	5M, 5F 5M, 5F 5M, 5F	5M, 5F 5M, 5F 5M, 5F
2	1.5 × 10 ⁹ , IM (rAAV9-DES-hGAA)	NA	2a (29 ± 1) 2b (57 ± 1) 2c (113 ± 2)	10/10 10/10 10/10	5M, 5F 5M, 5F 5M, 5F	5M, 5F 5M, 5F 5M, 5F
3	1.5 × 10 ¹⁰ IM (rAAV9-DES-hGAA)	NA	3a (29 ± 1) 3b (57 ± 1) 3c (113 ± 2)	10/10 10/10 10/10	5M, 5F 5M, 5F 5M, 5F	5M, 5F 5M, 5F 5M, 5F
4	1.5 × 10 ¹¹ IM (rAAV9-DES-hGAA)	NA	4a (29 ± 1) 4b (57 ± 1) 4c (113 ± 2)	10/10 10/10 10/10	5M, 5F 5M, 5F 5M, 5F	5M, 5F 5M, 5F 5M, 5F
5 ^d	1.5 × 10 ¹⁰ , IM (rAAV9-DES-hGAA)	1.5 × 10 ¹⁰ , IM (rAAV9-DES-hGAA)	5 (113 ± 2)	10/10	5M, 5F	5M, 5F
6	3.0 × 10 ¹⁴ vg/kg, IV (rAAV9-DES-hGAA)	NA	6 (29 ± 1)	10/10	5M, 5F	5M, 5F

^aDosing was performed by IM injection into the TA muscle of hind leg for group 1–5 mice, and dose is presented as vg/animal. For group 6 animals, dosing was performed by tail vein IV injection, and the dose was targeted to be closer to the IM middle dose as feasible (assuming a 25 g mouse with 25 mg muscle mass of TA).

^bA new ID number was assigned to a replacement animal.

^cThe day of initial vector dosing was designated as study day (SD) 1.

^dGroup 5 was to test the effect of preexisting immunity to AAV9 capsid.

biodistribution, and from the 5M/5F HP mice for assessment of histopathology.

For animals in groups 2–5, the vector, rAAV9-DES-hGAA was administered once on SD 1 via IM injection into the TA muscle in the right hind leg at the determined dose. In addition, group 5 animals received a second vector dose (rAAV9-DES-hGAA) via IM injection to the TA muscle in the left hind leg 4 weeks after the first vector dose (i.e., on SD 29). The dose volume for IM administration was 20 μ l. Group 6 mice was administered the vector rAAV9-DES-hGAA on SD 1 at a dose of 3.0 × 10¹⁴ vg/kg via tail vein injection (volume: 90 μ l for male and 70 μ l for female). Vehicle solution was administered to group 1 animals once on SD 1 in a manner identical to that used for administering vector for groups 2–4. The dose volume was 20 μ l.

Detailed necropsies were performed on all study animals. Necropsies consisted of a complete external and internal examination including the injection sites, body orifices (e.g., ears, nostrils, mouth, anus), and cranial, thoracic, and abdominal organs and tissues. Tissues harvested at necropsy are described in Table 3.

Biodistribution of the AAV9-DES-hGAA vector in blood and tissues was determined by qPCR assay. The vector DNA was quantified in blood and the following tissues: brain, liver, heart, spleen, lungs, kidneys, diaphragm, lumbar spinal cord,

popliteal lymph nodes (left and right, separately), TA (left and right, separately), testes/ovaries, and gross lesions.

ASSESSMENT OF THE SAFETY, BIODISTRIBUTION, AND IMMUNOGENICITY OF rAAV9-DES-hGAA ADMINISTERED ONCE OR TWICE IN MALE AND FEMALE RHESUS MACAQUES

The goals of the study in rhesus macaques were to (1) assess the safety and biodistribution of the vector following single and repeated IM administration, and (2) assess the efficacy of immunosuppressive agents to reduce the NAb response to the vector.

Experimental design

A total of 28 rhesus macaques (14 male and 14 female) divided into 8 groups were included in the study (Table 4). Six groups of animals (groups 2–5) received vector once, while three groups (groups 6–8) received vector twice. Group 1 (control) received vehicle according to the schedule for groups 6 and 7. Vector was administered by IM injection (5 × 10¹³ vg/animal) into the TA muscle. This dose was 2.5 × the clinical dose that was represented in the mid-dose group of the mouse multiple ascending dose study. For the first dose, the right TA

Table 3. Tissues collected

<i>Core tissues for histopathological examination</i>	
Brain ^{a,c}	Sternum with bone marrow
Heart ^{a,c}	Pancreas
Lung ^{a,c} with mainstem bronchi and caudal trachea	Spleen ^{a,c}
Liver ^{a,c} (sections of the right lobe, left lobe, and right portion of the median lobe)	Tibialis anterior (left and right, separately) ^{a,c}
Kidneys (bilateral) ^{a,c}	Testes (bilateral, males) ^{a,c}
Lymph node (popliteal, left and right separately) ^{c,d}	Ovaries (bilateral, females) ^{a,c}
Diaphragm ^{b,c}	Gross lesion ^c
<i>Additional tissues harvested and saved for potential future examination</i>	
Adrenal gland (bilateral) ^a	Lymph nodes (mesenteric, inguinal, ^d lumbar)
Jejunum (with Peyer's patch if possible)	Peripheral nerve (sciatic: longitudinal and cross sections, included with biceps femoris)
Cecum	Skeletal muscles (gastrocnemius, quadriceps femoris, and triceps brachii)
Cervix and vagina	Pituitary
Coagulating gland and seminal vesicle	Prostate
Colon	Salivary glands (parotid, submandibular, and sublingual)
Duodenum	Skin (inguinal/abdominal with mammary gland)
Epididymis	Spinal cord (cervical, thoracic, lumbar ^{b,c})
Esophagus	Stomach
Eyes (with Harderian gland and optic nerve)	Thymus ^a
Gallbladder	Thyroid/parathyroid gland
Ileum (with Peyer's patch if possible)	Urinary bladder
	Uterus ^a

^aTissues from histopathology mice ("HP mice") were weighed.

^bPortions of the tissue from biodistribution mice ("BD mice") were frozen at $\leq -60^{\circ}\text{C}$ until submitted to the sponsor for measurement of GAA activity.

^cTissues from BD mice were harvested using clean technique and frozen at $\leq -60^{\circ}\text{C}$ for vector biodistribution analysis by qPCR.

^dIf recoverable at necropsy. If the indicated lymph nodes were not recovered, they were documented.

received vector and the left leg received vehicle. The second administration of vector in groups 6 and 7 was 4 months (121 ± 5 days) after administration of the first dose. For the second dose administration, the left TA received vector and the right received vehicle. Vehicle was administered by IM injection to the TA of both right and left legs in group 1. Group 8 received one IV injection of vector (1×10^{14} vg/kg at each dose).

Immune modulation protocol

For the first vector injection, groups 3, 4, 7, and 8 received rituximab (Genentech) 750 mg/m^2 body surface area for two dose sessions to equal a total of 1500 mg/m^2 . These dose sessions occurred 10–14 days before vector administration and then on the day of vector administration. Groups 7 and 8 received rituximab (750 mg/m^2 of body surface area for one dose) on the day of the second vector dose administration. IV infusion of rituximab occurred over a target period of 1 to 1 hr 10 min (approx-

mately $0.26\text{--}0.36 \text{ ml/min}$) at 4 ml/kg/hr , using a Medex Medfusion infusion pump. Animals were sedated by IM injection of telazol administered intramuscularly at a dosage of 3 mg/kg ($2\text{--}6 \text{ mg/kg}$), with ketamine administered by IM injection as supplemental sedation ($1\text{--}5 \text{ mg/kg}$ as needed). The rituximab was infused through the cephalic or saphenous vein.

Sirolimus was delivered to the animals mixed with pudding. Sirolimus (Wyeth) was administered in groups 3, 5, 7, and 8 daily starting from 3 days before the first injection of AAV9 and continuing to 120 days for groups 3 and 5 and for 180 days in groups 7 and 8 (receiving a second vector dose). The dose was adjusted to achieve a whole blood 24 hr trough concentration of $2\text{--}5 \text{ ng/ml}$. Dosing began at $4 \text{ mg/m}^2/\text{day}$ and was adjusted to achieve the target 24 hr trough concentration range.

Endpoints

The efficacy of rituximab (infused intravenously) and sirolimus (oral) administered alone or in combination to reduce production of NAb against the AAV9 capsid or GAA was assessed in groups 3–5, 7, and 8.

In-life endpoints included assessment of clinical signs, body weight, clinical pathology (hematology and serum chemistry), NAb antibodies to AAV9, antibodies to GAA and AAV9 in serum, T-cell ELISPOT against GAA and AAV9 in peripheral blood monocytes, quantification of B lymphocytes in whole blood by flow cytometry, and sirolimus concentrations in whole blood.

All monkeys were euthanized on SD 181 ± 5 . Postmortem endpoints included gross pathology, organ weights, histopathology, vector concentration in blood and tissues, and GAA activity in brain, heart, diaphragm, lumbar spinal cord, and right and left TA muscles, and immunohistochemical analysis of lymphocyte populations in the spleen (Table 5).

EVALUATION OF READMINISTRATION OF RECOMBINANT ADENO-ASSOCIATED VIRUS ACID ALPHA-GLUCOSIDASE (rAAV9-DES-hGAA) IN PATIENTS WITH LATE-ONSET POMPE DISEASE

The proposed clinical study is designed to consider the following questions: (1) to test the hypothesis that administration and readministration of rAAV9-DES-hGAA vector delivered by IM injection into the TA in LOPD is safe. Safety will be tested by clinical laboratory tests, blood assay for vector genomes, antibodies against AAV and GAA, and T-cell ELISPOT against GAA and AAV, and (2)

Table 4. Experimental design

Dose group ^a	Question addressed	First injection ^b	Immune Rx ^c	Second injection ^d	Immune Rx
1, N=4	Control group for endpoint comparisons	Vehicle	None	Vehicle	None
2, N=4	Safety and biodistribution following one injection of vector	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	None	None	None
3, N=4	Efficacy of co-administration of rituximab and sirolimus on reducing any antibody production against vector and GAA	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	Rituximab and sirolimus	None	
4, N=2	Efficacy of rituximab alone before vector administration on any antibody production against vector and GAA	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	Rituximab	None	
5, N=2	Efficacy of sirolimus alone (repeated daily oral administration) on any antibody production against vector and GAA	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	Sirolimus	None	
6, N=4	Safety and vector biodistribution associated with repeat vector dosing in absence of immune suppression	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	None	Leg 1: vehicle; leg 2: rAAV9-DES-hGAA	
7, N=4	Efficacy of immune suppression administered only in conjunction with first vector dosing phase	Leg 1: rAAV9-DES-hGAA; leg 2: vehicle	Rituximab and sirolimus	Leg 1: vehicle; leg 2: rAAV9-DES-hGAA	Rituximab and sirolimus
8, N=4	Safety and biodistribution of vector administered intravenously twice with immune suppression	Intravenous rAAV9-DES-hGAA	Rituximab and sirolimus	Intravenous: rAAV9-DES-hGAA IV	Rituximab and sirolimus
Total, N=28					

^aThe dose is 2.5×10^{13} vg/animal per vector administration. This dose was based on data showing a TA dose of 1.25×10^9 vg/mg muscle provided a GAA activity level 20-fold over GAA activity in wild-type mice. The average TA muscle mass in a nonhuman primate was 20 g.

^bRituximab (infused) and sirolimus (oral) was administered as described in the Methods section.

^cSecond vector injections occurred 4 months (121 ± 5 days) after the first.

^dGroup 8 received test article 2 vector (4×10^{14} vg/animal per vector administration for a total dose of 8×10^{14} vg/animal.

Table 5. Endpoints

In-life endpoints	Schedule
Physical examination	Week -2 before any group receives vector for randomization into dose groups and within 2 days before euthanasia
Blood collection for hematology	All animals, twice prior rituximab or vector dosing, and on SD 8 ± 1 , 16 ± 2 , 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and at days 30 ± 5 and 60 ± 5 after the second vector administration (groups 1, 6, 7, and 8) or 121 ± 5 and 181 ± 5 for groups 2–5.
Blood collection for flow cytometry (B lymphocyte population)	Study groups 3–5, 7, and 8 once before rituximab or vector dosing, and on SD 8 ± 1 , 16 ± 2 , 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and at days 30 ± 5 and 60 ± 5 after the second vector administration (groups 7 and 8), or 121 ± 5 and 181 ± 5 for groups 3–5.
Blood collection for serum chemistry	All animals, twice prior rituximab or vector dosing, and on SD 16 ± 2 , 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and at days 30 ± 5 and 60 ± 5 after the second vector administration (groups 1 and 6–8) or 121 ± 5 and 181 ± 5 after first vector dosing for groups 2–5.
Blood collection for neutralizing antibodies to AAV9 in serum	All animals, SD 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and at days 30 ± 5 and 60 ± 5 after the second vector administration (groups 1 and 6–8) or 181 ± 5 for groups 2–5.
Blood collection for antibodies against AAV9 (ELISA) and GAA (ELISA) in serum	Prestudy and on SD 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and at days 30 ± 5 and 60 ± 5 after the second vector administration (groups 1 and 6–8) or 151 ± 5 after first vector dosing for groups 2–5.
Blood collection for quantitation of AAV9 by qPCR	SD 16 ± 2 , 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and on days 15 ± 2 , 30 ± 5 , and 60 ± 5 after the second administration for groups 6–8 and SD 121 ± 5 and 181 ± 5 after first vector dosing for all remaining groups.
Blood collection for measuring sirolimus levels	Blood was collected once weekly in groups 3, 5, 7, and 8 during the first month of sirolimus administration, and then monthly thereafter to confirm values are at target concentrations.
Blood collection for assessment of T-cell response to AAV9 and GAA in peripheral blood monocytes	Blood was collected from all monkeys at baseline, and at days 31 ± 5 , 61 ± 5 , and 91 ± 5 after the first vector administration, and on days 15 ± 2 , 30 ± 5 , and 60 ± 5 after the second administration for groups 6–8 and SD 121 ± 5 and 181 ± 5 for all remaining groups.
Body weights and detailed clinical signs	Detailed clinical observations were recorded for all monkeys on the day of randomization, on all assigned monkeys the day before each vector dosing or on the day of vector dosing, weekly for the first four weeks after each vector dosing, and monthly thereafter. Additional body weights may be obtained during blood collection procedures.
Muscle biopsies (T-cell immune response, GAA expression and activity, and vector concentration)	Muscle biopsy samples were taken to assess the concentration of vector and extent of GAA expression. Baseline concentrations of GAA in muscle will be measured in control animals on SD 15 ± 2 and at necropsy. Postdosing samples for assessment of GAA activity and AAV9 vector concentration were obtained for all animals in dose groups 2–8 at day 31 ± 5 after the first and day 30 ± 5 after the second vector administrations.

to test the hypothesis that administration and readministration of rAAV9-DES-hGAA vector injected IM into the TA in LOPD will have biochemical and functional impact.

Potential activity of the study agent will be tested using the following measures:

1. Clinical tests: balance test and muscle strength test
2. Muscle biopsy for biochemical and immunohistochemical assays.
3. Neurophysiological tests: surface and needle EMG to test the common fibular nerve and NMJ transmission
4. Magnetic resonance imaging (MRI)

Experimental design

This study is a within-participant, double-blind, randomized-start design (or delayed start design), phase I/II controlled study evaluating the toxicology, biodistribution, and potential activity of readministration of rAAV9-DES-hGAA injected IM into the TA. Six participants will be enrolled. The immune modulation strategy is to ablate B-cells (using rituximab and sirolimus) before the initial exposure to the study agent. Subjects will receive the vector in one leg and the subsequent exposure of the same vector to the contralateral leg after four months. Side of administration will be randomized at first injection. At each study agent dosing, the contralateral leg will receive excipient. Patients will act as their own controls. Repeated measures will assess the safety, biochemical, and functional impact of the vector. Any subject currently on GAA replacement therapy (Lumizyme or Myozyme) will continue protein replacement throughout the study.

Study population. The study population consists of 6 subjects, male or female, aged ≥ 18 years, who have been diagnosed with LOPD by mutational analysis, GAA assay by blood spot, and/or fibroblast culture as well as clinical symptoms. Subjects without residual ability to get up from a standard chair and perform three steps will be excluded.

Inclusion criteria. Participants must be male or female; 18 years of age or older; have a diagnosis of Pompe disease, as defined by protein assay and/or DNA sequence of the acid alpha-glucosidase gene; present with clinical symptoms of the disease; have residual ability to get up from a standard chair and perform 3 steps; and willing to discontinue aspirin, aspirin-containing products, and other drugs that may alter platelet function 7 days before dosing, resuming 24 hr after the dose has been administered.

Exclusion criteria. Participants must not be pregnant; have taken oral or systemic corticosteroids within 15 days before baseline screening; have a platelet count less than $75,000/\text{mm}^3$; have an international normalized ratio greater than 1.3; have transaminases and alkaline phosphatase more than 10 times the upper limit of normal at screening or day -1; have bilirubin and gamma-glutamyl transpeptidase levels greater than 2 times the upper limit of normal at screening or day -1; be currently, or within the past 30 days, participating in any other research protocol involving investigational agents or therapies; have a history of platelet dysfunction, evidence of abnormal platelet function at screening, or history of recent use of drugs that may alter platelet function, which the subject is unable/unwilling to discontinue for study agent administration; have received gene transfer agents within the past 6 months before screening; and have any other concurrent condition that, in the opinion of the investigator, would make the subject unsuitable for the study.

IM injection of rAAV9-DES-hGAA. Vector injections will be in the TA muscle and will be performed as an outpatient procedure at the Clinical Research Center at the University of Florida, Gainesville, Florida. The target injection site in the muscle will be determined from MRI images. The injection of 3 ml of vector will be made in the designated site following local anesthetic delivery. Clinical 2-D and Doppler ultrasound will be used with a sterile sheath to maintain sterility of the injection site. For each participant, the TA muscle mass will be determined from the baseline MRI images. TA muscle volume will be calculated by drawing the muscle cross-sectional area throughout the entire muscle length. Muscle mass will be derived using 1.05 g/cm^3 for muscle density.³⁵ The dose for both vector and placebo injections will be confirmed by mouse and nonhuman primate studies to be 1×10^9 vg per TA mg in 3 ml of excipient buffer.

Immune suppression protocol. Participants will receive rituximab (Genentech; dose: 750 mg/m^2 body surface area for two dose sessions to provide a total of 1500 mg/m^2) via IV infusion 10–14 days before vector administration and then on the day of vector administration, before vector was injected. For groups 7 and 8, rituximab (750 mg/m^2 of body surface area for one dose) is given on the day of the second vector dose administration. Premedication will be administered 30–60 min before rituximab infusion to ameliorate infusion-associated reactions, and will include acetaminophen (Tylenol) 650 mg

by mouth once and diphenhydramine (Benadryl) 25–50 mg by mouth once. In addition to rituximab, patients will also receive sirolimus (Wyeth; dose 4 mg/m²/day, adjusted to maintain a trough serum sirolimus level of 2–4 ng/ml) every day starting on day –3 (3 days before first injection of study agent) and continuing through day 150 (1 month after second injection of study agent). Subjects will be monitored for infection through physical examinations and periodic lab work and will be treated appropriately if deemed clinically necessary.

Discussion and clinical impact. In this article we describe the rationale and studies used to support a first-in-human study where AAV vectors will be administered twice. The design of the preclinical studies is to support a clinical program, which will establish the basis for AAV redosing. The study is not designed to establish this route of delivery for clinical use in Pompe disease but instead to look at a site that is accessible for imaging, muscle biopsy, and importantly to collect neurophysiology data. The opportunity to collect neurophysiology data will evaluate if the findings in humans are confirmatory of the non-clinical data the findings in preclinical studies, which establish a primary aspect of weakness in Pompe disease is because of motoneuron dysfunction. Ultimately, this clinical study will have implications beyond the immediate results generated, supporting additional exploration of AAV-mediated gene therapy where dose escalation studies or placebo-controlled crossover design may be useful. We anticipate that, following local gene transfer to muscle, the next step in our gene therapy research program will be systemic delivery of vector via IV infusion. Body-wide, systemic delivery has the potential to provide biochemical correction in all striated muscle, which could improve cardiorespiratory function, as well as restoring peripheral muscle strength. The results of this study may provide additional information for use in future gene therapy studies, since duration of gene expression and the need for re-dosing are major challenges in the field.

The clinical application of this approach will ultimately be most important in pediatric patients, who will be the most likely candidates for multiple dosing events over the course of their lifetime. Three basic principles support this conclusion. First, body mass increases up to 20-fold from infancy to adulthood. Second, many of the conditions for which early rAAV-mediated gene therapy is

being considered have increased rates of cell division or cell death because of the underlying disease. We have observed loss of vector genomes because of increased cellular turnover as a result of primary myopathy.³⁶ These factors alone or in combination will almost certainly lead to a decline in genome copy number per cell, leading to loss of efficacy over time. Third, early phase studies of this nature must include low-dose cohorts, and therefore not all subjects will receive a clinically effective dose.^{29,37} This may necessitate re-exposure to the therapeutic vector. In order to ethically manage such studies in rare disease populations, it is vital to have a strategy in place to mitigate the confounding effects of anticapsid immunity and allow such subjects the opportunity to enter subsequent gene therapy trials, in which a more effective dose is being studied. We expect that the results of these comprehensive endpoints will establish the basis for similar approaches using AAV-mediated gene transfer.

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AUTHOR DISCLOSURE

B.J.B., Johns Hopkins University, and the University of Florida could be entitled to patent royalties for inventions described in this article. B.J.B. is an inventor of intellectual property owned by the Johns Hopkins University and the University of Florida related to this research. He is also a founder of Applied Genetic Technologies Corporation and owner of founder equity. He is an unpaid member of the Scientific Advisory Board of Audentes Therapeutics and Solid GT, LLC, and Bristol-Meyers Squibb.

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