Review Article

Pompe disease gene therapy: neural manifestations require consideration of CNS directed therapy

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Abstract: Pompe disease is a neuromuscular disease caused by a deficiency of the lysosomal enzyme acid alpha-glucosidase leading to lysosomal and cytoplasmic glycogen accumulation in neurons and striated muscle. In the decade since availability of first-generation enzyme replacement therapy (ERT) a better understanding of the clinical spectrum of disease has emerged. The most severe form of early onset disease is typically identified with symptoms in the first year of life, known as infantile-onset Pompe disease (IOPD). Infants are described at floppy babies with cardiac hypertrophy in the first few months of life. A milder form with late onset (LOPD) of symptoms is mostly free of cardiac involvement with slower rate of progression. Glycogen accumulation in the CNS and skeletal muscle is observed in both IOPD and LOPD. In both circumstances, multi-system disease (principally motoneuron and myopathy) leads to progressive weakness with associated respiratory and feeding difficulty. In IOPD the untreated natural history leads to cardiorespiratory failure and death in the first year of life. In the current era of ERT clinical outcomes are improved, yet, many patients have an incomplete response and a substantial unmet need remains. Since the neurological manifestations of the disease are not amenable to peripheral enzyme replacement, we set out to better understand the pathophysiology and potential for treatment of disease manifestations using adeno-associated virus (AAV)-mediated gene transfer, with the first clinical gene therapy studies initiated by our group in 2006. This review focuses on the preclinical studies and clinical study findings which are pertinent to the development of a comprehensive gene therapy strategy for both IOPD and LOPD. Given the advent of newborn screening, a significant focus of our recent work has been to establish the basis for repeat administration of AAV vectors to enhance neuromuscular therapeutic efficacy over the life span.

Keywords: Pompe disease; neuropathology; gene therapy

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Introduction

Gene replacement strategies are best suited to single gene defects where augmentation of expression or complete restoration of wild type activity levels is required to impact the clinical phenotype. Of course, even achieving the heterozygous level of gene expression should have a therapeutic effect in Pompe disease since carriers are entirely asymptomatic. Directing a gene therapy vector to the primarily affected systems can be influenced by route of delivery; however, with the advent of systemic vector delivery, tissue restricted expression is mostly achieved through promoter selection. Early treatment would have

the benefit of preventing secondary manifestation of the disease. In addition, when progressive cellular dysfunction leads to cell death, early intervention is warranted. In this review we present evidence of the extensive neuropathology in Pompe disease in association with the metabolic myopathy classically considered the primary pathology. The neuropathology observed in three animal models of Pompe and in human autopsy studies are a key justification for gene therapy with the overarching goal of direct cell autonomous correction in the brain and spinal cord. In non-clinical studies, we have demonstrated that pathophysiology is directly related to *GAA* gene function and restoration of GAA activity leads to reversal of glycogen accumulation and improved cellular function. Another important consideration in recessive conditions and Pompe in particular is the impact of immune response to the transgene when there is an effective null phenotype. Such cases require both tissue restricted expression and strategies to induce immune tolerance to the transgene. Lastly, as a lysosomal protein, GAA selectively traffics to the lysosome and achieving cell autonomous correction takes advantage of the highly efficient system of endo-lysosomal transport rather than relying on relatively inefficient binding of the therapeutic protein to the extracellular mannose-6-phosphate receptor for internalization.

Because of the cardiac and skeletal muscle involvement in Pompe disease, the condition is often considered a metabolic myopathy and a member of the general category of muscular dystrophy. Acid alpha-glucosidase is however required for degradation of lysosomal glycogen in all tissues and therefore manifestations are systemic. One explanation for the differential manifestations across tissue beds is that the disease severity in an organ system may be related as much to the cellular program for glycogen synthesis in each tissue as to the rate of glycogen degradation. This consideration is supported by cases where a second mutation leading to increased glycogen synthesis is especially severe when there is hemizygous expression of GAA [Austin *et al.* (1) and Shebab in preparation]. Currently over 10 years of enzyme replacement therapy (ERT) experience has been established in Pompe disease (2-4) and children and adults on ERT now demonstrate a modified natural history compared to untreated historical controls. The findings from longitudinal registry studies will be important to continue to understand the strengths and weakness of individual therapies that have achieved marketing approval (5,6). Many observational studies in the treated patient population are defining the benefits and key limitations of ERT, especially related to the neurological manifestation of the disease (7-11).

Our group has emphasized the importance of establishing meaningful preclinical models to establish a better understanding of the pathophysiology in Pompe disease and allow for the most impactful human clinical studies for gene therapy (12-17). One goal of this review is to present the basis for gene therapy in Pompe disease by consideration of the non-clinical proof of concept data predominantly derived from the Pompe mouse model (18). The null ($Gaa^{-/-}$) mouse has been a useful model and matched the biochemical phenotype of infantile onset Pompe disease (IOPD). However, the lack of early mortality in the $Gaa^{-/-}$ mouse has led us to develop new murine models as well as rat and canine Pompe models to better enable adequately planning for clinical studies in both early- and late-onset disease.

In vitro studies supporting biochemical correction and cross-correction

Pompe patient derived cells were used initially to test the concept that gene augmentation would lead to sufficient expression of human GAA and that the vector-derived protein would reach the lysosome (19-21). Cross-correction was also demonstrated in these studies using a trans-well system. The same studies established the basis for ERT. New cell culture systems have also emerged as a way to model Pompe disease by induced pluripotent stem cells (iPSC) or specific cell types derived from iPSC (22-26). Targeting the murine *GAA* allele has produced two strains of mice which vary in clinical phenotype (18,27). In addition, differing genetic backgrounds has helped to emphasize the importance of modifier genes on disease severity.

Beyond the mouse model, we have now developed and characterized a knockout rat model of Pompe disease. The rat was created using a zinc finger nuclease (ZFN) to create a ten base pair deletion in the *Gaa* gene resulting in a global GAA knockout (Falk, in preparation, 2019). In this rat model, the observed cardiomyopathy is more representative of the human disease. In fact, the average age of death is 7.3 months in female rats and 4.9 months in male rats (Falk, in preparation, 2019).

The final model we have generated to facilitate the non-clinical studies supporting Pompe disease gene therapy is the canine model. Seppälä *et al.* (28) characterized the genealogy of a naturally occurring nonsense codon mutation in exon

16 of the canine *Gaa* locus (c.2237G>A; W746*). Carrier females were generated by crossing a purebred Finnish Laphund carrier male with a female black Labrador. Natural history data of clinical cases confirmed the rapid onset of cardiomyopathy and early mortality in affected animals. Importantly, lumbar spinal cord histology of untreated pups indicates pathology and glycogen accumulation in motoneurons (*Figure 1*), suggesting this model is representative of Pompe disease in humans. Treatment with systemic gene transfer is underway to evaluate longevity of expression. Initial findings show that rapid progression of motoneuron pathology is not reversible with AAV-mediated gene transfer when therapy is initiated after 6 months of age in the dog, which corresponds to 5–10 years of age in human (personal communication Byrne). Conversely, early treatment and immune management promote normal muscle development and strength. Further studies to establish the optimal regimen for immune tolerance induction are in progress.

Cardiac gene therapy in Pompe disease

In the pivotal studies to evaluate Myozyme and subsequently Lumizyme, all early-onset patients with severe missense mutations or nonsense mutations had severe and progressive cardiomyopathy leading to early mortality (29-34). Cardiorespiratory failure at an average age of eight months of age is the principle cause of death in the natural history study done in preparation for the first ERT treatment study (1). Evidence of early effects on cardiomyopathy are likely the primary reason for improved survival (31,35) and therefore a gene therapy strategy in IOPD must consider and specifically target myocardium. Also notable is that those with the most severe mutations have an incomplete response to ERT and increase in LV mass is observed in the face of anti-drug antibodies (36). A gene therapy strategy can result in direct cell autonomous correction of myocardium in non-clinical studies therefore leading to reduction in LV mass index and improved cardiac function.

The *Gaa*$^{-/-}$ mouse model (18) has been critical in developing new therapeutic strategies and understanding the pathophysiology in Pompe disease. Importantly, the model has also been the principal test bed for progress in gene therapy. The model has features of both early and late onset disease and lives a full life-span since the cardiac manifestations are less severe than in humans with a null mutation. Structural and functional abnormalities in the heart are apparent in *Gaa*$^{-/-}$ mice by 6 months of age by examining LV mass index and the electrocardiogram (18,37-39). Difficulty in rearing and feeding lead to weight loss after 18 months of age and are sometimes the cause for euthanasia.

We have demonstrated that high-field cardiac magnetic resonance imaging (MRI) can be used to monitor for left ventricular hypertrophy and impaired ejection fraction in *Gaa*$^{-/-}$ mice (15,16,37,40,41). We and others have established the basis for AAV-mediated expression of hGAA in cardiac tissue and clearance of glycogen following systemic AAV dosing or via cross-correction from liver-mediated production of GAA (15-17,21,37,40-47) (Mc Call, 2019, submitted). Importantly, the exposure of AAV9 to the cardiac musculature is far more efficient than in skeletal muscle. Indeed, a single intravenous adeno-associated virus (AAV) vector injection results in extensive transduction and high-level hGAA expression. The high level expression leads to restoration of cardiac function at all stages of disease progression (37,40,44,48,49). Systemic administration of AAV1-CMV-hGAA results in ~70% reduction in cardiac lysosomal glycogen one-year post-dosing (37). Other outcomes include correction of the common electrophysiological abnormality and increased LV mass (15,37). Beyond the experience with AAV1, we found that the strong tropism of AAV9 for myocardium and the brain make this a suitable candidate for human studies. For example, the level of gene expression following systemic delivery of AAV9-CMV-LacZ resulted in a 200 fold increase in expression versus the identical vector DNA packaged into AAV1 (41). The systemic administration of a an rAAV2/9 GAA vector with a modified promoter to restrict expression to muscle and neural cells has been subsequently used in a number of mouse, rat and canine studies (40,44). Even in the setting of existing cardiac and skeletal muscle abnormalities, treatment of newborn or adult *Gaa*$^{-/-}$ mice can result in physiological and electrical correction of cardiac and skeletal muscles. Quantitative glycogen measurement, PAS staining, and electron microscopy are the most favorable methods to demonstrate clearance of glycogen and correction of sub-cellular morphology (40). Studies in rhesus macaques were also conducted to confirm the novel finding of significantly increased activity in heart (5× over baseline after 6 months) (41). Additional evaluation of the safety and efficacy of AAV-GAA in primates have been complete with support of the NHLBI Gene Therapy Resource program (50). These studies confirm the initial observations with systemic delivery of AAV-GAA and go on

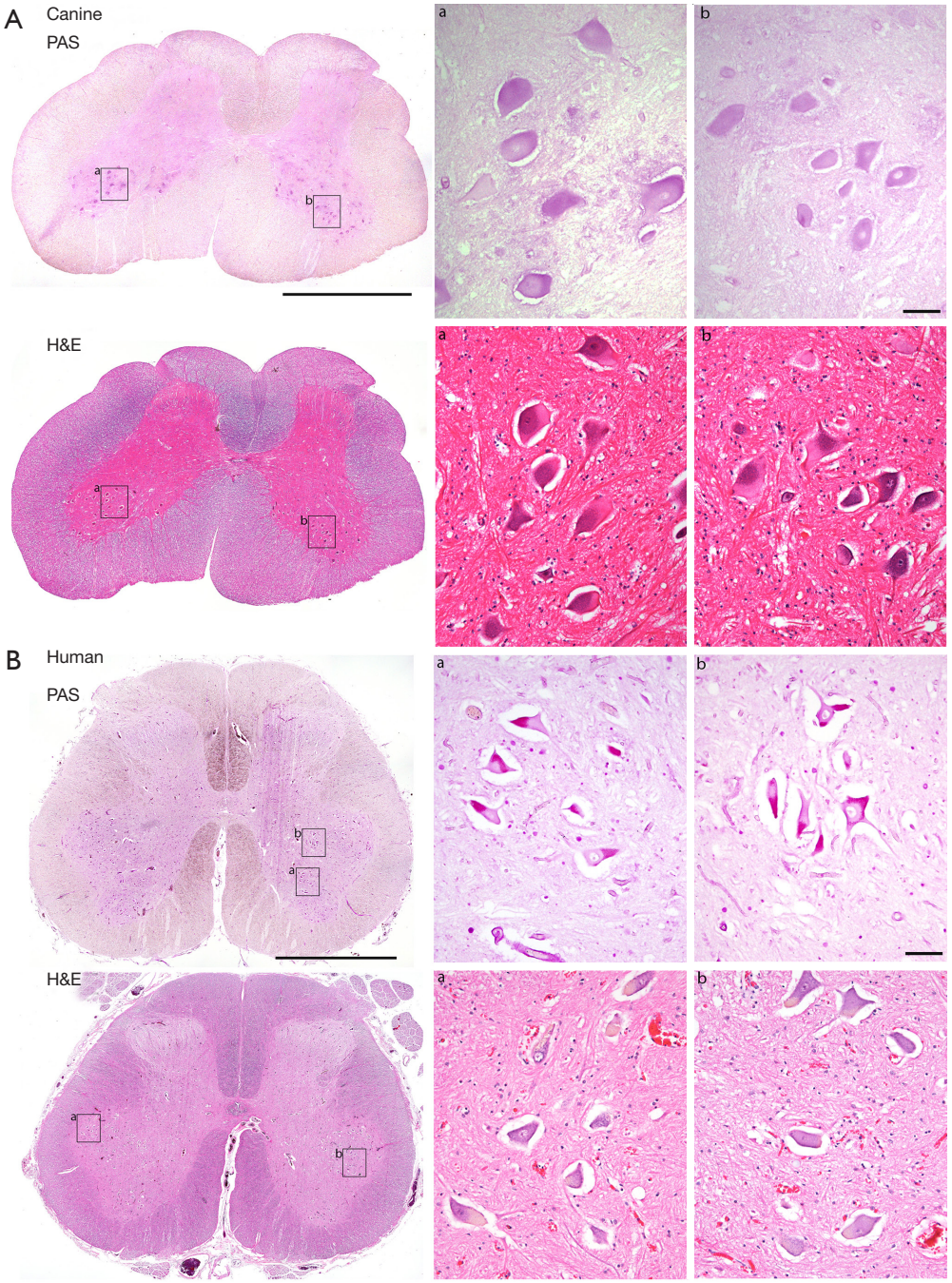

Figure 1 Lumbar spinal cord sections from an adult dog with early onset Pompe disease (A) and an adult human with late-onset Pompe disease (B). Tissues were paraffin embedded, sectioned at 7 µm, slide mounted and stained with Periodic acid-Schiff (PAS) to recognize glycogen (pink) or with hematoxylin and eosin (H&E). Panel A shows canine cord from an 8-month-old dos who was euthanized due to progression of neurological findings. Prominent PAS stating is evident in motor neurons throughout the ventral (anterior) horn. Many of these cells show the prototypical "ballooning" appearance associated with motor neuron histopathology in lysosomal storage diseases. The tissues in Panel B are from a 55-year-old patient with late-onset Pompe disease who succumbed to respiratory failure and neurological decline. Similar to the canine cord, PAS-positive staining is prominent in neurons throughout the ventral horn, including motor neurons. The scale bars indicate 2 mm on whole tissue sections and 50 µm on inset panels.

to establish the basis for immune protection versus the AAV capsid and transgene in Pompe gene therapy (51).

The set of studies described above demonstrate the efficacy of systemically delivered AAV-GAA and confirm extensive transduction in the heart and subsequent reduction of glycogen content with reversal of cardiac hypertrophy and dysfunction. Ongoing studies in the rat and canine models will determine the potential for benefit in the setting of severe cardiac hypertrophy as well as durability following systemic delivery.

AAV gene therapy for the muscle phenotype in Pompe disease

Since the initial description of the Pompe null mouse model (18) we have generated several derivative strains to better understand aspects of disease pathology. To study immune management, we generated a mouse line expressing human CD20 (52) by crossing human CD20 transgenic mice into the null GAA background. The resulting mice allow for testing anti-B cell strategies based on human CD20. Studies with this line have elucidated both the effect of prolonged administration of ERT (51,53,54) (a novel antigen in the KO mice) and for the administration of gene therapy vectors expressing the human gene (50,55). Additionally, mouse lines have been generated with deletion of floxed exon 2 (unpublished), ectopic expression of GAA (56) and a known human missense (54,57) mutation to evaluate the findings of gene transfer in the setting where the human vector derived protein is considered a self-protein.

We utilized isometric force-frequency relationships from diaphragm muscle and soleus muscle from the $Gaa^{-/-}$ line to demonstrate a progressive decline in contractile strength with age (58). The Pompe mice also show reduced ventilatory function as seen in juvenile and adult-onset patients (13). Given these baseline observations, we sought to show that systemic delivery of the AAV-GAA vector would have the maximal benefit in the context of gene therapy. It is important to emphasize that subcellular trafficking of lysosomal proteins has evolved a specific mechanism for retention of the lysosomal proteins to facilitate targeting to the lysosome. The strategy of cell autonomous correction should be highlighted as fundamentally different from liver-mediated cross correction (59). Specifically, the efficiency of cross-correction is influenced by the mannose-6-phosephate receptor density in the peripheral musculature and, therefore, is several logs less efficient than the intracellular trafficking of GAA since transit from trans-Golgi to the lysosome is the pathway which has evolved to capture bis-mannose-6-phosphate lysosomal proteins.

Mah *et al.* (37) showed that systemic delivery of rAAV2/1 to $Gaa^{-/-}$ neonatal mice led to correction of GAA enzymatic activity and glycogen clearance in striated muscle for up to one year. In addition, there was evidence of physiological correction by measurement of soleus and diaphragm force mechanics to 90% of wild-type peak diaphragm contractile strength and concomitant increase in ventilatory function. Adult $Gaa^{-/-}$ mice showed significantly reduced glycogen content in striated muscle and diaphragm at 4.5 months post-dosing with either rAAV2/8 or rAAV2/9 vectors where expression is restricted to striated muscle (44). Additional evidence of skeletal correction is derived from *ex vivo* force mechanics on excised $Gaa^{-/-}$ diaphragms following systemic rAAV2/9 GAA vector as well as X-ray analysis demonstrating a decrease in spinal scoliosis and kyphosis (40). The strong affinity of rAAV2/9 vectors for a variety of muscle fiber types as well as neurons and cardiac muscle is advantageous compared to other rAAV serotype vectors as a therapeutic vector for muscular dystrophies (41,44,48). Liver-directed delivery of rAAV2/5 and rAAV2/8 vectors using a cross-correction strategy can increase enzyme levels in the diaphragm and hind-limb muscles with concomitant reduction of glycogen content (17,42,59). Importantly, Puzzo *et al.* found 50% reduction in CNS glycogen content after one year of therapy (59) . The mechanism of glycogen reduction is presumably via hematopoietic cells that carry GAA across the blood-brain barrier, but it is unclear if this will be sufficient to provide clinical benefit in humans. To obtain a more direct effect, the same group has proposed an hybrid promoter which is active in muscle and the liver or CNS and the liver (60). Additional considerations for *in vivo* non-clinical studies include the age of the animal, species-specific specificity of the AAV receptor and the degree of expression of mannose-6-phosphate receptor in the affected tissues (46,47,49,58).

The only commercially approved therapy for Pompe disease is alglucosidase alfa, Lumizyme® (US) and Myozyme® (ex-US) which is a recombinant protein used for ERT. ERT improves ventilator-free survival rates in patients with infantile-onset disease, but longer-term follow-up showed a significant proportion showing progressive loss of ventilatory function (22 of the original 38 subjects now either require assisted ventilation or have died). All subjects have demonstrated functional deficits in respiratory function and some aspects of disease progression are not eliminated. Based on these findings we have intensified the effort to better

understand the incomplete response to ERT to identify additional therapeutic strategies using gene therapy.

AAV vectors can be delivered either systemically or directly in regional dosing for proof of concept. Direct diaphragm delivery was tested to focus on the reversibility of phrenic motor and diaphragm contractile function (43,61). Targeted administration of rAAV2/1-CMV-hGAA to the diaphragm of *Gaa*$^{-/-}$ mice resulted in near wild-type levels of diaphragm GAA activity and the clearance of accumulated glycogen, both in younger animals and 2-year-old mice with established disease. All treated mice had improved diaphragm contractile function and *in vivo* ventilatory capabilities, however disease progression impacts the potential for improvement and is influenced by the degree of neuronal loss with advanced age (62,63). It will be necessary to test these therapeutic strategies in human subjects at varying stages of disease severity to determine the degree of reversibility in either muscle pathology or neuronal cell dysfunction (58,61,64).

Evidence for CNS manifestations of Pompe disease amenable to gene therapy

The degree of weakness in Pompe disease patients has historically been attributed predominantly to muscle pathology (65). Yet, there is long-standing evidence that glycogen accumulates in the central nervous system (CNS) of Pompe patients (13,66-70) and in animal models of the disease (13,62,63,71,72). Neurological motor symptoms have been reported in case studies (11,70,73-76) and one report describes possible cognitive deficiencies (77). Studies in the *Gaa*$^{-/-}$ mouse demonstrate glycogen accumulation in phrenic motoneurons and diminished phrenic motor output leads to impaired ventilation (13,58,78-83). We have reported autopsy findings of cellular pathology in spinal motoneurons of a Pompe infant treated with Myozyme (13) and we have recently had the opportunity to evaluate CNS pathology in an adult (age 55) who directed that upon his death, an autopsy would be performed to evaluate CNS pathology (personal communication). *Figure 1* shows lumbar spinal cord sections from this individual who had been on long term ERT at the time of his death. The clear demonstration of motoneuron glycogen accumulation and histopathology are consistent with the observation that ERT does not effectively cross the blood-brain-barrier (39,84) and therefore cannot impact GAA deficiency in the CNS. It is possible that the variability in the success of enzyme replacement (85) could reflect persistent (untreated) CNS pathology. In fact, Muller *et al.* reported that children with Pompe disease remain at high risk for speech disorders despite ongoing ERT (86) and the same group has recently report neuroimaging findings in children with Pompe disease (87). *Figure 1* also presents lumbar spinal sections from the canine Pompe model, with similar evidence of motoneuron histopathology.

The principal focus of our group has been to establish AAV-mediated therapies which target both cardiac and skeletal muscle as well as the CNS (7,13,15,37,64,78-80,88-90). We have evaluated CNS targeting by direct administration, retrograde transport of vector, or after systemic or sub-arachnoid delivery (91,92). The first observation of retrograde transport was following administration of rAAV2/1 to the diaphragm of an adult *Gaa*$^{-/-}$ mouse leading to increased efferent phrenic nerve activity (58). Subsequent studies have shown robust transduction of hypoglossal motoneurons following intra-lingual delivery or leg motoneurons following tibialis treatment (78-81,83,93). Todd *et al.* observed that older animals treated with AAV-GAA can have restoration of muscle GAA activity and glycogen reduction but electrical stimulation of the peroneal nerve is unable to generate force at the ankle (81,83). In contrast, neuromuscular junction abnormalities can be restored with AAV-GAA in younger animals, and the contrast in therapeutic efficacy between young and old mice likely reflects loss of lower motoneurons over time. Consistent with that hypothesis, transcriptome analysis of the Gaa$^{-/-}$ mouse spinal cord indicated activation of molecular pathways associated with cell death, and neuronal apoptosis was confirmed with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (63).

Immunological considerations for Pompe disease gene therapy

The current standard of care IOPD is ERT with alglucosidase alfa (Lumizyme®). In LOPD there is an active debate about when to initiate therapy. For example, is preventative treatment in asymptomatic patients warranted? In the era of newborn screening, this debate will continue as more data on pre-symptomatic patients are collected (94). Other supportive data from imaging (87,94-97) and blood biomarker studies (98) will be important in establishing a basis for treatment and by what approach.

In IOPD with severe missense or nonsense mutations, ERT has resulted in high sustained anti-GAA antibody titers in most patients and in turn, there is a high rate of infusion

reactions (33,34,99). Similarly, *Gaa*$^{-/-}$ mice generate strong humoral immune responses to recombinant human GAA (53,54). Both IgG and IgE anti-drug antibodies have been observed, which likely causes the fatal anaphylactic reactions that occur in these animals after repeated ERT (100). While the acute response can be suppressed with anti-histamine drugs, the anti-drug antibodies are still able to bind the recombinant protein and cause aggregation which reduces efficacy.

It is possible to induce tolerance to GAA by pretreating with hepatic AAV-GAA gene transfer which prevents the predisposition to anaphylactic reactions (100). These observations are based on the pioneering work by Herzog *et al.*, where hepatocyte-derived expression induces transgene product-specific immune tolerance (101-103) in which regulatory T-cells actively suppress B and T cells (102,104,105). Additional approaches toward immune tolerance induction have been developed using immune suppressive drugs such as rapamycin in combination with B cell-depleting antibodies (36,50,101).

The anti-transgene response is influenced by the underlying *GAA* mutation. An additional consideration is the anti-capsid response in which the innate and adaptive immune system responds to the novel vector capsid protein thereby clearing the vector from the circulation and limiting efficacy. There is some evidence that AAV can induce a transient innate response accompanied by complement activation, TLR-9 signaling, and plasmacytoid dendritic cell activation which can be significant at the higher doses used in systemic therapy (106). Another important aspect of anti-capsid immunity is an environmental exposure, which would prime the individual to a secondary or amnestic response if exposed to a therapeutic vector of the same or related serotype (107). Important variables to be considered in regards to adaptive responses to AAV include the vector serotype, route of administration, dose, promoter and the targeted organ (42,49,103,108,109). We have also considered the use of liver-directed gene therapy (AAV2/8-LSP-hGAA) followed by ERT to reduce IgE mediated hypersensitivity (42,51,100). Strategies to transiently suppress the immune response have been used to circumvent innate and adaptive responses in gene therapy and ERT for Pompe disease and other inherited protein deficiency disorders (101,110). We have begun two studies to evaluate transient immune suppression in the context of AAV-medicated gene therapy and the initial results demonstrate the potential to increase efficacy and enhance safety (9,50,55).

Human clinical experience with AAV-GAA therapy

In the current era of an approved ERT for Pompe disease and the implementation of newborn screening across the US and Taiwan, there is a need for adjunctive therapy which will address the remaining unmet need in the current Pompe population and those identified by newborn screening. Therefore, a successful gene therapy strategy for Pompe disease should impact the disease manifestations which are defined by the current natural history of both treated and newly diagnosed patients. The first gene therapy in Pompe disease was an open label, Phase I/II study administering rAAV2/1-CMV-hGAA by direct intramuscular injection to the diaphragm of children (age 3–14) with ventilatory failure despite ERT (ClinicalTrials.gov Identifier: NCT00976352) (9,111,112). That study confirmed safety and indicated benefit to respiratory function in some patients (113). An ongoing trial assesses the ability to re-administer an AAV9 vector intramuscularly in patients with late-onset Pompe disease (ClinicalTrials.gov Identifier: NCT02240407). The study is a blinded crossover design to test safety and effectiveness of administration and re-administration of AAV9-DES-hGAA vector injected intramuscularly into the tibialis anterior (TA) muscle. In this study, the immune modulation strategy is to transiently ablate B-cells (Rituximab) and modulate T-cell response (Sirolimus) prior (and after) the initial exposure to AAV9 in one leg and the subsequent exposure of the same vector to the contralateral leg after four months. Preliminary results in 2 subjects show that immunomodulation successfully prevents antibody formation against both the AAV capsids and the transgene, and allows for repeated exposure to the vector. In addition, immunomodulation during AAV administration increases the efficacy and duration of the treatment (Corti oral communications). Based on preclinical and clinical results supporting the immunomodulation approach, a new clinical trial in children with Pompe disease will be initiated at the NIH Clinical Center, Bethesda, MD. The study is a Phase I–II trial of systemic delivery of rAAV9-DES-GAA in children with IOPD (3–5 years old) in conjunction with immunomodulation (Rituximab and sirolimus).

Conclusions

Currently, there is substantial evidence of neuropathology in Pompe disease, which influences the efficacy of treatment approaches based on ERT and liver-mediated gene therapy

strategies. Targeting of CNS and muscle using novel AAV serotypes will be critical for a successful therapy. Additionally, management of immune responses against the vector capsid and the transgene would be required to increase treatment safety and efficacy. The knowledge gained from many non-clinical studies and initial clinical trials is paving the way for a truly transformative therapy for the Pompe patient community.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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