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

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Abstract

Pompe disease is an inherited neuromuscular disease caused by deficiency of lysosomal acid alpha-glucosidase (GAA) leading to glycogen accumulation in muscle and motoneurons. Cardiopulmonary failure in infancy leads to early mortality, and GAA enzyme replacement therapy (ERT) results in improved survival, reduction of cardiac hypertrophy, and developmental gains. However, many children have progressive ventilatory insufficiency and need additional support. Preclinical work shows that gene transfer restores phrenic neural activity and corrects ventilatory deficits. Here we present 180-day safety and ventilatory outcomes for five ventilator-dependent children in a phase I/II clinical trial of AAV-mediated GAA gene therapy (rAAV1-hGAA) following intradiaphragmatic delivery. We assessed whether rAAV1-hGAA results in acceptable safety outcomes and detectable functional changes, using general safety measures, immunological studies, and pulmonary functional testing. All subjects required chronic, full-time mechanical ventilation because of respiratory failure that was unresponsive to both ERT and preoperative muscle-conditioning exercises. After receiving a dose of either 1×10^{12} vg ($n=3$) or 5×10^{12} vg ($n=2$) of rAAV1-hGAA, the subjects' unassisted tidal volume was significantly larger (median [interquartile range] 28.8% increase [15.2–35.2], $p<0.05$). Further, most patients tolerated appreciably longer periods of unassisted breathing (425% increase [103–851], $p=0.08$). Gene transfer did not improve maximal inspiratory pressure. Expected levels of circulating antibodies and no T-cell-mediated immune responses to the vector (capsids) were observed. One subject demonstrated a slight increase in anti-GAA antibody that was not considered clinically significant. These results indicate that rAAV1-hGAA was safe and may lead to modest improvements in volitional ventilatory performance measures. Evaluation of the next five patients will determine whether earlier intervention can further enhance the functional benefit.

Introduction

POMPE DISEASE (glycogen storage disease type II, acid maltase deficiency) is an autosomal recessive neuromuscular disease characterized by a deficiency of the enzyme acid alpha-glucosidase (GAA). GAA is responsible for degradation of glycogen within lysosomes (Raben *et al.*, 2002). A severe deficiency or absence of GAA results in accumulation of glycogen in all cells, leading to the most significant accumulation in striated muscle and motoneurons (Hirschhorn and Reuser, 2001; Raben *et al.*, 2002). There is marked heterogeneity in the clinical presentation; the age of onset and rate of

clinical progression are related to the degree of enzyme deficiency (Hirschhorn and Reuser, 2001; van der Ploeg and Reuser, 2008). The most severe phenotype presents clinically in infancy as cardiomyopathy, respiratory compromise, weakness, and hypotonia. Historically, these affected children experienced early mortality because of cardiorespiratory failure within the first 2 years of life (Kishnani *et al.*, 2006).

More recently, intravenous recombinant GAA enzyme replacement therapy (ERT) has been found to increase survival, partially correct cardiac function, and improve progression of developmental milestones in severely affected children (Kishnani *et al.*, 2007). Although ERT has become

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the standard of care for Pompe disease, the clinical benefits of ERT have been confounded by immune responses (Kishnani *et al.*, 2007) and progression of neuromuscular impairments (Nicolino *et al.*, 2009; Chakrapani *et al.*, 2010; van Gelder *et al.*, 2012). In particular, longer-term evaluations reveal progressive respiratory insufficiency and a requirement for invasive ventilation in many surviving subjects, despite the chronic use of ERT (Nicolino *et al.*, 2009; Chakrapani *et al.*, 2010). One potential reason for the limited efficacy of ERT in ventilator-free survival is that GAA is not transported across the blood–brain barrier (Kikuchi *et al.*, 1998) and therefore likely cannot correct neural glycogen accumulation, especially in motoneurons. As such, progressive accumulation of glycogen in the central nervous system (CNS) may lead to ongoing dysfunction or loss of motoneurons and progressive dysfunction of motor units (Rohrbach *et al.*, 2010).

In support of this hypothesis, neural dysfunction has been noted in animal models and in human subjects (Mancall *et al.*, 1965; Gambetti *et al.*, 1971; DeRuisseau *et al.*, 2009; Burrow *et al.*, 2010). Our preclinical work in a knockout mouse model (*Gaa*^{-/-}) revealed glycogen accumulation in phrenic motoneurons and diminished phrenic efferent activity (DeRuisseau *et al.*, 2009; Mah *et al.*, 2010). These data share notable similarities to recent autopsy reports of neuropathology in children treated with ERT (DeRuisseau *et al.*, 2009; Burrow *et al.*, 2010). This neuropathology is consistent with the postulate that systemically delivered ERT does not effectively alleviate GAA insufficiency in the nervous system.

Our laboratory showed that systemic (Mah *et al.*, 2007) and direct gel-mediated delivery of the recombinant adeno-associated virus GAA gene (rAAV1-hGAA) to the diaphragm robustly increased minute ventilation of treated animals, compared with untreated controls (Mah *et al.*, 2010). In addition, AAV has the capacity for robust retrograde movement to motoneurons (Elmallah *et al.*, 2012), and initial

results suggest increased phrenic neural output after diaphragm gene therapy (Mah *et al.*, 2010). These preclinical findings suggest that rAAV gene therapy can influence neural GAA activity in Pompe disease. In addition, others have reported an acceptable safety profile for AAV1-mediated gene delivery in human clinical trials (Brantly *et al.*, 2006; Mendell *et al.*, 2010). As such, we initiated a phase I/II clinical trial of rAAV1-hGAA intramuscular gene transfer to the diaphragm. The study hypothesis was that rAAV1-hGAA gene replacement therapy to the diaphragm would be safe and improve ventilatory function in ventilator-dependent children affected by Pompe disease.

Materials and Methods

Study design

The study design (Fig. 1) incorporated a baseline period of preoperative inspiratory muscle conditioning to determine whether the patients' ventilatory function could be strengthened by exercise alone. The study agent consisted of a clinical-grade adeno-associated virus vector serotype 1, with a cytomegalovirus promoter followed by the human GAA cDNA (rAAV1-hGAA), produced at the Human Applications Laboratory at the University of Florida. Respiratory muscle conditioning continued for 1 year after gene replacement. Safety labs, immunological tests, and pulmonary functional tests were conducted before gene delivery and through 180 days postprocedure. ERT administration was unchanged throughout the study.

Vector production

Three lots of 10 cell stacks each were produced by the standard CaPO₄ cotransfection method. Cell harvests were submitted to digestion with Benzonase followed by microfluidization in the presence of 1.0% octyl phenol ethoxylate.

FIG. 1. Schematic of the clinical trial timeline shows periodic safety and ventilatory testing before and up to 365 days after gene transfer. Throughout the study, patients received enzyme replacement therapy (ERT) and inspiratory muscle strength training (IMST) exercises.

The clarified lysate was loaded onto a hydroxyapatite (HA) column (CHT-HA; Bio-Rad), washed, and eluted at 15% elution buffer (75 mM phosphate). The HA eluate was next diluted 1:3 with 15 mM NaCl and 20 mM Tris buffer and loaded onto a Q Sepharose column (HiTrap Q HP; GE Healthcare) and eluted at 212 mM NaCl and immediately loaded onto a 150 ml Sephacryl S-300 column. The vector-containing peak (Purified Bulk) was captured, QC tested, filtered using a 0.22 μ m filter, and stored frozen at -70°C to -90°C . Purified bulk lots were combined and concentrated by passage through a 300,000 molecular weight cut off (MWCO) tangential flow cartridge. The final filtered concentrated bulks lots were combined, filtered using a 0.22 μ m filter, and filled into 1.2 ml cryovials. Final product vials were stored frozen at -70°C to -90°C .

Human subjects

Children enrolled in the clinical trial were found to have Pompe disease, as confirmed by mutational analysis, GAA assay in blood spot, and/or fibroblast culture. Patients had stable, chronic ventilatory failure that required full-time invasive ventilation assistance, and maintenance Myozyme ERT was continued throughout the trial. Parents provided informed consent for the study procedures, and subjects provided assent. The Institutional Review Board (IRB) at the University of Florida approved all procedures.

Exclusionary criteria included having gene transfer agents within the past 6 months; having evidence or history of abnormal platelet function/counts; having high INR; having abnormal chemistry profile at screening or day -1 , including transaminases and alkaline phosphatases greater than 10 times the upper limit of normal and/or bilirubin and gamma-glutamyl transpeptidase greater than two times the upper limit of normal; having required acute oral or intravenous antibiotic therapy or corticosteroids within 15 days before screening; participating currently or within the past 30 days in another research protocol involving investigational agents or therapies other than alglucosidase alfa ERT; weighing under the minimum weight limit of 10 kg; or having any concurrent condition that would make the subject unsuitable for the study as determined by the investigator.

Inspiratory muscle conditioning

Inspiratory muscle-conditioning exercises included inspiratory muscle strength training (IMST) as well as endurance exercise on reduced ventilatory support. An exercise prescription was issued at each study visit starting at the initial screening visit, based upon the patient's vital signs and test performance. The prescription was updated at each study visit.

Inspiratory muscle strength training. Patients received IMST by their caregivers for up to 3 months preoperatively and postoperatively through day 365. A similar training regime has been described in difficult-to-wean adults and infants (Martin *et al.*, 2011; Smith *et al.*, 2013). During the study screening, the caregivers were instructed how to use the training device, expected training schedule, and completion of training diaries. For IMST exercises, subjects were prescribed a training device suitable for small (ACCU-PEEP; Vital Signs, Inc.) or moderate (Threshold PEP; Phillips-

Respironics) unassisted tidal volumes. Devices were inverted to deliver inspiratory threshold loads.

IMST was prescribed 3 days per week. To complete the exercises, subjects were briefly disconnected from the ventilator, and the prescribed training device was connected directly to the tracheostomy tube. Exercise sessions consisted of four sets of 6–12 best-effort breaths against the highest load that produced opening of the device's training valve and maintained stable heart rate and saturation of peripheral oxygen (SpO_2). Between exercise sets, subjects rested on their usual level of daytime ventilator support for 3–5 min. For the initial 2 weeks of IMST, a study investigator monitored training sessions by telephone or Skype. After the first 2 weeks, the investigator continued to monitor progress once weekly, and the caregiver completed diaries of the IMST sessions. Heart rate and pulse oximetry were continuously monitored during home IMST sessions.

Endurance exercise on reduced ventilator support. Additionally, ventilator settings were reduced up to 5 days per week for bouts of endurance exercise. The goal of this exercise was to build muscle conditioning on lowered ventilator settings. Endurance exercise did not necessarily begin at enrollment, but was initiated only when vital signs (SpO_2 , end-tidal CO_2 [ETCO_2], respiratory rate [RR], heart rate [HR]) were stable for 15 min of reduced pressure support. The prescribed ventilator setting for exercise was customized to the levels of pressure support and ventilated rate that enabled the patient to increase spontaneous efforts yet maintain a stable ETCO_2 . The exercise settings and duration were monitored and progressed with the aid of a portable capnograph/oximeter (Capnograph II; Smiths Medical).

Gene transfer

The vector injection site was in the lateral aspect of each hemidiaphragm. Access to the diaphragm was obtained via thoracoscopy. The diaphragm injections were performed with direct observation to identify the injection sites, with the injection needle introduced through the chest wall. A beveled needle of appropriate gauge and length was determined by the surgeon and attached to a 1 ml syringe. Three sites were injected separately per hemidiaphragm, corresponding to the ventral, lateral, and dorsal costal regions bilaterally. Subjects received a fixed total dose of either 1×10^{12} vg (cohort 1) or 5×10^{12} vg (cohort 2) rAAV1-hGAA. Thus, the total dose per site was approximately 1.67×10^{11} vg (cohort 1) or 8.33×10^{11} vg (cohort 2) rAAV1-hGAA, in each 0.8 ml dose. After the thoracoscope retraction, the insufflated CO_2 was removed via the entry port, followed by skin closure. The procedure was repeated on the contralateral hemidiaphragm to complete the injection series.

Safety tests

Quantitative real-time polymerase chain reaction. Quantitative real-time polymerase chain reaction (PCR) was performed to assess the biodistribution of vector DNA in peripheral blood at baseline and days 1, 3, 14, 30, 60, 90, and 365 after administration (cohort 1) and days 1, 3, 14, 30, 60, and 90 after administration (cohort 2). Venous blood was snap-frozen in liquid nitrogen and stored at -80°C until genomic DNA (gDNA) was extracted according to a previously

described protocol (Song *et al.*, 2002; Poirier *et al.*, 2004). gDNA was isolated from blood using a DNeasy blood and tissue kit (Qiagen, Inc.) according to the manufacturer's instructions. gDNA concentrations were determined using an Eppendorf Biophotometer (Eppendorf). rAAV genome copies in the gDNA were quantified by real-time PCR using an ABI 7900 HT sequence detection system (Applied Biosystems) according to the manufacturer's instructions, and results were analyzed using the SDS 2.3 software. Briefly, primers and probe were designed to the CMV enhancer of the CMV-hGAA vector. A standard curve was performed using plasmid DNA containing the same SV40 poly-A target sequence. PCRs contained a total volume of 100 μ l and were run at the following conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 sec and 60°C for 1 min.

DNA samples were assayed in triplicate. To assess PCR inhibition, the third replicate was spiked with plasmid DNA at a ratio of 100 copies/ μ g gDNA. If a sample contained greater than or equal to 100 copies/ μ g gDNA, which is the limit of detection, it was considered positive for vector genomes. All samples were normalized to 1 μ g of gDNA.

Anti-AAV1 antibody measurement. Serum samples were assayed by ELISA for circulating antibodies to the AAV1 capsid proteins at baseline and days 14, 90, 180, and 365 and intact human GAA at baseline and days 14, 30, 60, 90, 180, 270, and 365 (cohort 2 through day 90 only). Ninety-six-well plates were coated with 1.2×10^9 AAV1 particles/well overnight at 4°C. A wash with phosphate-buffered saline (PBS)-Tween was followed by blocking for 2 hr at 37°C with 10% fetal bovine serum (Cellgro; Mediatech). After a 1 $\times$ wash with PBS-Tween, samples and a known positive human standard were diluted between 1:10 and 1:10,240 and allowed to bind overnight at 4°C. Washing was followed by addition of the detection antibody at a dilution of 1:50,000 (for standard samples: goat anti-human immunoglobulin, conjugated with horseradish peroxidase [HRP; Biosource International] and subject's samples: goat anti-rabbit IgG-conjugated w/peroxidase [Sigma cat# A0545]) for 2 hr at 37°C. Finally, the plate was washed and exposed to tetramethylbenzidine peroxidase detection substrate (KPL) and read at 450 nm with an ELISA plate reader (uQuant Plate Reader). Sample anti-AAV1 titers were then read relative to a human standard curve derived from the same plate.

Anti-hGAA antibody measurement. Immulon microtiter plates were coated overnight at 4°C with 100 μ l of 5 μ g/ml hGAA in PBS 0.1 M NaHCO₃, pH 8.4. Wells were washed three times with 300 μ l PBS containing 0.05% Tween 20 (PBS/T) and blocked with 300 μ l 10% fetal bovine serum in PBS for 2 hr at room temperature. Wells were washed three times and serum samples (diluted 1:10 in blocking reagent) were added to the wells in a total volume of 100 μ l and serially diluted. Serial dilutions of an anti-GAA antibody standard were used to derive the standard curve. Samples and standards were incubated overnight at 4°C. Washing was repeated and 100 μ l of sheep anti-human IgG-HRP conjugate (Amersham RPN 4101) diluted 1:20,000 in blocking buffer was added to all wells and incubated for 2 hr at room temperature. After incubation, washing was repeated and 100 μ l of tetramethyl benzidine was added to the wells for 3 min. The reaction was stopped with 100 μ l of 0.5 M

H₂SO₄ and absorbance was measured at 450 nm (BioTek uQuant Plate Reader).

Antigen-specific response assay. Anti-AAV1 and anti-hGAA antigen-specific lymphocyte proliferation responses were assessed as previously described (Hauswirth *et al.*, 2008), at baseline and days 14, 90, and 365. After isolation and purification from blood, lymphocytes were cultured at 1×10^5 cells per well of 96-well plates. Lymphocytes were separated into four groups with three control and six patient sample cultures per group: unstimulated (as negative control), stimulated with AAV1 (5,000, 500, and 50 particles/cell). After 5 days of incubation, the stimulation index (SI), defined as the mean counts per minute of [³H] thymidine from stimulated cells divided by the mean counts per minute of [³H] thymidine from unstimulated cells, was calculated. SI values greater than 2.0–3.0 are considered significant. The viability of each lymphocyte culture was confirmed by positive controls with mitogen-induced proliferation in response to phytohemagglutinin and recall antigen-induced proliferation to *Candida albicans*.

Ventilatory assessment

For ventilatory tests, the tracheostomy cuff was inflated and the patient was tested in the seated position with the head and trunk supported. The angle of the chair back was maintained during subsequent testing sessions. Ventilatory flow and timing were recorded with a pneumotachograph and capnograph (CO₂SMO; Philips-Respironics) connected in series with the ventilator circuit.

Maximal inspiratory pressure. A one-way inspiratory occlusion has been determined to be a valid test of maximal inspiratory pressure (MIP) in mechanically ventilated adults (Truwit and Marini, 1992) and children (Harikumar *et al.*, 2008). The subject was briefly removed from the ventilator, and a pressure transducer and one-way valve were attached directly to the tracheostomy tube. The one-way valve permitted exhalation, but prevented inspiratory airflow. The maneuvers lasted for 20 sec, in accordance with American Thoracic Society testing guidelines (American Thoracic Society/European Respiratory Society, 2002). The test was repeated three times, with a minimum 5 min break on resting ventilator settings between trials. The most negative pressure was recorded and compared with age- and sex-predicted reference values (Domenech-Clar *et al.*, 2003).

Maximal voluntary ventilation. Subjects were removed from the ventilator briefly and performed 15 sec of rapid, best-effort, unassisted breaths. Strong encouragement was provided to promote a maximal effort. Two sets were completed and a 5 min break was provided between test sets. The volume and timing of the final 10 breaths of the unloaded set were extrapolated to 1 min to obtain estimated maximal voluntary ventilation (MVV). Age and sex reference values for MVV do not exist for young children, and therefore a double-indirect method used height to estimate FEV₁ and predict MVV (Rosenthal *et al.*, 1993; Stein *et al.*, 2003; Zapletal and Chalupova, 2003). Results of the regression technique were compared with tests performed on age-matched healthy children in our lab.

Spontaneous ventilatory endurance. The tolerance to independent breathing was measured by disconnecting the patient briefly from the ventilator. The first fully unassisted breath was recorded as the zero time point. Heart and respiratory rate, blood pressure, ETCO_2 , EKG rhythm, and SpO_2 were continuously monitored. Patients were returned to the ventilator when they exhibited one or more physiological signs of weaning failure. These signs included increase in heart rate ≥ 30 bpm from resting; systolic blood pressure >150 mm Hg or <80 mm Hg; SpO_2 sustained $<92\%$ for at least 1 min; respiratory rate >45 bpm sustained for >1 min; ventricular dysrhythmias; evidence of impeding muscular fatigue (accessory muscle use, substernal retraction, sternomastoid activation, paradoxical breathing, nasal flaring); diaphoresis; or pallor changes. Additionally, assisted ventilation was restored if patients could not subjectively tolerate further unassisted breathing and requested to return to the ventilator. Patients who could not tolerate any off-ventilator time with full cuff inflation completed the test with a level positive end-expiratory pressure (PEEP). The spontaneous ventilatory endurance was calculated as the total volume of air patients moved from the first off-ventilator breath until the ventilator was restored.

Data analysis

To calculate the effect of preoperative muscle conditioning on muscle performance, the baseline data were expressed as percent of the value obtained at screening. After gene transfer, the difference in performance between days 0 and 180 was expressed as the percent of the value obtained at baseline. Data were analyzed with Friedman's two-way analysis of variance by rank. Median and interquartile range are reported, and differences were accepted to be statistically significant at $p < 0.05$.

Results

Patient characteristics at baseline

Seven patients (five males, age 18–180 months) were enrolled into the trial thus far (Table 1). Subject 202 was unable to complete screening procedures and was withdrawn from the study. Subject 203 enrolled and completed 10 weeks of preoperative muscle conditioning. He was then withdrawn at the request of his local IRB and medical providers to enroll

in a Genzyme-sponsored study for continuation of ERT. Three patients from cohort 1 (3 males, age 66–108 months) and two patients from cohort 2 (1 male and 1 female, age 18–180 months) completed enrollment and 180-day post-procedure safety assessments. The patients were chronically treated with ERT. Patients required 12–101 continuous months of full-time, chronic invasive ventilatory support, and initial tolerance of breathing without any ventilator assistance ranged from <0.5 to 8 min. Only one of the patients could independently control head or trunk posture, and all required augmentative communication devices or strategies.

The baseline respiratory muscle function was profoundly impaired in the subjects. MIP was reduced by 60%–90% (Fig. 2A) as compared with expected age- and sex-matched unaffected children, and even larger deficits were apparent in MVV (Fig. 2B). The body-mass-corrected, maximal-effort unassisted tidal volumes fell well below expected values for resting ventilation in unaffected individuals (Fig. 2C). The causes of impaired ventilatory performance were likely related to chronically elevated lung and upper airway mechanical resistance, decreased lung/chest wall compliance, and chronic orthopedic restrictions, in conjunction with the underlying progressive neuromuscular disease (Allen, 2010; Ferrari *et al.*, 2010). The initial findings were consistent with chronic ventilator dependence and severe phrenic neuromuscular dysfunction.

Effect of preoperative IMST on ventilation

Preoperative respiratory muscle conditioning consisted of pressure-threshold IMST and reduced or off-ventilator endurance exercise. Only one patient (101) was able to tolerate endurance exercises before rAAV-hGAA replacement. Individual responses were variable to preoperative IMST (7–12 weeks), but there was no appreciable change in MIP (-3.13% [-11.5 to 19.7]) or unassisted tidal volume (-5.53% [-32.3 to 13.3]) (Fig. 3).

Gene transfer procedure

The rAAV1-hGAA vector was delivered using a thorascopic approach. Insufflation with CO_2 was used to achieve adequate observation of the diaphragm. During the gene transfer procedure, diaphragmatic electromyography (EMG) recordings revealed spontaneously active phrenic motor units interspersed with fibrillation potentials and positive

TABLE 1. BASELINE CHARACTERISTICS OF THE STUDY PARTICIPANTS

	Patient 101	Patient 102	Patient 103	Patient 201	Patient 202	Patient 203	Patient 204
Sex	M	M	M	M	F	M	F
Age at diagnosis (months)	6.5	15	17	18	0	3.5	6
Age ERT started (months)	8	15	18	108	96	4.5	6
Age-invasive ventilation started (months)	42	7	29	28	96	20	18
Age at enrollment (months)	96	108	66	180	179	75	30
MIP, cm H_2O (% predicted)	-25.1 (26)	-1.6 (2)	-6.4 (8)	-2.6 (3)	N/A	-4.3 (5)	-35.5 (40)
MVV, liter/min (% predicted)	7.0 (14.9)	1.0 (2.1)	2.1 (6.9)	0.5 (0.8)	N/A	1.6 (7.0)	2.1 (12.7)
Off-ventilator tolerance (min/day)	8	0.5	1.1	0.4	N/A	0.9	1.5
Initial weight (kg)	27	35	18.5	47	29	24.5	13

M, male; F, female; ERT, enzyme replacement therapy; MIP, maximal inspiratory pressure; MVV, maximal voluntary ventilation; N/A, not available.

FIG. 2. Ventilatory function of subjects at study enrollment. **(A)** Although all subjects required full-time invasive ventilation, baseline maximal inspiratory pressure (MIP) varied appreciably between children. MIP values were >60% reduced from expected age- and sex-matched unaffected children. Solid and dashed lines represent average and upper/lower limits of age-predicted normal values for MIP. **(B)** Maximal voluntary ventilation (MVV) is influenced not only by respiratory muscle strength, but also by pulmonary mechanics, upper airway patency, and chest wall restrictive disease. MVV of the subjects was reduced >80% from healthy references. Unfilled bars represent the age, sex, and height-predicted reference value for each child. **(C)** The maximal-effort, unassisted tidal volumes of subjects fell short of the expected range of resting tidal volume in unaffected individuals (dashed lines).

sharp waves, suggesting a functional denervation in some recorded motor units. The HR, BP, and ETCO₂ remained stable throughout the dosing procedures. The chest tube was removed on postprocedure day 1. Subject 103 required evacuation of residual capnothorax on postprocedure day 2. In addition, subject 102 experienced right lung contusion and delayed chest tube removal (grade II toxicity) related to a preexisting lung adhesion, which complicated placement of the thoroscope. None of the patients experienced acute worsening of ventilatory function during the first 14 postoperative days (Fig. 3).

Vector dissemination throughout the diaphragm and peripheral organs

Vector DNA was detected in the blood at day 1 post-administration and was undetectable by day 90 in all three patients in cohort 1 and both patients in cohort 2 (Fig. 4A). These findings are consistent with the preclinical observa-

tions in mouse and rabbit studies. The diaphragm is highly vascular and some vector entered the blood stream with up to 3.6×10^3 (subject 101) vector genomes per μg gDNA 1 day after injection in cohort 1 (1×10^{12} vg dosed) individuals and up to 2.5×10^6 genomes in cohort 2 (5×10^{12} vg dosed, subject 204) at day 1. There were no observed consequences of dissemination of vector DNA in preclinical studies or in the human subjects in this study.

Anti-AAV1 and anti-hGAA circulating antibody and T-cell-mediated responses

Humoral antibody responses to AAV1 and human GAA were compared with baseline values (Fig. 4B and C). All patients developed Ig antibody responses to the AAV1 capsid proteins by day 14 and were found in excess of 3,700-fold over baseline in subject 101. No significant response was detected against the transgene in cohort 1. One subject, 201, in the high-dose group consistently presented with a 2–3-fold increase in anti-GAA antibodies after the injection procedure. Available peripheral blood mononuclear cells (PBMC) preparations at baseline and days 14 and 90 postinjection were challenged with whole AAV1 capsids and recombinant human GAA protein with no response observed to either antigen by day 365 in cohort 1 (Fig. 4D and E). Subject 201 was found to have a positive antigen-specific response to GAA approaching the limit of detection (SI=2.09). To date, antibody responses remain stable in cohort 1 approaching day 365 and cohort 2 at day 90, and no relationship was identified between immune responses and ventilation responses in any patient.

Adverse events

Two serious adverse events were considered related or possibly related to the study procedure or agent. One event, right lung contusion with apical pneumothorax, was related to the study procedure and resolved. The second event, pleural effusion, was considered possibly related at the time of the event and was subsequently determined to be unrelated. Specifically, subject 201 reported increased difficulty

FIG. 3. Acute ventilatory function during the preoperative and 14-day postoperative periods. To date, five children have completed study procedures through the 180-day postoperative assessment. **(A)** During the preoperative period between the screening and baseline tests, subjects underwent preoperative inspiratory muscle strength training (IMST). Exercise alone did not acutely change the maximal inspiratory pressure (MIP) or the best-effort, unassisted tidal volume (VT). **(B)** Further, acute changes were minimal between the baseline tests performed the day before gene transfer and the day 14 postoperative test session (median [interquartile range]).

FIG. 4. Presence of vector DNA and immune responses. (A) Quantitative real-time polymerase chain reaction was performed to assess the biodistribution of vector DNA in peripheral blood at baseline and days 1, 3, 14, 30, 60, 90, and 365 after administration. Serum samples were assayed by ELISA for circulating antibodies to the AAV1 capsid proteins (B) and intact human acid alpha-glucosidase (GAA) (C) at baseline and days 14, 30, 60, 90, 180, 270, and 365. Antigen-specific response assays challenged PBMCs with either vector (D) or intact transgene (E) product at baseline and days 14 and 90.

completing exercise training sessions at day 75 postdosing. A chest X-ray and computed tomography scan revealed bilateral pleural and pericardial effusions (grade 3 toxicity). Pleurocentesis was performed at day 77 postdosing and chylous fluid was obtained. The fluid was analyzed to contain 95% lymphocytes and elevated triglyceride. Antibody and ELISPOT testing of the peripheral blood and pleural fluid showed no reactivity to either the AAV capsid or GAA transgene. At the time of screening, we noted that the existing indwelling left-sided infusaport catheter was obstructed. A right antecubital peripherally inserted central venous catheter (PICC) was placed. At the time of study agent dosing, the infusaport was removed. Additional imaging studies confirmed subclavian vein (SVC)/thoracic duct obstruction related to PICC line placement. This adverse event was considered to be unrelated to the study procedure. The effusions resolved with pleurocentesis, steroid treatment, and PICC line removal. During the recovery period (days 75–120), the patient rested on full, control-mode mechanical ventilation and did not engage in conditioning exercises. Exercises were gradually started, beginning day 120.

All subjects experienced grade II toxicity of transient pain related to the study procedure. Pain was managed with appropriate analgesia. All subjects reported that pain resolved before discharge from the inpatient setting on post-procedure day 3. Subject 101 and subject 102 reported transient edema and tenderness to the right chest (grade I toxicity).

180-day ventilatory outcomes

The resting and unassisted breathing pattern, MVV, and MIP were measured on days 14, 90, and 180. The resting breathing pattern was unchanged on the usual resting levels of ventilator support. However, by day 180 some patients were able to wean to lower pressure support and rate settings during some or all of their waking hours.

The best-effort unassisted tidal volume (Fig. 5A) improved significantly (28.8% change [15.2–35.2]) at 180 days after dosing ($p < 0.05$). The gains that most patients achieved in MVV (17.0% change [–12.5 to 55.2]) did not reach significance (Fig. 5B). Likewise, no clear changes were detected in MIP (–9.6% change [–54.2 to 4.3]), and in some cases, pressure declined (Fig. 5C). At day 90, all ventilatory variables were below baseline values for patient 201, following an acute hospitalization as reported above. He resumed a reduced muscle-conditioning routine by day 120. At day 180, his ventilatory function had improved, though some parameters had not yet reached baseline levels.

On days 0, 90, and 180, the spontaneous ventilatory endurance was measured as the total volume of air each subject could generate (Fig. 5D). Patient 204 consistently became tearful with combined tracheostomy cuff inflation and disconnection from the ventilator. For this patient, the resting PEEP setting was maintained for the spontaneous endurance tests. She was also able to tolerate several minutes of fully unsupported ventilation by day 180, while using a Passy-

FIG. 5. Ventilatory muscle function, following the gene transfer procedure. Patient performance was expressed in relation to the baseline function (dotted line at 100% reflects baseline function), measured the day before gene transfer. **(A)** The maximal unassisted tidal volume was significantly increased by day 180 (* $p < 0.05$). No appreciable change was detected in **(B)** maximal voluntary ventilation or maximal inspiratory pressure **(C)**. Although most patients achieved large relative gains in spontaneous ventilatory endurance **(D)**, they did not reach statistical significance ($p = 0.08$).

Muir valve. Although the majority of subjects achieved large gains in spontaneous ventilatory endurance (425% change [103–851]), this difference did not yet reach statistical significance ($p = 0.08$).

Discussion

Here we demonstrate that GAA gene replacement therapy to the diaphragm is feasible and safe in children with chronic ventilator dependence because of Pompe disease. Further, rAAV-hGAA appears to facilitate gains in unassisted tidal volume and spontaneous ventilatory endurance. Each patient was dependent upon total ventilatory support at baseline, which presented an opportunity to evaluate the safety of intramuscular delivery to the diaphragm. Since the retrograde transport of AAV1 is limited, we were able to determine safety outcomes following a local delivery approach. The fixed dose was based upon a clinically manageable volume for intramuscular delivery and the biodistribution of dose ranges derived from preclinical studies. Importantly, the doses were selected for evaluating safety and feasibility of this first-in-human diaphragm delivery (Mah *et al.*, 2007, 2010). A chronic, fully ventilator-dependent patient population was also selected to favor safety and to reflect the long-term prevalence of assisted ventilation in the majority of participants from the seminal pediatric ERT clinical trials (Nicolino *et al.*, 2009). Therefore, in light of the dose range, severity of the baseline impairments and duration of pre-existing ventilator dependence, the safety data, and measured ventilatory gains were consistent with our hypothesis.

The primary risks of gene replacement therapy are the systemic spread of the AAV vector and immune responses to the GAA protein. These immune responses have been reported previously during other gene therapy clinical trials or ERT-related studies and did not result in serious adverse

events (Kishnani *et al.*, 2007; Brantly *et al.*, 2009; Ponder, 2011). Similar to previous patients injected with rAAV vectors either intramuscularly or vascularly, each individual had a transient dissemination of vector DNA above the limit of detection out to 14 days postinjection with 1×10^{12} vg of rAAV1-hGAA. One patient in cohort 1 (subject 102) did, however, have recurring detectable vector genomes at days 30 and 90, but not day 60. The presence of vector genomes at day 1 was increased in both subjects in cohort 2 when dosed with 5×10^{12} vg of rAAV1-hGAA and remained positive in subject 204 at day 90. Each patient experienced a sustained humoral antibody response to the intact AAV1 capsid that remained 50- to 4,000-fold over baseline at the day 180 time point in cohort 1 and day 90 in cohort 2 and well above the population range (99 samples from healthy controls). Importantly, one subject showed a higher T-cell-mediated response to GAA than other subjects but was below the threshold considered as a positive test; there were no adverse events related to this finding, either for the vector capsid or for the transgene.

All of the patients had long-standing ventilatory failure that was unresponsive to preoperative conditioning exercises. We recognize that mechanical ventilation itself has been found to atrophy and weaken the human diaphragm (Levine *et al.*, 2008; Jaber *et al.*, 2011). Specific physiological training responses to IMST have been identified in patients (Ramirez-Sarmiento *et al.*, 2002; Huang *et al.*, 2003), yet significant gains in muscle strength or reduced ventilator dependence typically occur within the first 2–4 weeks of instituting IMST exercises in neurally intact ventilated patients (Martin *et al.*, 2011; Smith *et al.*, 2011). The intraoperative EMG assessments indicate the study patients likely suffered from both muscular and neural pathology (Bolton *et al.*, 1992; Zifko *et al.*, 1997; Dhand, 2006), and neither IMST nor ERT are thought to reverse phrenic

functional denervation. Thus, in the absence of additional restorative therapies, exercise alone may be unlikely to offer any functional ventilatory benefit in severely affected, ventilator-dependent patients with Pompe.

Ventilatory gains were exclusively detected after vector delivery. The lack of any functional change to preoperative exercise excludes the possibility that conditioning exercises alone accounted for the measured efficacy post rAAV1-hGAA administration. After gene transfer, the ventilatory muscles were capable of generating larger tidal volumes without ventilator assistance, and this improved volume capacity trended toward breathing unsupported for longer periods. In fact, with the exception of patient 201, who had to resume controlled MV support and refrain from conditioning exercises for ~6 weeks at day 75, patients more than tripled their spontaneous breathing endurance. Moreover, most children used lower ventilator pressure and rate settings during daytime hours, which may reduce the risks associated with chronic, high-pressure invasive ventilation (Baydur and Kanel, 2003). Although patients continued to require >22 hr/day of ventilator support, the gains in ventilatory endurance yielded functional benefits for children and their families that facilitated daily care. Gains in routine care enabled parents to transfer children within their home or to the car, bathe, and complete activities of daily living, and even leave the home for brief periods without using the ventilator.

We can only speculate why gene replacement therapy did not improve MIP. Significant changes in MIP can be detected early in mild to moderate respiratory muscle weakness, but it has been found less sensitive to changes in severe disease (DePalo and McCool, 2002; Nicot *et al.*, 2006). Further, the particular reliance of the MIP test on fast motor unit recruitment could have limited its ability to detect change. In small mammals, fast-fatigable motor units are typically recruited only during occlusion (i.e., MIP maneuvers) or with defensive reflexes (Sieck and Fournier, 1989; Mantilla *et al.*, 2010). It is thought that ordered recruitment also occurs in the human diaphragm. Further, both brief (Levine *et al.*, 2008) and extended periods of mechanical ventilation (Sieck, 1994) have been shown to preferentially atrophy diaphragmatic fast, glycolytic fibers. While fiber type differences in intracellular trafficking of GAA and autophagy have been noted in Pompe (Fukuda *et al.*, 2006; Shea and Raben, 2009), fiber type did not appear to influence transduction of the AAV1 vector in murine or macaque models (Louboutin *et al.*, 2005; Mah *et al.*, 2005). We cannot determine by our methodology whether fiber type influenced rAAV-hGAA transduction in the sample.

The gains in respiratory motor output appeared specific to ventilation. After 180 days, three of the subjects could not yet initiate a functional volitional cough, and the remaining two could not consistently clear all secretions without assistance. These findings underscore the strong influence of extra diaphragmatic and upper airway neuromuscular dysfunction on achieving independent ventilation and managing clearance of oral and tracheal secretions. Addressing these influences may be necessary to achieve greater functional gains in ventilation, speech, and airway defensive reflexes (Lee *et al.*, 2011).

There are limitations to the approach used to administer GAA gene transfer. Although the vector was delivered to three sites in each hemidiaphragm, it may have transduced

to only a small region of the diaphragm muscle. Further, while not specifically assessed, the efficiency of AAV1-mediated retrograde transport has not been thoroughly studied and may be more effective in addressing the muscle component of the motor unit. To more effectively treat the component of neural dysfunction, it may be necessary to use other AAV serotypes that can transduce neural tissue more efficiently (DiMattia *et al.*, 2012) or adopt a regional delivery approach (Falk *et al.*, 2012).

In summary, intramuscular rAAV-hGAA to the diaphragm was safe in ventilator-dependent children with Pompe disease, and the safety doses appeared to also offer a modest functional benefit to ventilatory performance. Next steps of this trial will examine whether higher doses and earlier intervention can result in a greater functional benefit. Further work is needed to determine whether local dosing to the lingual muscles or regional delivery strategies will augment the therapeutic effect.

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