

# **Gene Therapy for Pompe Disease Using AAV9 and AAVB1 Encoding the GAA Gene**

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 27, 2017

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## **ABSTRACT**

Pompe disease, a storage disease caused by a lack of lysosomal acid alpha-glucosidase (GAA), results in systemic lysosomal glycogen accumulation and respiratory and muscle destruction. We propose that viral gene therapy can lower glycogen accumulation and relieve symptoms in a  $GAA^{-/-}$  mouse model. Two viruses were engineered to contain plasmids encoding GAA (AAVB1-GAA or AAV9-GAA), and were injected IV into 90-day old  $GAA^{-/-}$  or WT mice. Preliminary data with respiratory, behavioral, and histological assays suggest that both vectors improve the survival and pathology.

# TABLE OF CONTENTS

Signature Page .....	1
Abstract .....	3
Table of Contents .....	4
Acknowledgements .....	5
Background .....	6
Project Purpose .....	15
Methods .....	16
Results .....	20
Discussion .....	27
Bibliography .....	29

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Mai El Mallah (UMMS) for allowing me the opportunity to work in her laboratory, and for the direction, insight, and time offered to me this year. I would also like to thank Allison Keeler-Klunk and Kaitlyn Desrochers for instructing me in various lab techniques and their assistance with my project. Finally, I would like to thank Dr. David Adams for the input and guidance he provided me with throughout the entire MQP.

# BACKGROUND

## Lysosomal Diseases

Lysosomal storage disorders (LSDs) are a group of rare genetic diseases caused by a lack of proteins involved in lysosomal pathways. They result in the accumulation of substances within the lysosomes. As lysosomes exist in all cells except for red blood cells, diseases that affect the lysosomes present with symptoms involving many body systems (Bruni et al., 2007). Many LSDs present with similar features, including organomegaly, skeletal abnormalities, central nervous system dysfunction, coarse hair, hepatosplenomegaly, facies, and varying levels of mental retardation (Meikle et al., 1999; Bruni et al., 2007). LSDs are grouped into different categories by the type of substrate that accumulates in the lysosome. These groups include PMSs, lipidoses, glycogenoses, and oligosaccharidoses.

LSDs share many characteristics, from their method of inheritance, to the range in severity of symptoms. Most LSDs are inherited in an autosomal recessive pattern; the three exceptions are Danon disease, Fabry disease, and Mucopolyccharidosis, each of which are X-linked recessive (Meikle et al., 1999). Almost all of these diseases have a broad range of both clinical severity and the range at which symptoms present. Even the same mutations in the same gene will not always cause disease presentation in the same manner or at the same age (Meikle et al, 1999). Due to the many differences in disease presentation between different individuals with the same genotype, it is likely that environmental and other genetic background have roles in the progression of LSDs (Meikle et al., 1999).

There are at least 41 different LSDs, and they have a broad distribution of prevalence. A study analyzing data from Australia from 1980 through 1996 determined a combined occurrence of 1 child with an LSD in 7,700 live births, ranging from 1 in 57,000 with Gaucher Disease, to 1 in 4.2 million with sialidosis (Meikle et al., 1999).

There are a variety of treatments for LSDs, with different levels of efficacy. Bone Marrow Transplantation (BMT) replaces the deficient enzyme activity with IV infusion of normal hematopoietic progenitor cells. Since transplants require a donor with specific requirements, this therapy is only available to those able to find a suitable donor. BMT is only effective when performed before the diseased individual is three years old (Bruni et al., 2007). Another available treatment is Enzyme Replacement Therapy (ERT) which involves IV injection of the deficient enzyme. However, ERT does not cross the blood-brain barrier so is unable to treat symptoms of LSDs involving the central nervous system (Bruni et al., 2007). In addition, Substrate Reduction Therapy slows the formation of the lysosomal substrate so that the residual enzyme is able to degrade the substrate without buildup in the lysosome. This therapy only works in instances where the enzyme is still present, and does not treat individuals with complete enzyme deficiency (Bruni et al., 2007). Chemical Chaperone Therapy works to stabilize the enzyme by aiding in the stabilization and function of misfolded enzymes. This stabilization increases enzyme activity and thus decreases the accumulation of substrate in the lysosomes. Since this treatment works by fixing an existing enzyme, it only works for genotypes that produce the enzyme in question. Gene Therapy treats disease by inserting the normal gene into cells, therefore allowing the normally defective enzyme to be produced (Bruni et al., 2007).

## **Acid Alpha-Glucosidase**

Acid alpha-glucosidase (GAA) is a lysosomal enzyme that degrades the glycogen within lysosomes into glucose which then exits the lysosome. Specifically, GAA functions in the acidic environment of lysosomes to degrade alpha 1-4 and alpha 1-6 glycosidic linkages in glycogen, maltose, and isomaltose (Brown and Brown, 1970; Muller-Felbert et al., 2007). Discovered in 1961 by H. G. Hers, GAA was found to be the cause of the LSD Pompe Disease (Hers et al., 1963).

Located on chromosome 17q23, the structural gene that encodes GAA is 28 kb in length, and includes 20 exons (Nickel et al., 1982; Martiniuk et al., 1991). The cDNA is 3.6 kb with a 2,856 bp coding sequence that has a 5' UTR of 218 bp and a 3' UTR of 555 bp (Mariniuk et al., 1986; Hoefsloot et al., 1988). The polypeptide produced is 952 amino acids, and has a predicted molar mass of 105 kDa (Hasilik and Neufeld, 1980). It is processed along a similar pathway as other lysosomal enzymes; the polypeptide is co-translationally glycosylated in the endoplasmic reticulum (ER), its mannose residues are phosphorylated, and it is further processed in the ER and Golgi to different species, predominantly fragments of 76 and 70 kDa (Hasilik and Neufeld, 1980; Kornfeld, 1986).

## **Pompe Disease**

Pompe Disease, also called Glycogenosis Type II, Glycogen Storage Disease Type II, and Acid Maltose Deficiency, is a LSD caused by a deficiency of acid alpha-glucosidase (GAA). Categorized by JC Pompe in 1932, it was the first LSD to be discovered (Kishnani et al., 2006). Pompe Disease is an autosomal recessive inherited disorder with progressively worsening symptoms from the onset of the disease. The

deficiency of GAA leads to an accumulation of glycogen within the lysosomes, especially in cardiac tissue, skeletal muscle, and smooth muscle (Winkel et al., 2004). This accumulation results in cellular dysfunction, structural disorganization, and abnormal autophagy (Fukuda et al., 2006). Pompe is extremely rare, with prevalence ranging from 1 in 33,000, to 1 in 300,000, with a combined incidence of 1 in 40,000 (Kishnani et al., 2006). The occurrence depends on both geographic region and ethnicity (Martiniuk et al., 1998; Ausems et al., 1999; Chien et al., 2008). There are two types of Pompe Disease, the quickly fatal infantile onset, and slowly progressive late onset (Martiniuk et al., 1991). Infantile onset Pompe Disease is more common in African-Americans and Chinese populations, and Late Onset Pompe Disease (LOPD) has a higher incidence in The Netherlands (Kishnani et al., 2006).

Pompe disease results from a large variety of allelic mutations. Different mutations cause different levels of GAA mRNA or enzyme activity. A study (Martiniuk et al., 1998) found five out of 10 infantile onset patients with a complete lack of mRNA for GAA, and two out of 4 LOPD patients with mRNA of abnormal size. Another study found two thirds of infantile onset patients lacked any detectable mRNA (van der Ploeg et al., 2010). Other patients who still produce GAA have normal GAA activity without any abnormalities in the structure or function of the enzyme. These patients have reduced enzyme activity, but the enzyme activity that is present is normal (Reuser et al., 1978; Schram et al., 1979). The reduced levels of GAA activity most often occur because of defects in the synthesis or processing of the enzyme, and not from an increase in degradation of mature GAA (Reuser and Kroos, 1982). However, one study found 2 patients whose GAA deficiency resulted from rapid degradation of either the precursor to



GAA or GAA itself (Steckel et al., 1982). There does not appear to be any correlation between the genotype causing the disease and the resulting phenotype (Muller-Felbert et al., 2007).

Infantile onset Pompe Disease occurs when symptoms start within the first year of life. The average onset of clinical symptoms in these individuals occurs at three months old, and untreated patients die between six months and 18 months (Bijvoet et al., 1998). Deaths are most often caused by either cardiac failure or respiratory failure (Muller-Felber et al., 2007). Symptoms include cardiomegaly, severe generalized muscle weakness, and respiratory dysfunction, with most patients requiring invasive ventilation (Muller-Felbert et al., 2007; Wang et al., 2007).

LOPD occurs when the symptoms begin after the first year of life. There is large variability in the progression and clinical symptoms of this disease; the age of disease onset does not correlate with the severity of progression (Muller-Felbert et al., 2007). Symptoms affect almost all body systems, with large amounts of cardiac, muscular, and respiratory involvement. The most frequent presenting sign is progressive limb-girdle weakness (Muller-Felbert et al., 2007). A large number of LOPD patients become wheelchair bound (Winkel et al., 2005). The probability of an untreated individual with LOPD needing a wheelchair increases by thirteen percent every year after the diagnosis (Hagemans et al., 2005). In patients whose symptoms presented during childhood, deformities and delays in motor development often occur (Barba-Romero et al., 2012). Respiratory dysfunction develops in more than 70% of LOPD patients. Vital capacity is reduced by an average of 1.5% every year after diagnosis (Vand der Beek et al., 2009). A large number of individuals with LOPD require assisted ventilation; the likelihood of

needing invasive or non-invasive ventilation increases by eight percent every year after diagnosis (Hagemans et al., 2005). Cardiac glycogen accumulation affects the ability of the heart to function properly. Some LOPD individuals have Wolff-Parkinson-White syndrome, spontaneous activity, or myopathic EMG patterns. Others show no cardiac symptoms (Muller-Felbert et al., 2007). There are a variety of other symptoms that can occur in LOPD.

The current treatment for Pompe Disease is Enzyme Replacement Therapy (ERT). Approved in both the United States and Europe, ERT involves injections of recombinant human GAA from rabbit milk (Van den Hout et al., 2000; Toscano and Schoser, 2013). This treatment is recommended every two weeks for the rest of the patient's life. Due to the amount of times treatment is needed and its high cost, ERT can cost a patient in the United States up to \$300,000 each year for the rest of their life (Brady, 2006).

In infants, ERT leads to increased survival and improvements in both skeletal and cardiac muscle (Klinge et al., 2005). It also delays the age at which assisted ventilation becomes necessary and markedly improves cardiac symptoms (Kishnani et al., 2009; Nicolino et al., 2009). Many patients are able to reach developmental and functional milestones such as walking and running that were never before possible (Kishnani et al., 2009; Spiridigliozzi, 2012). Patients with a complete lack of GAA activity may develop antibodies against ERT, with one study finding 20% of infantile onset patients developing these antibodies (Amalfitano et al., 2001). Additional therapies of immunomodulation allow these GAA immune responses to be eliminated and those patients to effectively be treated with ERT (Messinger et al., 2012). However, infantile

onset patients treated with long-term ERT do not live normal lives. They present with dysphagia and other speech disorders, increased likelihood of skeletal fractures, muscle deterioration, and hearing loss (Kamphoven et al., 2004; Case et al., 2007; Case et al., 2012; van Gelder et al., 2012).

ERT stabilizes the progression of Pompe Disease in late onset patients but does not provide a cure. Twenty-one studies with a total of 368 LOPD patients found that about one half of the patients were stabilized or had improved muscular and/or respiratory function after starting ERT (Toscano and Schoser, 2013). Of the 115 patients with impaired ambulatory status at the start of ERT, 6.1% improved with ERT. Of these, one was able to use a wheelchair after being bedridden, three were able to walk short distances after previously only being mobile in a wheelchair, one wheelchair patient became able to walking with assistance, and the last was able to walk without assistance after needing a wheelchair before treatment (Toscano and Schoser, 2013). Seven studies with 122 patients found that 77.9% improved, 8.2% stabilized, and 13.9% declined in the six minute walk test, another measure of muscular performance (Toscano and Schoser, 2013). Respiratory function was tested using forced volume vital capacity (FVC) and by tracking any assisted ventilation used. For 124 LOPD individuals on ERT, FVC improved in 51.6%, declined in 34.7%, and stabilized in 13.7%. Of 66 patients on ventilatory support before starting treatment, 59.1% improved, 36.4% stabilized, and 4.5% declined (Toscano and Schoser, 2013). There was no correlation between a longer duration of ERT and greater improvements in motor or respiratory function. This lack of correlation suggests that ERT does not improve the symptoms of LOPD but merely stabilizes the progression of the disease (Toscano and Schoser, 2013).

## **Gene Therapy**

Gene therapy is the insertion of nucleic acids such as DNA into cells in order to replace missing or defective genes and correct genetic disorders. It is a fairly recent type of therapy, with the first human gene therapy trial being performed in 1989 (Rosenberg et al., 1990). The death of Jesse Gelsinger in 1999 due to a reaction to the adenovirus-based vector that was supposed to be treating him caused a backlash against this method of treatment (Marshall, 1999). Subsequent lack of confidence in the efficacy of gene therapy limited research and trials. In 2004, China approved the first gene therapy for commercial production (Pearson et al., 2004). As of April 18, 2017, gene therapy is not approved for use in the United States except in research (American Society of Gene and Cell Therapy, 2017).

For this project, a mouse model for Pompe Disease was used. The mouse model is a homozygous knockout for GAA. This model produces tissues and cultured cells that are completely deficient in GAA (Bijvoet et al., 1998). It presents with lysosomal glycogen accumulation that progresses over the course of its lifetime. The progressive glycogen storage causes similar cellular pathology in the mice as in humans (Bijvoet et al., 1998). The mouse model presents with symptoms that represent both infantile onset and late onset Pompe Disease. Similar to humans with infantile onset Pompe Disease, the mice have virtually complete GAA deficiency without detectable mRNA synthesis, cardiac pathology, and glycogen accumulation in motor neurons (Bijvoet et al., 1998). However, clinical symptoms do not present before adulthood, and the mice survive much longer than humans with infantile onset Pompe Disease. Thus, this knockout mouse model is a good representative for both types of Pompe Disease (Bijvoet et al., 1998).

This project uses adeno-associated virus (AAV) to deliver the GAA gene. AAV viruses were discovered in 1965 by two groups simultaneously, one was Bob Atchison and the other was composed of M. David Hoggan and Wallace Rowe (Hastie and Samulski, 2015). AAV replication only occurs when the infected cells also contain adenoviruses. Even when it replicates, AAV does not cause any known pathology in mammals (Flotte, 2013). This non-enveloped virus has linear ssDNA genome with a length of 4,680 base pairs with 145 bases of IRT (Vector BioLabs, 2016). It attaches to cells via heparin sulphate proteoglycans and its internalization is aided by co-receptors on the cell surface (Vector BioLabs, 2016). When used for gene therapy, AAV allows for long-term expression with high yields of more than 100,000 particles per cell of the inserted gene (Hastie and Samulski, 2015; Vector BioLabs, 2016). This persistence, combined with the ability to infect a large variety of tissues makes AAV a popular choice for gene therapy (Pacak et al., 2008).

The specific AAV serotypes and promoter were chosen for this project with the target organs in mind. Target tissue in the mouse model was respiratory tissue, skeletal muscle, brain, and spinal cord. AAV9 is known to effectively transduce the central nervous system, heart, liver, lung, and skeletal muscle (Addgene, 2017). AAVB1, which was generated in 2016 by S. Choudhury, transduces the brain, muscle, beta cells, pulmonary alveoli, and retinal vasculature. AAVB1 has better transduction than AAV9 to the brain, spinal cord, muscle, pancreas, and lung (Choudhury, 2016). The Desmin promoter was chosen for its generic targets. This promoter works well for systemic transgene delivery, and specifically targets cardiac muscle, skeletal muscle, and neurons (Pacak et al., 2008).

## **Interests of the El Mallah Lab at UMMS**

The El Mallah Lab is part of the Gene Therapy Center at UMASS Medical School, and investigates using gene therapy to characterize and treat genetic disorders that affect the respiratory system. Other than this project on Pompe Disease, the El Mallah lab also works on potential treatments for amyotrophic lateral sclerosis (ALS) and very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD).

## **PROJECT PURPOSE**

This MQP was designed to assess the ability of two AAV viruses, AAV9 and AAVB1, to serve as potential vectors for delivering GAA to GAA<sup>-/-</sup> mice by measuring GAA levels in GAA-KO mice using IHC microscopy, and measuring functional recovery in GAA<sup>-/-</sup> mice by respiratory function, hang strength, and survival time.

## **METHODS**

### **Viral Design**

AAVB1 and AAV9 were obtained from Miguel Sena-Esteves, PhD, at the UMASS Gene Therapy Center. Plasmids encoding GAA under the control of a desmin promoter were designed and inserted into the AAV viruses at the University of Florida.

### **Mice**

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. B6;129-GaaTm1Rabn/J mice were acquired from Jackson Laboratory (Bar Harbor, ME). Littermate male knockout GAA<sup>-/-</sup> and wild type GAA<sup>+/+</sup> were used after genotyping with standard PCR methods to confirm genotype. The GAA<sup>-/-</sup> genotype was confirmed by 251 bp product, and the GAA<sup>+/+</sup> genotype was indicated by a 131 bp product.

### **Viral Infections**

Male knockout GAA<sup>-/-</sup> and wild type GAA<sup>+/+</sup> mice were injected with either PBS, or  $1 \times 10^{12}$  vector genomes of AAV9-GAA or AAVB1-GAA through the tail vein at 90 days of age. At the indicated time point, physiological tests were run on the mice. At



270 days of age, the additional respiratory tests were run on the mice and tissues were collected for histological studies.

### **Grip Strength**

Four limb grip strength tests were performed at 90 days, 120 days, 180 days, and 270 days of age to determine muscular function over time. The mice were individually placed on a grip strength meter and pulled back steadily by the tail to determine the amount of force that could be sustained with all four of the mouse's limbs. This test was repeated using only the mouse's front two limbs to determine the amount of force able to be sustained by the forelimbs.

### **Hang Test**

Mice were individually placed on an inverted screen. The amount of time that the mice were able to hold themselves up for with a maximum of 120 seconds was determined over two attempts. This test was repeated at 90 days, 120 days, 180 days, and 270 days of age to determine muscular function over time.

### **Airway Responsiveness**

Barometric plethysmography using whole body plethysmography (WBP) was used to test the airway responsiveness of the mice to increased respiratory demand. The chamber was calibrated with a two second injection of 20 mL air into the main chamber.

The pressure differences between the WBP containing the mouse and a reference chamber were measured and used to determine Peak Inspiratory Flow (PIF), Peak Expiratory Flow (PEF), Tidal Volume (TV), Minute Volume (MV), and Frequency (F). These measurements were determined over a ten minute period of normoxia (21% oxygen, 0% carbon dioxide) and a ten minute period of hypercapnia (21% oxygen, 7% carbon dioxide). Responsiveness was determined by calculating the percent change from normoxia to hypercapnia.

A FlexiVent<sup>TM</sup> piston system (SCIREQ, Montreal, Canada) was used to determine bronchial airway responsiveness of the mice at 270 days of age. The mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (90mg/kg, Animal Health International) and xylazine (4.5mg/kg, Propharma). They were then intubated and connected to the FlexiVent<sup>TM</sup> via the tracheal cannula. Mice were ventilated with room air at a frequency of 150 breaths/minute with a volume of 10 mL/kg. FlexiVent program was used to take measurements by analysis of pressure and volume signals. Baseline measurements were taken. Additional measurements were obtained after incremental doses (3.125, 6.25, 12.5, 25, 50, and 100 mg/mL) of aerosolized methacholine, receiving two deep lung inflations between each dose. Measurements included respiratory system resistance (Rrs), resistance of central airways (Rn), small airway and tissue resistance (G), respiratory system elastance (Ers), and small airway and tissue elastance (H) and were taken every 15 seconds with 12 measurements per dose of methacholine.

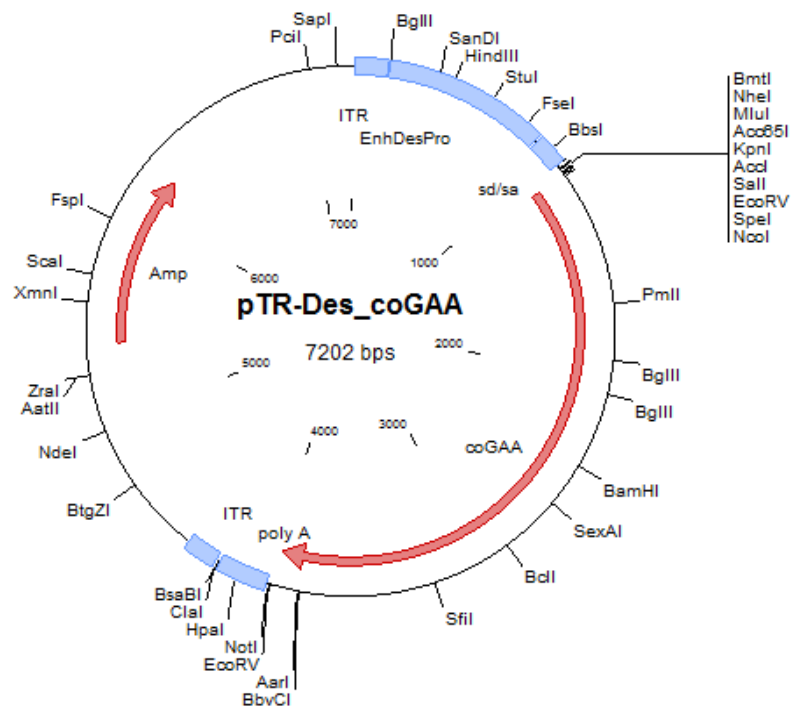
## **Immunohistochemistry of GAA**

Levels of GAA enzyme present in various tissues were assayed by Immunohistochemistry (IHC). Sections of the medulla, cervical spine, and thoracic spine were embedded in OCT. 40  $\mu\text{m}$  sections were cut. To determine presence of GAA, sections were stained using an antibody against GAA and visualized by staining with 3,3'-Diaminobenzidine (DAB).

# RESULTS

## Vector Design

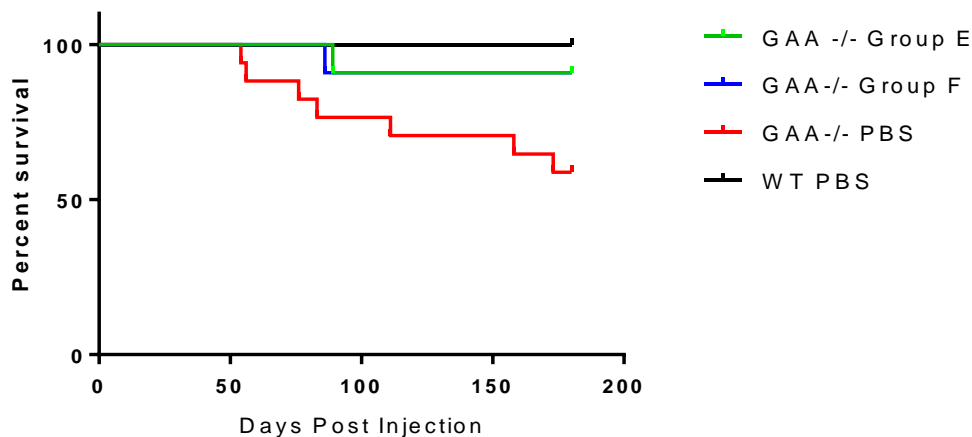
The plasmid vector encoding GAA was designed by the University of Florida (**Figure-1**). A desmin promoter (blue, upper right in the diagram) was used to control expression of the GAA gene from the plasmid (red, lower right in the figure). The plasmid vector was placed into AAV9 and AAVB1 serotypes and recombined with the viral DNA genome to create AAV9-GAA and AAVB1-GAA at the University of Florida.



**Figure 1: Map of the Plasmid Vector Inserted Into AAV.** Shown is the plasmid vector inserted into AAV9 and AAVB2. This vector was designed and inserted into the AAV viruses by the University of Florida. The GAA gene is denoted in red (lower right), and the desmin promoter controlling GAA expression is shown as blue (upper right).

## Survival

Male GAA<sup>+/+</sup> and GAA<sup>-/-</sup> mice were injected with either PBS or  $1 \times 10^{12}$  vector genomes of AAV9-GAA or AAVB1-GAA at 90 days old. There were 10 GAA<sup>+/+</sup> mice, 11 GAA<sup>-/-</sup> Group E (blind virus-1), 11 GAA<sup>-/-</sup> Group F (blind virus-2), and 17 GAA<sup>-/-</sup> PBS mice. The mice were harvested at 180 days. The survival of the mice up to the harvest point was tracked and plotted on a Kaplan-Meier graph (**Figure-2**). An analysis was performed and concluded that the GAA<sup>-/-</sup> PBS group was statistically different (lower survival) from the three other groups (p value of  $<0.05$ ), indicating that each viral treatment improved the survival of GAA<sup>-/-</sup> mice.

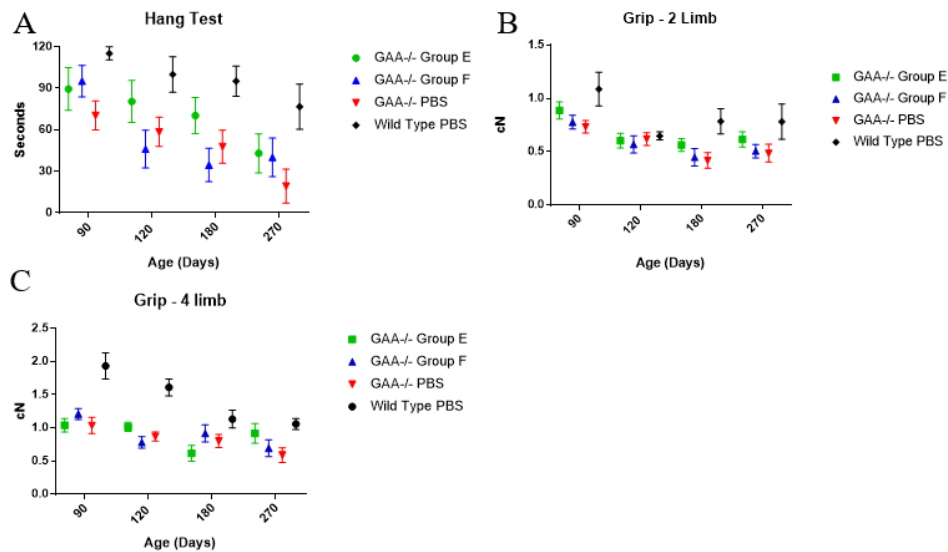


**Figure-2: Mouse Survival Curves.** Shown are the survival means of 4 treatment groups shown in the legend (N = 10-17). Groups E and F represent blinded viral treatments. The GAA<sup>-/-</sup> PBS group (red) is statistically different from all other groups (p value of  $<0.05$ ). Group E, Group F, and GAA<sup>+/+</sup> are not statistically different from each other.

## Musculoskeletal Function

An inverted hang test and two- and four-limb grip strength tests were performed on the mice at time of injection, and 30 days, 90 days, and 180 days post-injection (PI) to determine musculoskeletal function over time (**Figure-3**). Statistical significance was

determined using One-Way ANOVA and Multiple Comparisons Test with a p value of 0.05. The GAA+/+ PBS group was statistically different (stronger performance) from all the GAA-/- groups in the inverted hang test at all time points, the two-limb grip for at all times except 120 days, and the four-limb grip at 90 days and 120 days. The three GAA-/- groups were not statistically different from each other at any time point of the inverted hang, two-limb grip test, or four-limb grip test, suggesting that neither viral treatment measurably improved musculoskeletal function.

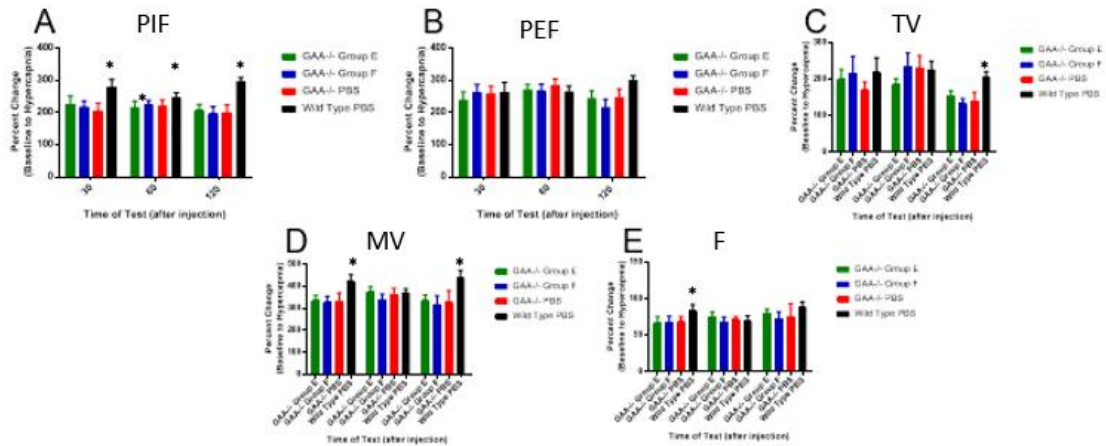


**Figure-3: Assay of Musculoskeletal Function.** Panel-A: Inverted hang test performed at 90, 120, 180, and 270 days of age. Injection point was 90 days. Panel-B: Two-limb grip strength test conducted at 90, 120, 180, and 270 days. Panel-C: Four-limb grip strength test performed at 90, 120, 180, and 270 days.

### Respiratory Function and Airway Responsiveness

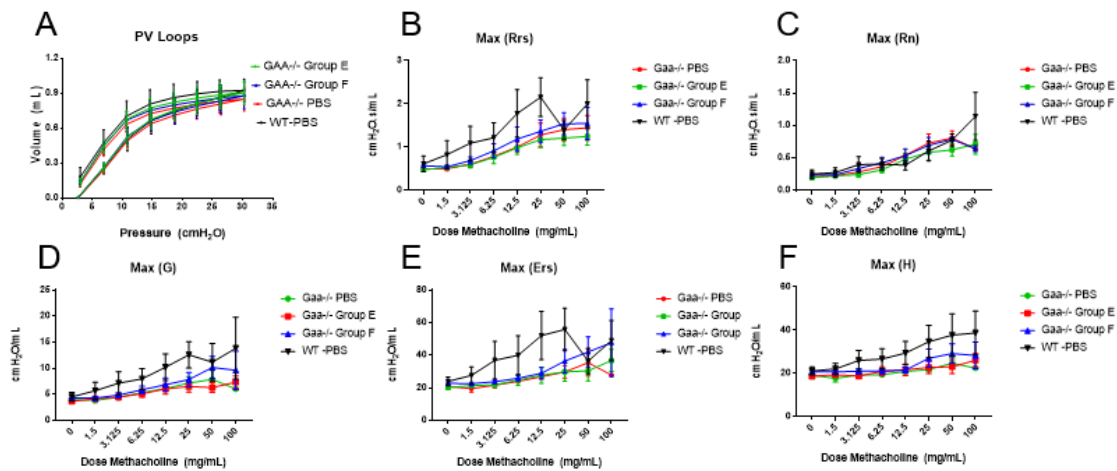
Respiratory function was determined at 120, 180, and 270 days using plethysmography, and airway responsiveness was determined at 270 days using FlexiVent™. Plethysmography was used to calculate the percent change from normoxia

to hypercapnia of PIF, PEF, TV, MV, and F (**Figure-4**). The results indicated that none of the viral treatments significantly altered respiratory function or airway responsiveness.



**Figure-4: Plethysmography.** Whole body plethysmography performed at 120, 180, and 270 days old. Percent of normoxia was calculated for each measurement. An asterisk shows a measurement statistically different from the GAA-/- PBS group ( $p=0.05$ ). A. Peak Inspiratory Flow (PIF). B. Peak Expiratory Flow (PEF). C. Tidal Volume (TV). D. Minute Volume (MV). E. Frequency (F).

To test airway resistance and elastance, FlexiVent<sup>TM</sup> was used on the mice at 270 days. Pressure volume (PV) loops were determined, then additional resistance and elastance of the respiratory system, central airway, and small airway and tissue were determined using increasing doses of nebulized methacholine (**Figure-5**).



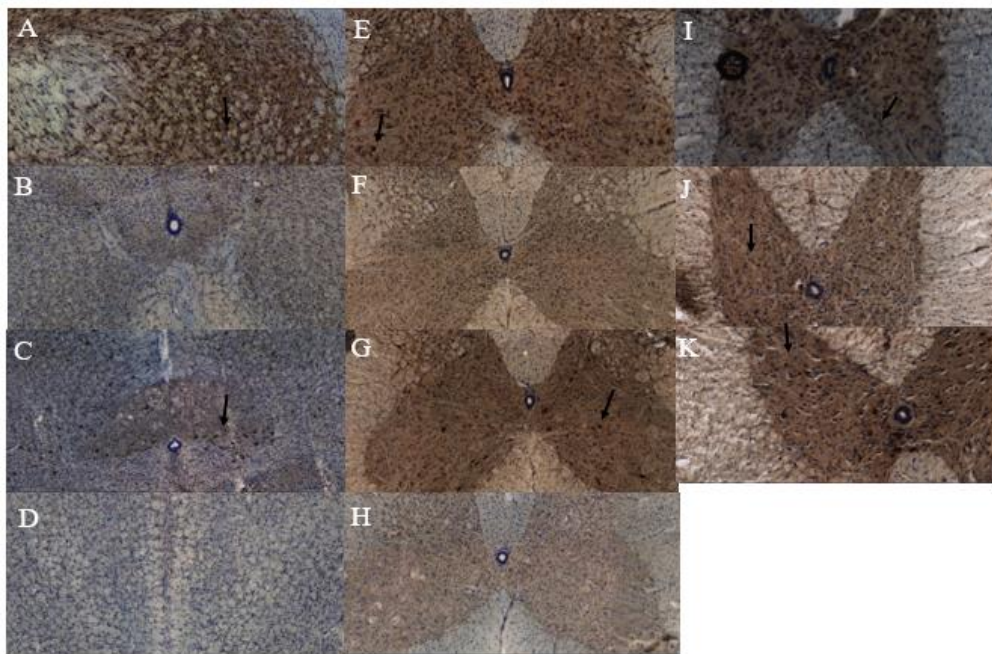
**Figure-5: Airway Responsiveness.** FlexiVent<sup>TM</sup> was used to determine PV loops, airway resistance, and airway elastance with increasing doses of nebulized methacholine. A. PV Loops. B. Respiratory system resistance (Rrs). C. Resistance of central airways (Rn). D. Small airway and tissue resistance (G). E. Respiratory system elastance (Ers). F. Small airway and tissue elastance (H).

The multiple comparisons test was used ( $p = 0.05$ ) to determine if differences between groups were statistically significant. The PV loops show no difference between any of the groups (Figure 5A). The GAA<sup>+/+</sup> group was different from all three GAA<sup>-/-</sup> groups at all doses except 0, 1.5, and 50 mg/mL methacholine for Rrs, and none of the GAA<sup>-/-</sup> groups were different from each other (Figure 5B). For Rn, the groups were all the same except for at 100 mg/mL methacholine, at which the GAA<sup>+/+</sup> group was significantly different (Figure 5C). Similarly, all the groups were the same for G except for the GAA<sup>+/+</sup> group at 25 and 100 mg/mL methacholine (Figure 5D). The GAA<sup>+/+</sup> group had a different Ers from the GAA<sup>-/-</sup> groups at 6.25, 12.5, and 15 mg/mL methacholine, and a different H at 50 and 100 mg/mL methacholin (Figures 5E and 5F). There was no significant difference between any of the GAA<sup>-/-</sup> groups for any of the airway resistance or elastance measurements.



## GAA Presence in Central Nervous System

The presence or lack of GAA in the motor neurons was determined by immunohistochemical staining (IHC). IHC was performed on 40  $\mu\text{m}$  sections of the medulla, cervical spine, and thoracic spine using an antibody against GAA and DAB to visualize. The sections were photographed under a microscope at 8X (**Figure-6**).



**Figure-6: IHC of GAA on Medulla, Cervical Spine, and Thoracic Spine Sections at 8X.** Shown are examples of motor neurons containing stained GAA. Black arrows denote positive areas of GAA staining. A. GAA+/+ PBS medulla. B. GAA-/- Group E medulla. C. GAA -/- Group F medulla. D. GAA-/- PBS medulla. E. GAA+/+ PBS cervical spine. F. GAA-/- Group E cervical spine. G. GAA-/- Group F cervical spine. H. GAA-/- PBS cervical spine. I. GAA+/+ PBS thoracic spine. J. GAA-/- Group E thoracic spine. K. GAA-/- Group F thoracic spine.

All sections of GAA+/+ PBS medulla, cervical spine, and thoracic spine had a large number of motor neurons containing GAA (Figure 6A, Figure 6E, and Figure 6I).

No GAA<sup>-/-</sup> PBS sections contained any motor neurons that had GAA (Figure 6D and Figure 6H). Group E (viral-1 treated) sections showed no GAA-positive motor neurons in the medulla or cervical spine, and a small amount of GAA-positive motor neurons in the thoracic spine (Figure 6B, Figure 6F, and Figure 6J). Group F (viral-2 treated) contained motor neurons with GAA staining in all three areas. There were fewer GAA-containing motor neurons in the medulla and cervical spine than in the thoracic spine (Figure 6C, Figure 6G, and Figure 6K). For all spinal sections, the GAA<sup>+/+</sup> PBS group had the greatest amount of motor neurons containing GAA.

## DISCUSSION

Both AAV9-GAA and AAVB1-GAA increased survival in GAA<sup>-/-</sup> mice. Neither group measurably improved musculoskeletal or respiratory function. Since the increase in survival was not due to improvements in either the musculoskeletal or respiratory systems' functions, this project was unable to determine the cause of increased survival. Both AAV9-GAA and AAVB1-GAA successfully delivered the GAA gene to the central nervous system, as AAV9-GAA induced GAA expression in motor neurons of the thoracic spine, and AAVB1-GAA caused GAA expression in motor neurons of the medulla, cervical spine, and thoracic spine.

The sample sizes for all four groups were fairly small. The GAA<sup>+/+</sup> PBS group had 10, the GAA<sup>-/-</sup> AAV9-GAA and GAA<sup>-/-</sup> AAVB1-GAA both had 11, and the GAA<sup>-/-</sup> PBS groups had 17. These small sample sizes could have skewed the results, especially for the respiratory function tests which had large amounts of variance.

The GAA<sup>-/-</sup> PBS mice that died before they reached end-point were not autopsied to determine cause of death. Knowing the cause of death could have led to insights of how AAV9-GAA and AAVB1-GAA increased survival.

The next steps for this project include determining GAA expression and glycogen accumulation for more tissues to determine where the two AAV vectors were able to induce GAA expression and thus lessen glycogen accumulation. It would be interesting if both vectors successfully induced GAA expression in the skeletal muscles and respiratory tissue but did not improve musculoskeletal or respiratory function.

For the future, it would be interesting to test a combined therapy of ERT and gene therapy, since ERT has been shown to stabilize musculoskeletal and respiratory function. By combining ERT with gene therapy, the glycogen accumulation in the CNS would be lessened and musculoskeletal and respiratory functions might be stabilized. This combined treatment would hopefully perform better than ERT alone.

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