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Comparisons of Infant and Adult Mice Reveal Age Effects for Liver Depot Gene Therapy in Pompe Disease

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Pompe disease is caused by the deficiency of lysosomal acid α-glucosidase (GAA). It is expected that gene therapy to replace GAA with adeno-associated virus (AAV) vectors will be less effective early in life because of the rapid loss of vector genomes. AAV2/8-LSPhGAA (3×10^{10} vector genomes [vg]/mouse) was administered to infant (2-week-old) or adult (2-month-old) GAA knockout mice. AAV vector transduction in adult mice significantly corrected GAA deficiency in the heart ($p < 0.0001$), diaphragm ($p < 0.01$), and quadriceps ($p < 0.001$) for >50 weeks. However, in infant mice, the same treatment only partially corrected GAA deficiency in the heart ($p < 0.05$), diaphragm ($p < 0.05$), and quadriceps ($p < 0.05$). The clearance of glycogen was much more efficient in adult mice compared with infant mice. Improved wire hang test latency was observed for treated adults ($p < 0.05$), but not for infant mice. Abnormal ventilation was corrected in both infant and adult mice. Vector-treated female mice demonstrated functional improvement, despite a lower degree of biochemical correction compared with male mice. The relative vector dose for infants was approximately 3-fold higher than adults, when normalized to body weight at the time of vector administration. Given these data, the dose requirement to achieve similar efficacy will be higher for the treatment of young patients.

INTRODUCTION

One of the main obstacles to the development of gene therapy targeted to the liver is the gradual loss of episomal adeno-associated virus (AAV) vector genomes, which is accelerated early in life.[1–4] Although AAV vectors have advanced to successful clinical trials based on liver transgene expression,[5] the loss of vector genomes is greater than the rate expected solely from cell division in the liver.[1,3] Approaches to this problem have included higher vector dosages[2,6] and early readministration of the vector, prior to the formation of anti-AAV antibodies.[4] However, these approaches have not comprehensively addressed the effects of the loss of vector genomes upon efficacy in animal models for genetic disease following neonatal treatment.

Gene therapy for Pompe disease is under evaluation in multiple clinical trials.[7] Current trials do not enroll young infants, despite the need for early treatment in the infantile form of Pompe disease. One previous clinical trial enrolled 5 boys (aged 18–180 months old) in a study of a recombinant AAV serotype 1 (rAAV1) injected in the diaphragm, and showed stable effects on tidal volume for 180 days.[8] Although promising, this approach has been deemed too invasive and too localized in its benefits to be developed for the treatment of all patients.

Preclinical data have suggested that early treatment might be successful in Pompe disease. For example, administration of an rAAV1 vector to neonatal acid α-glucosidase (GAA) knockout (GAA-KO) mice achieved sustained correction of GAA deficiency and substantially cleared accumulated glycogen stored in the heart and diaphragm for up to 1 year, which was accompanied by functional improvement with regard to both cardiac and respiratory involvement.[9,10] Those studies featured intramuscular transgene expression from an immediate early cytomegalovirus (CMV) promoter, which was not dependent on sustained liver transduction for efficacy. A more recent 4-month study of an rAAV9 vector containing a tandem promoter with dual tissue specificity for both muscle and liver achieved supraphysiologic GAA activity and complete clearance of glycogen from the heart, whereas physiologic GAA activity and partial clearance of glycogen was demonstrated in the diaphragm.[11] Similar to the case with the rAAV1 vector containing the CMV promoter,[9,10] the tandem promoter relied on stable transduction of muscle for its efficacy following the treatment of neonatal mice with Pompe disease.

The current study evaluated the long-term efficacy of liver depot gene therapy,[12] directly comparing the efficacy of a clinically appropriate

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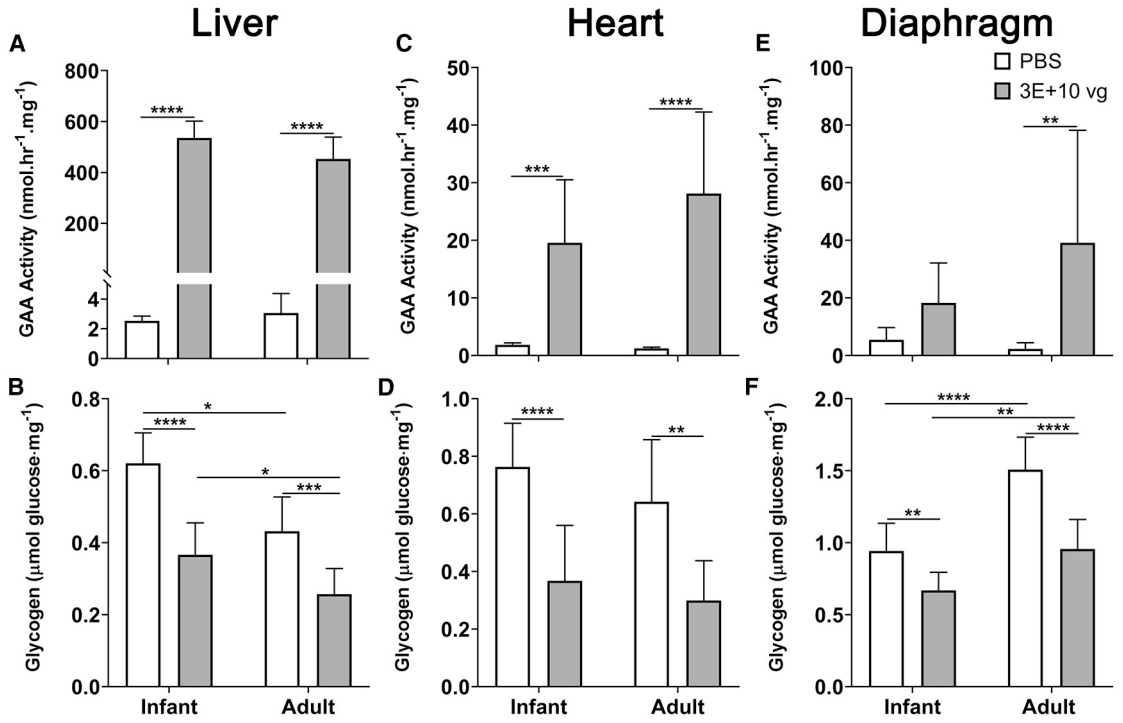

Figure 1. Short-Term Evaluation of Vector Administration to Infant or Adult Mice

Groups were as follows: infant PBS (n = 10), infant 3E+10 vg (n = 10), adult PBS (n = 8), and adult 3E+10 (n = 10). (A–F) Biochemical correction based on biochemical assay for (A) liver GAA activity, (B) liver glycogen content, (C) heart GAA activity, (D) heart glycogen content, (E) diaphragm GAA activity, and (F) diaphragm glycogen content. The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

dose of an rAAV8 vector in infant and adult GAA-KO mice. Biochemical correction and muscle function were evaluated 50 weeks following intravenous administration of the same absolute vector dosages at 10 days or 2 months of age to assess the effects of gene therapy either early or later in life. The vector is currently under evaluation in an active phase I clinical trial (ClinicalTrials.org: NCT03533673), and these experiments will be relevant to later phase clinical studies in the pediatric population of Pompe disease patients.

RESULTS

Liver Transduction in Infant and Adult Mice

The effects of age were evaluated in an experiment with AAV2/8-LSPhGAA,[13] an AAV2 vector genome cross-packaged as AAV8 that contains a liver-specific promoter to express human GAA. Liver transduction was analyzed 2 weeks following vector administration (3E+10 vector genomes [vg]) to either 2-week-old (infant) or 2-month-old (adult) GAA-KO mice. Liver transduction was similar for both groups, as demonstrated by vector DNA quantification (4.5 ± 3.2 vg/nucleus for infants and 4.3 ± 0.7 vg/nucleus for adults). Liver GAA activity was equivalent for the groups (535 ± 66 nmol/h/mg for infants and 453 ± 86 nmol/h/mg for adults), and both vector-injected groups had significantly higher liver GAA than PBS-injected controls (Figure 1A). Liver glycogen was higher and was decreased to a greater extent in infant mice (decreased by 41% from 0.62 ± 0.09 to 0.37 ± 0.01 μmol glucose/mg; Figure 1B) in comparison with adult mice (decreased by 31% from 0.43 ± 0.10 to 0.26 ± 0.07 μmol glucose/mg). The biochemical correction of the heart was similar following vector administration to infant and to adult mice (Figures 1C and 1D). Biochemical correction of diaphragm was slightly greater for adult mice, as shown by a significant increase in GAA activity, in comparison with PBS-injected controls. Increased GAA activity in the diaphragm was not observed in infant mice (Figure 1E). Moreover, diaphragm glycogen content was decreased more in adult mice (by 37% from 1.50 ± 0.2 to 0.96 ± 0.2 μmol glucose/mg; Figure 1F) in comparison with infant mice (by 29% from 0.94 to 0.19 μmol glucose/mg). However, diaphragm glycogen content was decreased to the lowest concentration in infant mice following vector administration (Figure 1F). Thus, short-term evaluation of vector administration revealed very similar effects in infant and adult GAA-KO mice.

Subsequently, liver transduction was analyzed 50 weeks following vector administration to either infant or adult GAA-KO mice. Groups of infant and adult mice were injected intravenously with either a lower (3E+9 vg) or higher (3E+10 vg) dose of vector. Liver transduction was quantified by liver GAA activity that was elevated in each vector-treated group in comparison with phosphate-buffered saline (PBS)-injected mice (Figure 2A). Liver GAA activity was significantly higher in adult-treated mice for both the high and low vector dosages (496 ± 59 or 252 ± 160 nmol/h/mg, respectively) in comparison with infant-treated mice (202 ± 78 or 60 ± 43 nmol/h/mg). Notably, the

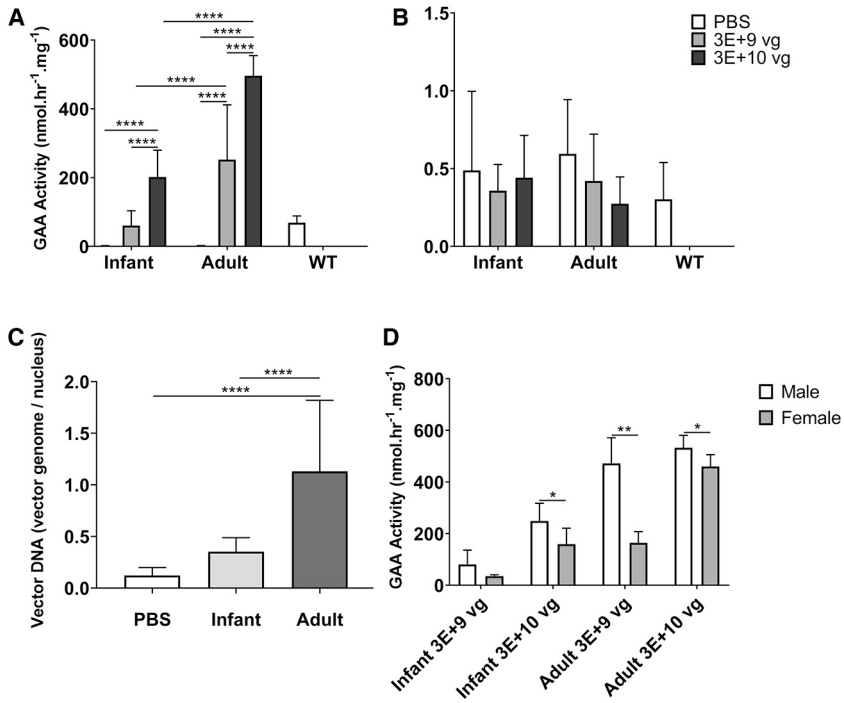

Figure 2. Long-Term Liver Correction following Vector Administration to Infant or Adult Mice

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls and evaluated 50 weeks later. Groups were as follows: infant PBS (n = 6), infant 3E+9 vg (n = 10), infant 3E+10 vg (n = 17), adult PBS (n = 10), adult 3E+9 (n = 7), adult 3E+10 (n = 10), and WT (n = 9). (A and B) Biochemical correction based on biochemical assay for (A) GAA activity and (B) glycogen content. (C) Vector genome quantification determined by qPCR detection of the human GAA cDNA. (D) Liver GAA activity in male and female mice. The p values are indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3E+10 vg dose produced GAA activity for infant-treated mice that was 40% of the value achieved for adult mice.

Liver glycogen content remained unchanged following vector administration, which is consistent with the presence of mainly cytoplasmic glycogen that is not increased in Pompe disease (Figure 2B).[14] Vector genomes were quantified by quantitative polymerase chain reaction (qPCR) of total liver DNA, which revealed significantly increased vector genomes in adult mice treated with 3E+10 vg in comparison with infant-treated mice or PBS-injected controls (Figure 2C). Although vector was administered by two different routes, retro-orbital injection for infant mice and tail vein injection for adult mice, equivalent vector genome delivery to the liver was confirmed in adult mice for these methods (retro-orbital = 2.8 ± 1.1 vg/nucleus; tail vein = 3.2 ± 1.7 vg/nucleus). The relevance of sex was demonstrated by significantly increased GAA activity in male mice from each vector-treated group in comparison with females from the same group (Figure 2D). The only group with no sex-related difference detected was the infant group treated with 3E+9 vg, which also had the lowest GAA activity (Figures 2A and 2D).

Heart Correction in Infant and Adult Mice

The heart accumulates lysosomal glycogen because of GAA deficiency in Pompe disease, which leads to cardiac enlargement.[15] The GAA activity of the heart was quantified 50 weeks following vector administration in infant and adult mice. Adult GAA-KO mice that were injected with 3E+10 vg had increased GAA activity in the heart in comparison with PBS-controls or GAA-KO mice injected with only 3E+9 vg (Figure 3A). Similarly, infant GAA-KO mice that were injected with 3E+10 vg had increased GAA activity in comparison with PBS-injected controls or mice injected with 3E+9 vg (Figure 3A). However, mice treated with 3E+10 vg as infants had approximately 10-fold lower GAA activity in the heart in comparison with mice treated as adults with the same vector dose (infant versus adult activity: 1.2 ± 0.7 versus 11.9 ± 10 nmol/h/mg).

The most sensitive measure for biochemical correction of muscle in Pompe disease is glycogen content.[16] Glycogen content was quantified by a sensitive biochemical assay that has advantages over glycogen staining.[17] Adult GAA-KO mice that were injected with 3E+10 vg had decreased glycogen content in the heart in comparison with mice injected with PBS or with 3E+9 vg (Figure 3B). Furthermore, adult mice injected with only 3E+9 vg also had decreased glycogen content in the heart in comparison with PBS-injected controls ($p < 0.0001$; Figure 3B). Similar trends were observed for mice injected with vector as infants (Figure 3B). However, mice treated with 3E+10 vg as infants had approximately 11-fold higher glycogen content in the heart in comparison with mice treated as adults with the same vector dose (infant versus adult glycogen: 0.85 ± 0.48 versus 0.08 ± 0.03 μmol glucose/mg).

Decreased glycogen content has correlated with lower ventricle mass in treated GAA-KO mice.[18] Left ventricle mass was significantly decreased by administration of 3E+9 vg in comparison with PBS-injected controls (Figure 3C), both in groups of mice treated as infants ($p < 0.001$) and as adults ($p < 0.001$). Treatment with 3E+10 vg further decreased left ventricle mass in comparison with 3E+9 vg (Figure 3C), both in groups of mice treated as infants ($p < 0.01$) and as adults ($p < 0.05$). Treatment with the higher dose essentially normalized left ventricle mass to the level observed in wild-type (WT) controls (infant versus adult 3E+10 vg mass: 4.1 ± 0.8 versus 3.5 ± 0.4; WT mass: 3.7 ± 0.8 mg/g). Vector treatment had no effect on body weight (Figure 3D). Overall, heart GAA activity, heart glycogen content, and left ventricle mass demonstrated correction of the heart to the greatest extent following administration of the higher dose in infant or adult GAA-KO mice, although mice treated as adults demonstrated higher biochemical correction than mice treated as infants.

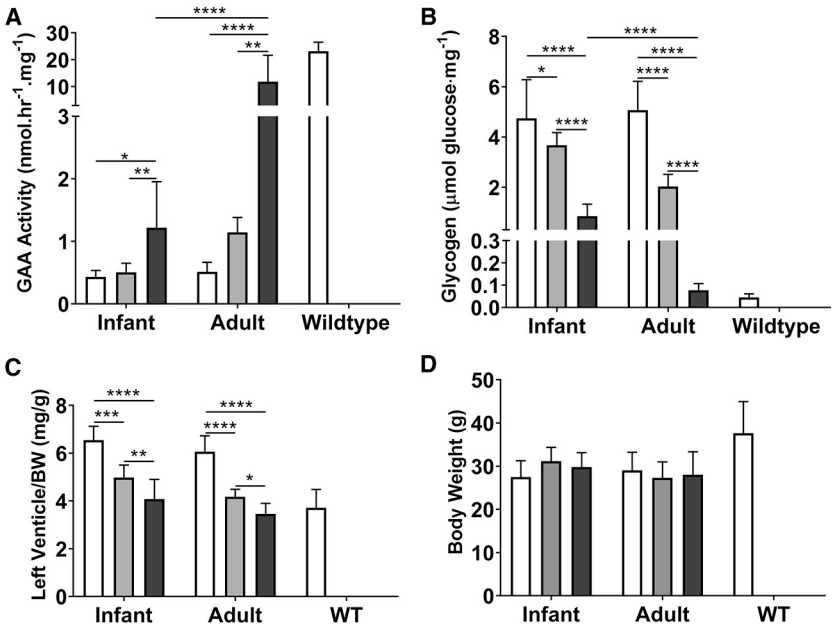

Figure 3. Correction of Heart Involvement in GAA-KO Mice Treated as Infants or Adults

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls. Groups were as follows: infant PBS (n = 6), infant 3E+9 vg (n = 10), infant 3E+10 vg (n = 17), adult PBS (n = 10), adult 3E+9 (n = 7), adult 3E+10 (n = 10), and WT (n = 9). (A and B) Biochemical correction based on biochemical assays for (A) GAA activity and (B) glycogen content in the heart. (C) Left ventricle (LV) mass normalized to body weight (BW). (D) BW. The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Correction of Diaphragm and Breathing in Infant and Adult Mice

GAA-KO mice that were injected with 3E+10 vg as adults had increased GAA activity in the diaphragm in comparison with PBS-injected controls or vector-injected infant mice (Figure 4A). Adult mice treated with 3E+10 vg had decreased glycogen content in the diaphragm in comparison mice injected with PBS or with 3E+9 vg (Figure 4B). A similar trend was observed in GAA-KO mice treated as infants (Figure 4B), although glycogen content was approximately 2.4-fold higher for mice treated as infants with 3E+10 vg in comparison with mice treated as adults (infant versus adult glycogen content: 2.2 ± 0.5 versus 0.9 ± 0.6 µmol glucose/mg).

Plethysmography was performed following the 12-month observation period to assess ventilation as described.[19] Breathing frequency was significantly lower following a respiratory challenge with hypoxia and hypercapnia in PBS-injected control GAA-KO mice in comparison with WT controls (Figure 4C). Administration of 3E+10 vg significantly improved the frequency of breathing for both infant-treated ($p < 0.05$) and adult-treated ($p < 0.05$) GAA-KO mice in comparison with PBS-injected controls (Figure 4C). Vector administration did not significantly alter other plethysmography endpoints (Figure S1). Treatment with the higher vector dose essentially normalized breathing frequency following the hypoxia/hypercapnia challenge to the level observed in WT controls (infant versus adult 3E+10 vg frequency: 304 ± 22 versus 309 ± 23 breaths/min [bpm]; WT frequency: 312 ± 21 bpm). Thus, administration of the higher vector dose achieved a greater degree of biochemical correction following injection of adult mice in comparison with injection of infant mice; however, a defect of breathing frequency was corrected regardless of age at the time of treatment.

Correction of Skeletal Muscle and Weakness

Skeletal muscle typically responds less efficiently to GAA replacement in comparison with the heart or diaphragm.[20] The biochemical correction of the quadriceps muscle was analyzed to assess the age-related skeletal muscle response. GAA-KO mice that were injected with 3E+10 vg as adults had increased GAA activity in the quadriceps, in comparison with PBS or with 3E+9 vg (Figure 5A). A similar trend was observed in GAA-KO mice treated as infants (Figure 5A), although GAA activity was approximately 2.9-fold lower for mice treated as infants with 3E+10 vg in comparison with mice treated as adults (infant versus adult activity: 0.7 ± 0.1 versus 2.0 ± 1.1 nmol/h/mg). Adult mice treated with 3E+10 vg had decreased glycogen content in the quadriceps, in comparison with PBS or with 3E+9 vg (Figure 5B). No change in glycogen content of quadriceps was demonstrated following vector administration to infant mice. Muscle strength was assessed by the wire hang test, which revealed significantly increased latency for adult mice treated with 3E+10 vg, consistent with the significant biochemical correction of skeletal muscle observed in those mice (Figure 5C). Glycogen staining revealed decreased glycogen-filled vacuoles in the adult GAA-KO mice treated with 3E+10 vg (Figure 5D, ii versus iii) and a decreased severity score reflecting fewer vacuoles and nuclei (Figure 5E). Overall, involvement of the quadriceps was improved to the greatest extent by treatment of adult mice with the higher vector dose.

Sex-Related Response to Gene Therapy

Studies of AAV vector-mediated liver transduction have consistently reported improved responses in adult male mice.[21,22] Sex-related differences of biochemical correction were analyzed, which revealed consistently higher GAA activity in male mice treated as adults in comparison with adult female mice (Figure 6A). However, adult-treated females did achieve significantly elevated GAA activity in comparison with PBS controls, both in the heart ($p < 0.01$) and the quadriceps ($p < 0.05$). In contrast, female mice treated during infancy did not have increased GAA activity in any muscle (Figure 6A). Glycogen content analysis did not reveal sex-related differences in the degree of biochemical correction, with the exception of quadriceps glycogen content that was significantly decreased only in females

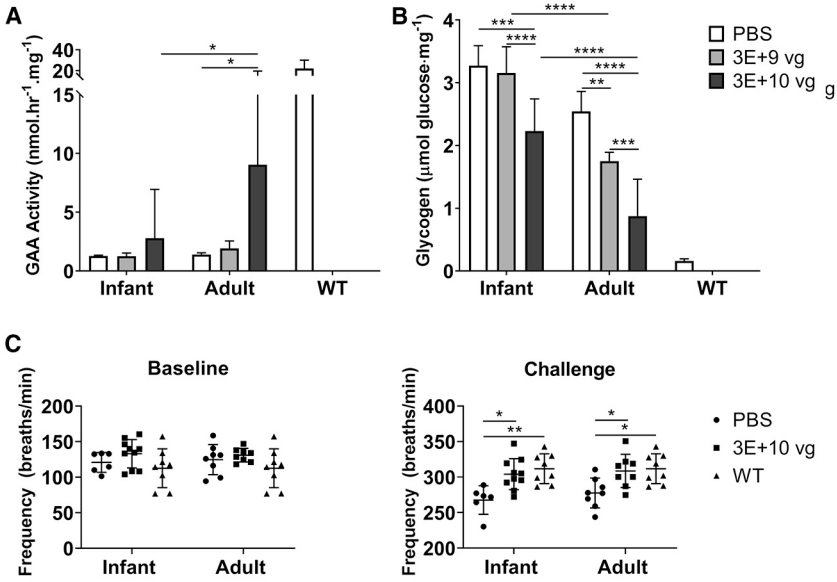

Figure 4. Correction of Diaphragm Involvement in GAA-KO Mice Treated as Infants or Adults

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls. (A and B) Biochemical correction based on biochemical assays for (A) GAA activity and (B) glycogen content. Groups were as follows: infant PBS (n = 6), infant 3E+9 vg (n = 10), infant 3E+10 vg (n = 17), adult PBS (n = 10), adult 3E+9 (n = 7), adult 3E+10 (n = 10), and WT (n = 9). (C) Breathing frequency. Groups were as follows: infant PBS (n = 6), infant 3E+10 vg (n = 10), adult PBS (n = 8), adult 3E+10 (n = 8), and WT (n = 8). The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

treated as adults (Figure 6B). The latter observation implied that the skeletal muscle benefits from vector administration might be greater in females, despite the trends toward higher GAA in male GAA-KO mice.

Functional testing was further analyzed for sex-related differences. Although mice treated as infants demonstrated improved breathing frequency in both sexes ($p < 0.05$), only females treated as adults had improved frequency (Figure 6C). Wire hang latency increased significantly in females treated as infants ($p < 0.05$) or as adults ($p < 0.05$), whereas wire hang performance did not improve in response to vector administration in males (Figure 6D). Thus, functional testing confirmed a favorable response to vector administration at the higher dose in female mice, despite the lower muscle GAA activity observed in females versus males.

Urine and Plasma Biomarkers Detected the Response to Vector Administration

The urinary glucose tetrasaccharide (Glc_4) has correlated with the treatment of Pompe disease in preclinical and clinical studies.[23,24] Glc_4 decreased in the urine of mice treated with 3E+10 vg as adults ($p < 0.0001$) or as infants ($p < 0.001$) in comparison with PBS-injected controls (Figure 7A). Furthermore, Glc_4 decreased in the urine of mice treated as adults with the 10-fold lower vector dose, 3E+9 vg ($p < 0.0001$; Figure 7A). Glc_4 was significantly lower in adult-treated mice at the higher dose in comparison with infant-treated mice ($p < 0.0001$; Figure 7A). An alternative, non-invasive biomarker in the form of plasma GAA activity increased significantly for adult mice treated with 3E+10 vg in comparison with PBS-injected controls or GAA-KO mice injected with only 3E+9 vg or infant-treated mice (Figure 7B). Sex-related differences were observed in urinary Glc_4 that decreased significantly in infant-treated male mice, but not in infant-treated female mice (Figure 7C). However, both sexes of adult-treated mice had significantly decreased urinary Glc_4, reflecting greater efficacy in adult mice. Furthermore, plasma GAA was significantly increased in adult-treated males, but not in adult-treated females or in infant-treated mice (Figure 7D). These biomarkers for Pompe disease generally detected higher efficacy with regard to biochemical correction in adult-treated mice and in male mice.

DISCUSSION

This study investigated the long-term correction of infantile Pompe disease mice with liver depot gene therapy in comparison with adult mice. Unsurprisingly, the degree of biochemical correction was greater in the adult-treated mice, because AAV vector transduction is more stable in older animals that have completed the rapid growth phase of infancy.[1–4] However, benefits were demonstrated related to the biochemical correction of muscle of infant-treated mice, including decreased left ventricle (LV) mass, decreased breathing frequency, and increased wire hang latency. Improvements in function were greater in female mice with regard to breathing frequency and wire hang, despite the lower degree of transduction in females overall, which might be attributed to unknown sex-related differences in the response to GAA replacement. These long-term benefits of gene therapy in infant mice with Pompe disease confirm the potential value of treatment early in life.

The question of how to effectively treat young patients with gene therapy has not been definitively answered for Pompe disease or other conditions treated by liver-targeted gene therapy. The rationale for liver transduction to achieve a depot of GAA production in Pompe disease includes greater efficacy at low vector dosages in comparison with the direct transduction of muscle. For example, earlier studies demonstrated long-term correction of skeletal muscle in neonatal mice with Pompe diseases from a vector dose range of 6E+12 to 1.0E+14 vg/kg body weight.[10,11,25] The higher dose of 3E+10 vg in the current study achieved long-term biochemical correction and functional improvement from a vector dose of only 3E+12 vg/kg in infant mice, which is at least 50% lower than the effective dose in previous studies of neonatal GAA-KO mice. An important difference in

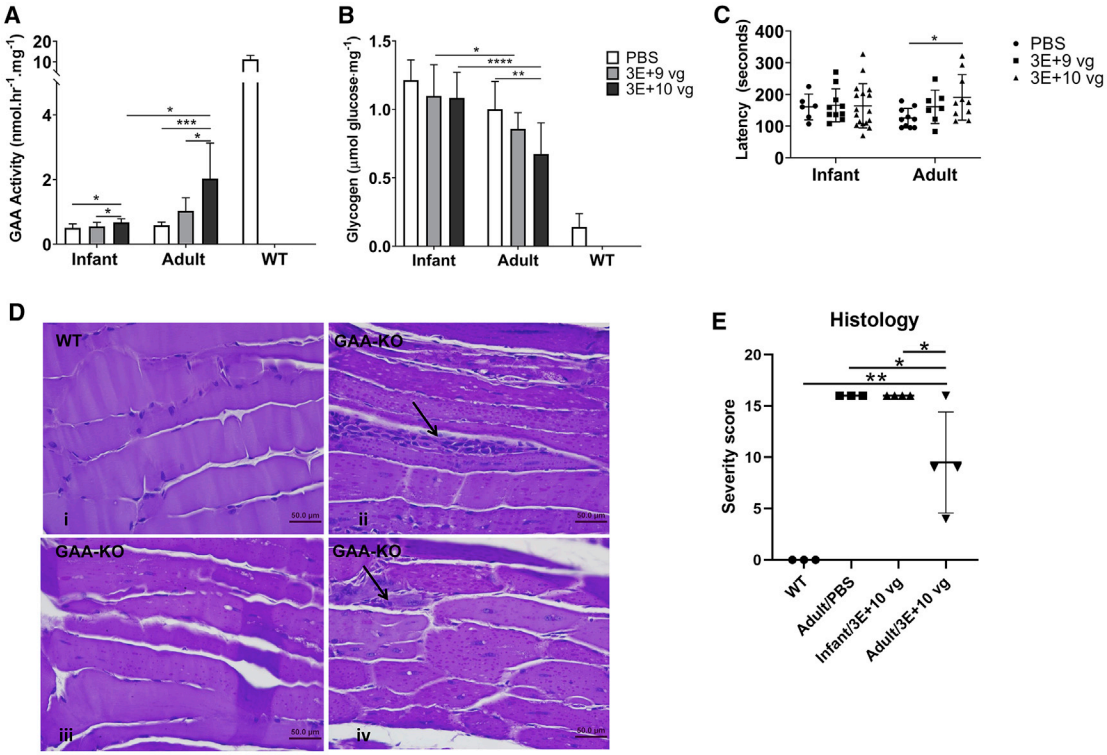

Figure 5. Correction of Skeletal Muscle Involvement in GAA-KO Mice Treated as Infants or Adults

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls. Groups were as follows: infant PBS (n = 6), infant 3E+9 vg (n = 10), infant 3E+10 vg (n = 17), adult PBS (n = 10), adult 3E+9 (n = 7), adult 3E+10 (n = 10), and WT (n = 9). (A and B) Biochemical correction based on biochemical assays for (A) GAA activity and (B) glycogen content in quadriceps. (C) Wire hang latency. (D) Muscle histology. (i) Photomicrograph of muscle from mouse in WT control group. Note lack of PAS-positive material and vacuoles in myofibers. (ii) Quadriceps muscle from mouse in GAA-KO adult group treated with PBS. Note numerous PAS-positive vacuoles and myofiber degeneration (arrow). (iii) Photomicrograph of muscle from mouse in the GAA-KO adult group treated with 3E+10 vg. Note diminution in PAS-positive material and vacuoles in myofibers relative to photomicrograph in (ii). (iv) Photomicrograph of muscle from mouse in GAA KO infant group treated with 3E+10 vg. Arrow depicts myopathic change. PAS stain, original magnification ×400. (E) Severity scores for WT and GAA-KO mice for the following groups: adult/PBS, infant/3E+10 vg, and adult/3E+10 vg. The latter group corresponds to the photomicrograph in (iii). The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

age of treatment should be noted, because the 2-week-old infant mice used here have developed to a stage more equivalent to human infants, including eye opening and the presence of hair. In contrast, the developmental of neonatal mice corresponds to fetal development in humans. A previous study of gene therapy in infant GAA-KO mice confirmed the long-term efficacy from whole-body correction of GAA deficiency at 5E+12 vg/kg, but this study utilized the PHP.B capsid that does not transduce human tissues efficiently, whereas the more clinically relevant rAAV9 vector was inefficacious in that study.[26] The current study demonstrated stable transduction of the liver in infant mice at 40% of the GAA activity that was achieved by the same number of vector genomes in adult mice. The 3E+10 vg dose corresponds to a 3-fold lower dose in the older mice of 1E+12 vg/kg, when normalized to body weight. These observations suggest that, at least in part, the need to treat young patients can be achieved by increasing the relative vector dose.

Another important consideration for liver depot gene therapy in Pompe disease is the induction of immune tolerance to GAA that avoids the complication of anti-GAA antibodies. Prior studies demonstrated a lack of efficacy from gene therapy following the formation of anti-GAA antibodies, which were linked with higher muscle glycogen content and hypersensitivity reactions during enzyme replacement therapy (ERT).[20,27–29] The prevention of antibody responses is emphasized due to the availability of standard-of-care ERT that might be needed to supplement efficacy from gene therapy, and provoking anti-GAA would reduce the efficacy from subsequent ERT.[20,30] Furthermore, immune responses to universal expression of GAA driven by a CMV enhancer neutralized the efficacy from gene therapy in association with cytotoxic T cell responses.[13,28] At least in adult GAA-KO mice, liver-specific GAA expression has required lower dosages due to the more efficient transduction of liver with rAAV and the lack of immune responses to GAA.[16,28,31]

Adjunctive therapy with β_2 agonists could improve outcomes following gene therapy in Pompe disease through enhancing the receptor-mediated uptake of GAA, which could enhance efficacy following vector administration at a young age. Treatment with

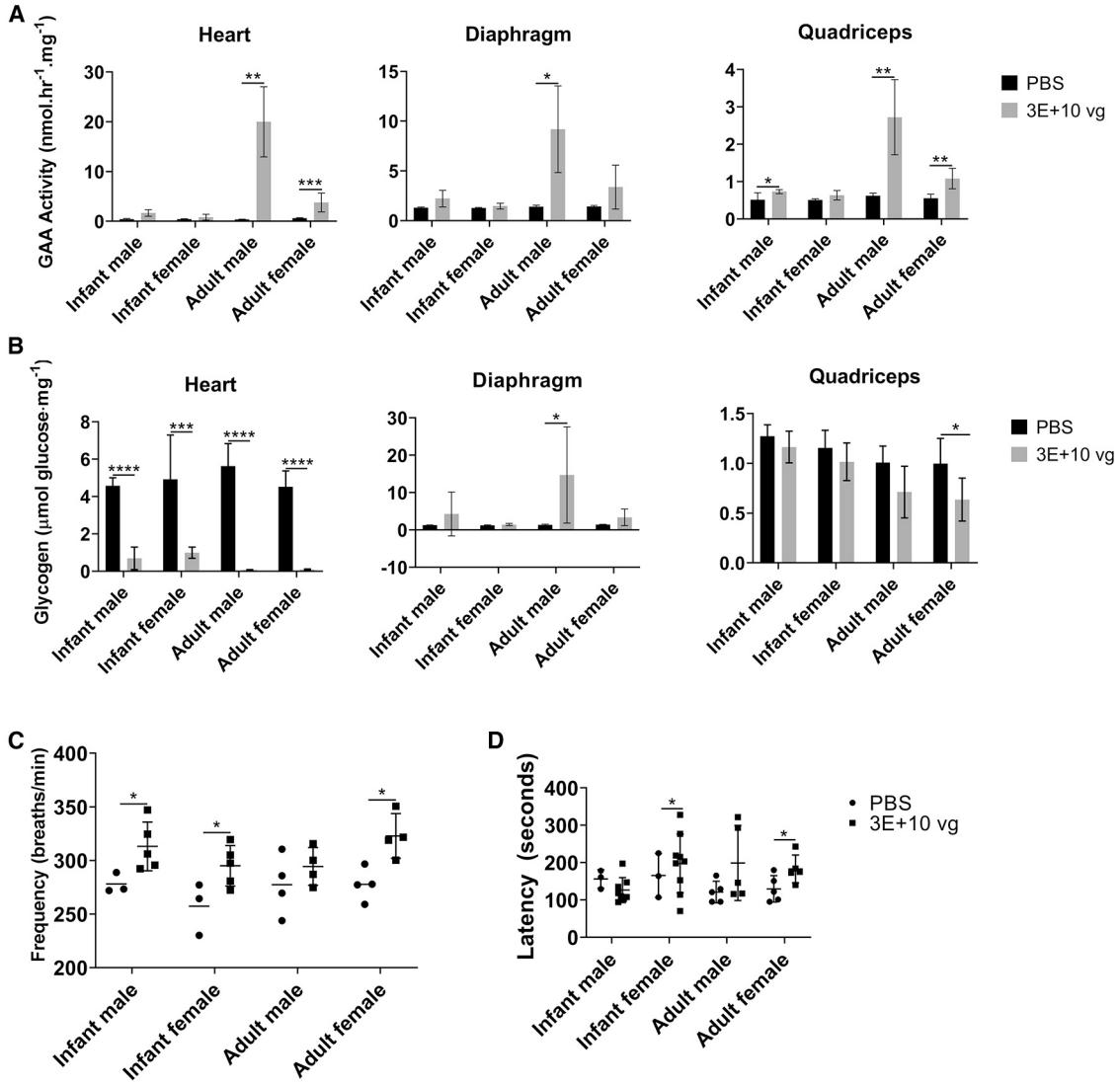

Figure 6. Effect of Sex on Correction in GAA-KO Mice Treated as Infants or Adults

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls. Groups were as follows: infant male PBS (n = 3), infant female PBS (n = 3), infant male 3E+10 vg (n = 8), infant female 3E+10 vg (n = 9), adult male PBS (n = 5), adult female PBS (n = 5), adult male 3E+10 vg (n = 10), and adult female 3E+10 vg (n = 5). (A and B) Biochemical correction as determined by GAA activity (A) and by glycogen content (B). (C) Breathing frequency. (D) Wire hang latency. The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

clenbuterol increased the biochemical correction of muscle following the administration of low-dose liver depot gene therapy, which correlated with increased cation-independent mannose-6-phosphate receptor (that binds GAA and traffics it to the lysosome).[32] Another long-acting β_2 agonist, salmeterol, improved the cardiac response to the direct transduction of striated muscle.[33] Furthermore, salmeterol improved the biochemical correction of skeletal muscle and enhanced muscle strength in combination with liver depot gene therapy.[34] Although direct transduction of muscle might provide more stable transduction, an AAV vector containing a muscle-specific promoter provoked antibody responses that interfered with the uptake of GAA in uncorrected myofibers.[35] Immunosuppression was beneficial, because glycogen clearance was clearly enhanced by treatment with a nondepleting anti-CD4 monoclonal antibody along with GAA expression in cardiac muscle.[35,36] Finally, anti-CD4 treatment along with clenbuterol achieved synergistic therapeutic efficacy in both cardiac and skeletal muscle.[35] These studies demonstrated the potential benefits of increased receptor-mediated uptake of GAA and suppression of anti-GAA antibodies, especially in the context of partial efficacy from gene therapy. Alternatively, GAA can be modified with an alternative signal peptide to increase its secretion from transduced liver cells with an accompanying increase in the

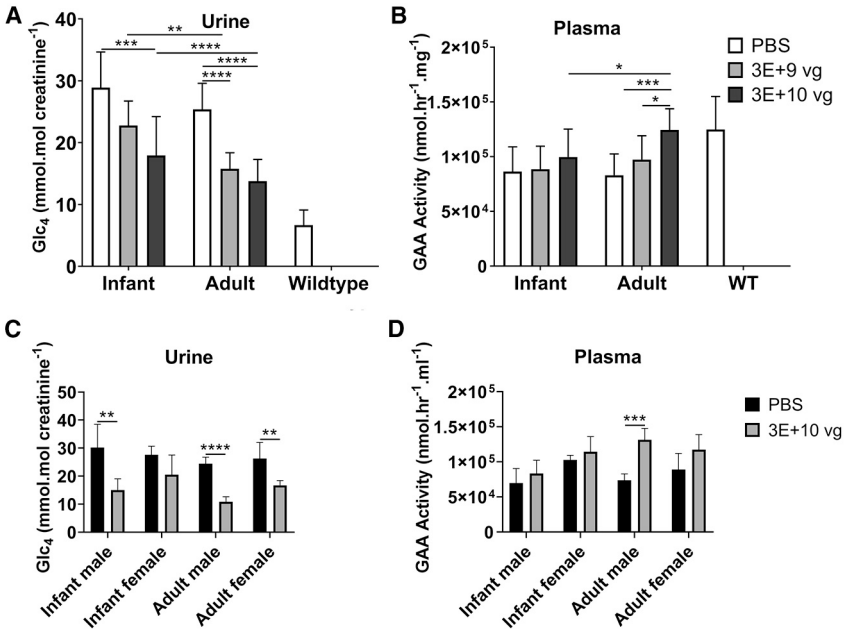

Figure 7. Biomarker Analysis

Mice were injected with vector at 2 weeks or 2 months of age, or injected with PBS as controls. Groups were as follows: infant PBS (n = 6), infant 3E+9 vg (n = 10), infant 3E+10 vg (n = 17), adult PBS (n = 10), adult 3E+9 (n = 7), adult 3E+10 (n = 10), and WT (n = 9). (A) Urinary biomarker, Glc_4. (B) Effect of sex on Glc_4 concentration. (C) Plasma GAA. (D) Effect of sex on plasma GAA. The p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

biochemical correction of untransduced muscle, while inducing immune tolerance to GAA through liver-specific expression.[37,38]

This report of long-term efficacy in GAA-KO mice treated in infancy supports the treatment of Pompe disease and other inherited metabolic disorders at an early age. AAV vector-mediated gene therapy effectively treated mice with urea cycle disorders at doses of 1.7E+13 to 4E+13 vg/kg (the latter in two divided doses).[2,4] Higher vector dosages can now be considered, given the high dosages administered in the clinical trial of rAAV9 vector-mediated gene therapy in patients with otherwise fatal spinal muscular atrophy (SMA) type 1. These patients were administered a lower dose (6.7×10^{13} vg/kg) in cohort 1 and a high dose (2.0×10^{14} vg/kg) in cohort 2. Long-term survival and continued achievement of motor milestones in the second cohort were accompanied by a lack of serious adverse events. The only significant safety concerns were elevated serum aminotransferase levels that occurred in four patients and responded to immunosuppression with prednisolone, which is a standard regimen for suppressing T cell-mediated immune responses to the AAV vector capsids.[5] The success of the SMA clinical trial has established a much higher threshold for dosing AAV vectors in clinical trials. Other studies have validated the safety of high dosages of AAV vectors, including those conducted in hemophilia. A clinical trial in hemophilia A with an rAAV5 vector demonstrated the cessation of bleeding events that indicated a beneficial replacement of factor VIII only for the high-dose (6.7×10^{13} vg/kg) cohort.[39] Similar to the SMA clinical trial mentioned above, the only significant adverse events were elevations of alanine aminotransferase that prompted the initiation of prophylaxis with prednisolone immunosuppression in the high-dose cohort. These studies validated the finding that higher dosages of AAV vectors were needed to achieve benefits, and that safety was acceptable for those higher dosages. On the other hand, recent concerns regarding toxicity at the highest vector dosages emphasize the need for using the minimal effective dose.[40]

The current study increases the understanding of gene therapy for children with Pompe disease, which is important due to the need for improved treatment of infantile and late-onset Pompe disease.[41,42] The relatively low vector dosages, long-term efficacy, and induction of immune tolerance to GAA support the further development of liver depot gene therapy for both pediatric and adult patients with Pompe disease.

MATERIALS AND METHODS

In Vivo Evaluation of AAV Vector-Mediated Efficacy

The AAV vector was prepared as described and administered intravenously to GAA-KO mice with a C57BL/6 background.[21,43,44] Age- and sex-matched GAA-KO mice were housed in groups of 3 to 5, and mice from different groups were co-housed when possible. Wire hang testing was performed as described previously.[32] Plethysmography was performed as described with modifications.[19] In brief, unanesthetized, unrestrained mice are placed in a Plexiglas chamber (Buxco FinePoine) and exposed to normoxic air (fraction of inspired air [Fi] O_2: 0.21, N_2 balance) for 1.5 h during which baseline data were recorded in 2-s intervals. Then the mice were exposed to a hypercapnic and hypoxic air (FiO_2: 0.10, $FiCO_2$: 0.07, N_2: balance) challenge for 10 min. GAA activity and glycogen content were analyzed as described previously.[21] All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Muscle Histology

Quadriceps muscle was collected from mice and immersion fixed in glutaraldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin and periodic acid-Schiff (PAS). Slides stained with PAS were scored by a pathologist (J.I.E.) semiquantitatively without knowledge of treatment group allocation. A vacuolar score was assigned by multiplying a severity score (0–4) × area involved score (0–4). A separate scoring of nuclear number was assigned based on 0–4 (negative = WT control): minimal (0%–10% increase), mild (10%–25% increase), moderate (25%–50% increase), and marked (>50% increase) in five random fields counted at ×400.

Viral Vector Genome Copy Number Analysis

Total DNA was extracted from approximately 100 mg of frozen liver tissue by using the MagNA Pure 96 DNA and viral NA small volume kit (Roche Diagnosis, Basel, Switzerland) according to the manufacturer's instructions. Viral vector genome copy number (VGCN) measured by qPCR was normalized by the copies of titin gene measured in each sample. qPCR was performed on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) using SYBR Green mix (Thermo Fisher Scientific, Waltham, MA) and the following specific primers and probes: GAA forward 5′-AGATCCCCCAGACA GTGCTG-3′, reverse 5′-TTCCTGCTGGCAGTGGTGCTGA-3′; titin forward 5′-AAAACGAGCAGTGACGTGAGC-3′, reverse 5′-TT CAGTCATGCTGCTAGCGC-3′.

Statistical Analyses

Multiple comparisons were assessed with two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test or with multiple t tests using Prism software (GraphPad, La Jolla, CA, USA). A p value <0.05 was considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.11.020.

AUTHOR CONTRIBUTIONS

S.-o.H., A.M., B.A., S.L., and H.Z. performed research and analyzed data. J.I.E. interpreted histologic data. S.P.Y., M.K.E.M., and D.D.K. analyzed data and wrote the paper.

CONFLICTS OF INTEREST

D.D.K. has developed the technology that is being used in the study. If the technology is commercially successful in the future, the developers and Duke University may benefit financially. D.D.K. has received research/grant support from Sanofi Genzyme Corporation in the past.

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