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REVIEW

# Pompe Disease: From Basic Science to Therapy

Lara Kohler[1] · Rosa Puertollano[1] · Nina Raben[1]

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**Abstract**
Pompe disease is a rare and deadly muscle disorder. As a clinical entity, the disease has been known for over 75 years. While an optimist might be excited about the advances made during this time, a pessimist would note that we have yet to find a cure. However, both sides would agree that many findings in basic science—such as the Nobel prize-winning discoveries of glycogen metabolism, the lysosome, and autophagy—have become the foundation of our understanding of Pompe disease. The disease is a glycogen storage disorder, a lysosomal disorder, and an autophagic myopathy. In this review, we will discuss how these past discoveries have guided Pompe research and impacted recent therapeutic developments.

**Key Words** Glycogen storage · lysosome · autophagy · myopathy · enzyme replacement therapy · newborn screening

## Background and History

Pompe disease, a severe metabolic myopathy, is caused by mutations in the gene coding for acid alpha-glucosidase (GAA), the enzyme that breaks down glycogen in acidic milieu of the lysosome. Once in the lysosome, glycogen can escape following complete degradation by GAA in the form of glucose. A deficiency of the enzyme leads to lysosomal accumulation of glycogen in multiple tissues, but cardiac and skeletal muscles are most severely affected. The disease also goes by the name "Type II glycogen storage disease (GSDII)" or "Acid maltase deficiency." It is named after a Dutch pathologist, Johannes Cassianus Pompe, who described an autopsy of a 7-month-old girl diagnosed with "idiopathic myocardial hypertrophy" and generalized muscle weakness [1]. Dr. Pompe provided an insight into the underlying biology of the disease—massive vacuolar glycogen storage in virtually all tissues. The same year, 1932, similar cases were described [2, 3].

Decades later, basic science breakthroughs led to the discovery of the metabolic pathway of glycogen [4] and a new cellular organelle, the lysosome, a ubiquitous membrane-bound vesicle which contains hydrolytic enzymes and an acidic intraluminal pH [5]. The glycogen-degrading enzyme, acid alpha-glucosidase, that normally resides in the lysosome and is missing in Pompe disease was discovered by a Belgian biochemist Henri-Gery Hers in 1963 [6]. Furthermore, Dr. Hers predicted that "other deposition diseases might be explained on the basis of the absence of other lysosomal enzymes," thus triggering the search for enzymes responsible for the storage compounds in other lysosomal storage diseases (LSDs). Pompe disease has the distinction of being the first documented lysosomal storage disease; there are now more than 60 such disorders.

## Biosynthesis of Acid Alpha-Glucosidase and Genetic Defects

The enzyme is synthesized as a 110 kDa precursor, which undergoes extensive posttranslational modifications in the rough endoplasmic reticulum (ER) on the way to the lysosomes; the activity of the enzyme is increased during the process [7]. The glycosylation and proper folding in the ER are crucial for transport to the Golgi where the enzyme acquires a mannose 6-phosphate (M6P) lysosomal targeting signal. The phosphorylation through the addition of M6P groups is a prerequisite for binding of the enzyme to the mannose 6-

✉ Rosa Puertollano
puertolr@mail.nih.gov

✉ Nina Raben
rabenn@mail.nih.gov

[1] Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

phosphate receptor (M6PR). The M6PR-captured enzyme is contained in a vesicle that pinches off from the Golgi to endosomes, where the enzyme dissociates from the receptors. The enzyme is then delivered to the lysosome, whereas the receptors cycle back for the next round of sorting, with some diverging and ending up at the plasma membrane. On the way to the lysosome, the enzyme is proteolytically cleaved at both the amino- and carboxyl-termini, a process critical for catalytic activation of the enzyme [7–10].

Pompe disease presents as a continuum of clinical phenotypes that differ by age of onset, severity, and organ involvement. The clinical course is largely dependent on the specific mutation and the resulting level of residual GAA activity, although genetic background and modifying factors may play a role [11]. A potential role of angiotensin-converting enzyme polymorphism in modulating the clinical outcome was shown in a group of patients with the late-onset form of the disease [12]. Hundreds of mutations have been described in the *GAA* gene on chromosome 17 q25 [13–15]. A database containing all the reported mutations and polymorphisms and information about their severity can be found on the homepage of the Pompe Center at Erasmus University in Rotterdam: www.pompecenter.nl.

Most mutations are found in a single family or a small population, and most patients are compound heterozygotes. The mutations are spread throughout the gene and affect different steps involved in the complex process of generating fully functional GAA including protein synthesis, posttranslational modifications, and lysosomal trafficking and maturation. Several mutations are commonly found in patients of certain ethnic backgrounds. Among these, c.-32-13T>G (IVS1) is the most common defect in Caucasians; this leaky mutation allows for the generation of low levels of normal enzyme [16–18]. Most often, this mutation is found in compound heterozygotes in combination with a second far more severe *GAA* mutation. Individuals homozygous for the IVS1 mutation were not expected to show any symptoms, but this long-standing assumption turned out to be incorrect. A recent report described myalgia, exercise-induced fatigue, and an increase in creatine kinase (CK), a marker of muscle damage, in patients harboring two IVS1 mutations [19] (see also section "Newborn Screening").

c.del525, del exon18, and c.925G>A (p.Gly309Arg) are frequent mutations in the Netherlands, but are also found in other populations [20–23]. Chinese patients from Taiwan share a common c.1935C>A (p.Asp645Glu) mutation [24]. The fascinating story about the origin of the most common African-American mutation, c.2560C>T (p.Arg854Ter), brings us back to the population of north Central Africa and to the slave trade to the Americas [25].

Two sequence variants, c.1726G>A and c.2065G>A, are known to cause pseudodeficiency, a condition associated with low levels of GAA activity but not with clinical disease. The relatively high frequency of pseudodeficiency in Asian populations may increase false-positive results in newborn screening (see below) [26–28].

## Clinical Manifestations, Diagnostic Tools, and Differential Diagnosis

Pompe disease affects people of all ages with varying degrees of severity. The continuum of phenotypes creates some ambiguity when it comes to classifying different subtypes. Two broad types are recognized based on the onset of symptoms and the presence or absence of cardiomyopathy. The most severe form, referred to as classic infantile onset Pompe disease (IOPD), is characterized by the age of onset at ≤ 12 months, rapidly progressive hypertrophic cardiomyopathy, left ventricular outflow obstruction, hypotonia and muscle weakness, respiratory distress, and progressive loss of independent ventilation. Breathing difficulties, feeding problems, and macroglossia are common manifestations. Motor development is significantly delayed, and major developmental milestones, such as the ability to roll over, sit, or stand, are often not achieved. Only a small percentage of untreated patients survive beyond 1 year of age; the main cause of death is cardiac and respiratory failure [29, 30]. A subset of patients with similar clinical presentations during the first year of life but less severe cardiomyopathy (and absence of left ventricular outflow obstruction) is referred to as nonclassic IOPD [31]. If left untreated, severe muscle weakness leads to respiratory failure by early childhood. The term "atypical" IOPD is used by some clinicians to describe the patients who present within the first year of life without cardiomyopathy; however, the same terminology is often used to describe nonclassic IOPD. No doubt, this inconsistency will be resolved at some point.

Less devastating late-onset Pompe disease (LOPD) manifests any time after 12 months of age, usually without significant cardiac involvement. Late-onset patients commonly present with the symptoms of proximal limb-girdle myopathy. The progression of the symptoms is relatively slow but ultimately leads to profound muscle weakness and wasting, wheelchair dependency, and respiratory failure due to the involvement of the diaphragm. A history of "not being able to keep up with others" during physical activities may help clinical diagnosis in teenagers or adults. The introduction of enzyme replacement therapy (ERT) and a growing scientific interest brought more attention to the disease, and many additional symptoms came to light: dysarthria and dysphagia, osteoporosis, scoliosis, sleep apnea, small fiber neuropathy, hearing loss, impaired gastric function, urinary tract and anal sphincter involvement, and pain and fatigue, as well as a risk of cardiac arrhythmia and cerebral and intracranial aneurysms [32]. These findings emphasize the multisystem nature of Pompe disease.

### Diagnostic Tools

Generally, the serum CK activity is elevated in Pompe patients, but a normal CK value in LOPD does not exclude the diagnosis. Other enzymes, such as aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), are often elevated [29, 33]. Most Pompe disease patients have elevated urinary glucose tetrasaccharide ($Glc_4$) levels which are higher in infants than in adults. This test can be useful for supporting the diagnosis and for monitoring the effects of ERT. Chest X-rays reveal massive cardiomegaly in IOPD, and cardiac evaluation includes an electrocardiogram (ECG) and echocardiography (Echo). ECG shows a short P-R interval, tall QRS complexes, and increased QT dispersions; Echo reveals increased left ventricular wall thickness and mass with or without left ventricular outflow tract obstruction [34].

The pulmonary function in late-onset cases is evaluated by measuring maximum inspiratory pressure (MIP), maximum expiratory pressure (MEP), forced vital capacity (FVC), and vital capacity (VC). The vital capacity is measured in the upright and supine positions; the latter helps evaluate the degree of diaphragmatic deficiency [34, 35].

Magnetic resonance imaging (MRI) can be helpful for evaluating the extent and localization of muscle changes in patients with LOPD. Although the enzyme activity is deficient in all muscles, some muscle groups are relatively well preserved even during advanced stages of the disease. MRI can also help identify the site for a muscle biopsy.

By histology, muscle biopsies show vacuolar myopathy, the extent of which usually correlates with the severity of clinical symptoms. Vacuoles are diastase sensitive and positive for periodic acid-Schiff (PAS) and acid phosphatase, a combination which confirms the nature of the storage material and its lysosomal origin. The diagnostic value of muscle biopsies in adult patients is rather limited and rightly questioned because different muscle groups, and even fibers within the same muscle group, exhibit highly variable pathology [36]. However, since LOPD often presents the diagnostic challenge, muscle biopsy may be helpful. Of note, histological identification of acid phosphatase-positive lipofuscin inclusions was suggested as a new diagnostic marker, particularly in adult patients [37, 38].

Most importantly, the diagnosis is established by demonstration of deficiency of GAA enzymatic activity. The activity can be measured in blood, dried blood spots, cultured skin fibroblasts, or in a muscle biopsy. In classic IOPD, the enzyme activity is absent or almost absent (< 1%), whereas low levels of residual activity, up to approximately 30% of normal, are usually measurable in all other clinical forms [39, 40]. Nowadays, most academic and nonacademic institutions perform GAA mutation analysis not only to confirm the diagnosis but also to assess the genotype–phenotype correlation, to identify carriers within families, and to provide genetic counseling.

### Differential Diagnosis

A number of rare diseases presenting with cardiomyopathy, hypotonia, and myopathy in infancy should be considered. These include Werdnig–Hoffman disease, Danon disease, glycogenoses types III and IV, nemaline myopathy, myofibrillar myopathy, and mitochondrial myopathies. Importantly, newborn screening eliminates the need for differential diagnosis in IOPD: clinical findings plus a decreased enzyme activity are sufficient to confirm the diagnosis.

The diseases that may resemble LOPD include limb-girdle muscle dystrophy, Duchenne muscular dystrophy and Becker muscular dystrophy, facioscapulohumeral muscular dystrophy (FSHD), scapuloperoneal syndromes, rigid spine syndrome, myasthenia gravis, polymyositis, fibromyalgia, chronic fatigue syndrome, and glycogenoses types V and VI [32].

## Pathogenesis of Muscle Damage

The loss of muscle structure and muscle force have long been attributed to the progressive enlargement of glycogen-filled lysosomes in the intermyofibrillar space followed by lysosomal rupture, accumulation of cytoplasmic glycogen, and displacement of the myofibrils [41, 42]. In retrospect, this view seems overly simplistic and inadequate because it does not take into consideration any of the secondary events that may occur as a result of accumulation of unmetabolized substrates in the lysosomes. Recently, it became abundantly clear that a number of pathogenic mechanisms, such as autophagy, calcium homeostasis, oxidative stress, and mitochondrial abnormalities, all contribute to tissue damage in Pompe disease as well as in other LSDs.

Defective autophagy has emerged as a critical common feature of many LSDs [43]. Autophagy (the term literally means "self-eating") is a recycling system of lysosomal delivery and degradation of intracellular components. At least three autophagic pathways have been described based on the route by which the cargo enters the lysosome [44]. In microautophagy, a direct invagination of the lysosomal membrane brings the cargo into the lysosomal lumen [45]; in chaperone-mediated autophagy, molecular chaperones deliver a subset of cytosolic proteins to the lysosome through the lysosome-associated membrane protein type 2A (LAMP-2A) [46]; macroautophagy involves sequestration of various cytosolic constituents into newly formed double-membrane vesicles (autophagosomes) which fuse with and discharge their content into lysosomes for breakdown and recycling [47]. Macroautophagy (traditionally referred to as "autophagy") is thought to be the predominant form of

autophagy, and it is profoundly dysregulated in Pompe disease. Multiple methods and markers for studying autophagy are now available [48], but perhaps the most important is the protein, MAP1LC3, commonly referred to as LC3, which is a highly specific marker of autophagosomes [49].

The morphological evidence for abnormal autophagy in muscle biopsies from adult Pompe disease patients was first reported by Dr. Engel [50]. However, this pathology and its contribution to the pathogenesis of the disease have largely been ignored. Later on, in preclinical trials, poor skeletal muscle response to ERT was, at least in part, linked to the presence of large areas of autophagic accumulation (autophagic build-up) reminiscent of those described by Engel in adult Pompe patients [51]. The extent of this pathology became clear when single muscle fibers were immunostained for LAMP1 (a marker for lysosomes) and LC3; the core of the fibers contained large areas composed of numerous autophagosomes, clustered late endosomes and lysosomes with broken borders, and autofluorescent material, as well as other cellular debris of unknown origin. In addition, the area is filled with undigested autophagic substrates, such as p62/SQSTM1 and potentially toxic ubiquitinated protein aggregates [52, 53]. The presence of large pools of autophagic debris in skeletal muscle and the impact of this pathology on therapy warrant the classification of Pompe disease into a group of disorders known as autophagic myopathies [54]. Furthermore, it has been shown that the autophagic build-up affects the trafficking and delivery of the recombinant enzyme to the lysosome [55, 56]. Thus, in Pompe disease, a profoundly disordered intracellular recycling system appears to be an important contributor to muscle weakness and incomplete response to treatment.

Impaired autophagy is directly related to mitochondrial abnormalities since damaged mitochondria are removed through the autophagic pathway, a process known as mitophagy [57]. Indeed, mitochondrial alterations are observed in muscle biopsies in the majority of Pompe patients [42, 58]. A profound dysregulation of $Ca^{2+}$ homeostasis and multiple mitochondrial defects, such as a decrease in mitochondrial membrane potential, mitochondrial $Ca^{2+}$ overload, an increase in reactive oxygen species, and an increase in caspase-independent apoptosis, were reported in GAA knockout (KO) mice and in primary muscle cells from Pompe disease patients [59]. Accumulation of excess $Ca^{2+}$ in electron-dense globular bodies within the area of autophagic build-up was also reported on computerized tomographic (CT) images of severely affected muscles in children with Pompe disease [60]. These electron-dense globular bodies, lipofuscin inclusions, are commonly found in diseased muscle [38, 58]. Progressive deposition of lipofuscin in the lysosomes and autophagosomes further diminishes the degradative capacity of the lysosomes leading to a decrease in the autophagic turnover of damaged mitochondria, generation of reactive oxygen species, and formation of oxidized proteins and aggregates, thus perpetuating the "vicious circle."

Thus, large clusters of noncontractile material, such as glycogen-laden lysosomes, lakes of cytoplasmic glycogen, autophagic debris, and lipofuscin, interrupt the contractile machinery leading to muscle damage and decline of muscle function [61, 62]. Yet another layer in the pathogenic cascade is a dysregulation of the mTOR (mammalian target of rapamycin) signaling pathway in diseased cells. mTOR kinase is a potent anabolic regulator, and the lysosome serves as a platform for its activation [63]. Furthermore, this kinase exerts control over muscle mass [64]. The diminished mTOR activity and the failure to shuttle to and from the lysosomes in response to cellular stress were shown to contribute to muscle wasting in Pompe disease [65].

## Enzyme Replacement Therapy

### ERT in Infants

A breakthrough in treating LSDs came with the serendipitous discovery of the lysosomal enzyme secretion–reuptake pathway: Neufeld and colleagues demonstrated that cultured fibroblasts from patients with two different lysosomal storage disorders, Hunter and Hurler's disease, were able to correct each other [66, 67]. Subsequent studies showed that secreted lysosomal enzymes can enter the endocytic pathway and reach lysosomes *via* the cation-independent-M6P receptors (CI-MPR) on the cell surface [68]. This naturally occurring metabolic cross-correction suggested that LSDs may be amenable to therapy with exogenously administered functional enzymes, a concept which became known as enzyme replacement therapy.

The success of ERT for the non-neuropathic form of Gaucher disease made ERT an obvious approach to explore for other LSDs. Pompe disease, however, is the only lysosomal storage disorder in which muscle is the primary target. The first ERT clinical trial in infants was conducted by the Rotterdam group using recombinant human acid alpha-glucosidase (rhGAA) from transgenic rabbit milk [69, 70] followed by the Duke group using the enzyme produced and purified from CHO (Chinese hamster ovary) cells [71]. The production of the milk product was later discontinued, and all surviving patients were transitioned to CHO cell-derived enzyme. Several other trials including two pivotal company-sponsored multicenter, multinational, open-label studies of rhGAA safety and efficacy in infants younger than 6 months (18 pts; AGLU 1602) and infants and children between the ages 3 to 43 months with cardiac involvement and onset of symptoms during infancy (21 pts; AGLU 1702) led to the approval of the first specific treatment for Pompe disease. In 2006, human recombinant acid alpha-glucosidase (alglucosidase alfa; marketed as Lumizyme within the USA and as Myozyme outside of the USA; Sanofi Genzyme,

Cambridge, MA) received broad-label marketing approval in Europe and in the USA. ERT is currently the standard of care to treat Pompe disease, and it is the first instance of using recombinant enzyme to treat skeletal muscle.

The results of the clinical trial of 18 nonventilator-dependent infants (< 6 months of age) treated for 52 weeks clearly demonstrated that the therapy markedly improved cardiomyopathy and cardiac function, dramatically reduced the risk of death (by 99%) and the risk of invasive ventilation (by 92%) compared to the outcomes in an untreated historic cohort. However, significant gross motor milestones were achieved only in a subgroup of patients [72]. During a longer follow-up period of up to 3 years, the survival rate dropped to 67.5%, the number of patients who became ventilator dependent rose to 50%, and 40% of those who initially learned to walk continued to do so [73]. Similarly, the second trial of 21 patients showed that the drug significantly improved cardiac function, reduced the risk of death (by 79%), and reduced the need for invasive ventilation (by 58%) [74].

The outcome of 20 infantile patients treated in the UK from 2000 to 2009 was poorer than in the pivotal clinical trials: 35% died at a median age of 10 months and 30% were ventilator dependent [75]. Another retrospective analysis of 23 patients with classic infantile form treated for a period of at least 30 months in Germany between 2003 and 2010, again, yielded more sobering results: 60% of the patients died or became ventilator dependent; 30.5% made no motor progress and approximately half of the patients with a positive initial response deteriorated during the course of the disease [76].

Apart from the variability in the results of clinical trials, the studies outside the clinical trials, and multiple case reports on the effect of ERT in patients with classic infantile form, a number of common findings can be drawn. No question, the therapy has changed the natural course of the disease and has significantly extended the lifespan of infants; all patients had striking and sustained improvement in cardiac parameters with marked decreases in left ventricular mass index and left ventricular wall thickness, correction of abnormal ECG parameters, and improvement of cardiac function; many patients achieve major milestones of motor development. It is, however, equally clear that most long-term survivors still carry the burden of the disease. The emerging new phenotype includes gross motor weakness, hearing loss, ptosis, facial muscle weakness, speech difficulties, dysphagia that can lead to aspiration risk, arrhythmias, recurrent pneumonias, osteopenia, and orthopedic deformities [76–78].

These data suggest that infantile Pompe disease still remains a life-threatening condition. Many patients do not survive ventilator free beyond 3 years of age, and respiratory infections and invasive ventilation can be life threatening. A recent study reported improved outcomes in four patients receiving higher and more frequent dosing of the drug (40 mg/kg/week) instead of the currently recommended 20 mg/kg/every other week [79]. Nevertheless, even the most optimally treated infants tend to develop motor problems [28]. On top of that, brain magnetic resonance imaging (MRI) and neuropsychological tests revealed cerebral white matter abnormalities and different degrees of cognitive decline in long-term survivors; these are the results of a new prospective study of a group of ERT-treated patients with classic IOPD [80]. These data underscore yet another limitation of ERT—the inability of the recombinant enzyme to cross blood-brain barrier.

Another common thread that emerges from multiple studies is that the therapy is negatively affected by immune responses. Nearly all Pompe patients develop antibodies to the exogenous protein, but the impact of the immune response is particularly detrimental in classic infantile patients who do not produce any endogenous acid alpha-glucosidase. These patients, referred to as cross-reactive immunologic material negative (CRIM-negative), develop high antibody titers associated with clinical decline often leading to death despite ongoing therapy [81, 82]. In a retrospective study of the influence of CRIM status on outcomes in patients receiving the drug, all 21 CRIM-negative patients were deceased or invasively ventilated by age 27.1 months [83]. Several protocols have been introduced for tolerance induction in CRIM-negative patients [84, 85]. The most common is a combination of rituximab with methotrexate with or without intravenous gamma globulins. The addition of bortezomib (Velcade) to immunomodulatory regimens was shown to be an effective and safe treatment strategy in a group of infantile patients with an established immune response associated with clinical decline [86]. High antibody titers were also reported in CRIM-positive adult patients [87, 88].

The consensus is that the timing of ERT initiation is of critical importance for the outcome of therapy—the earlier the better. The start of therapy in IOPD before 6 months of age has long met the definition of "early." However, this paradigm was changed with the introduction of newborn screening program, and the current view is that the best time frame for the initiation of treatment is within the first days after birth [89]. Newborn screening (NBS), which marks a new era in Pompe field, will be discussed in more detail (see below).

## ERT in Children and Adults

The only randomized, double-blind, placebo-controlled phase III clinical trial of alglucosidase alfa for the treatment of LOPD was performed in children and adults (over 8 years of age). This multicenter, multinational study involved 90 patients, 60 of whom received the drug and 30 received placebo over the course of 78 weeks (late-onset treatment study/LOTS) [90]. All patients were ambulatory and free of invasive ventilation. Inclusion criteria were the ability to walk at least 40 m and FVC in upright position between 30 and 80% of normal. This trial also included a prior observational study

[91] and an extension study [92]. The alglucosidase alfa-treated patients showed improvements in walking distance [a mean increase of 28.1 m on the 6-minute walk test (6MWT)] and stabilization of respiratory function. The trial also revealed a great deal of variability in the response to treatment, and some patients continued to deteriorate despite therapy.

Since the introduction of ERT, a number of observational open-label studies and individual or small series case reports on the effect of therapy in LOPD have been published [93–96]. These studies provided evidence of a beneficial effect of ERT at a group level, but the response to therapy varied significantly among patients. In most patients, the treatment was associated with improved ambulation, some degree of modest motor function improvement, an increase in distance walked on the 6MWT, and a modest increase or stabilization of pulmonary function measured by FVC. During the follow-up period, many adult patients maintained a plateau after the initial improvement. The results of these studies are summarized in a review by Toscano and colleagues [97], which covers 21 studies and provides an overview of clinical data from 368 Pompe patients (2 years of age or older) treated for a period of time from less than a year to over 3 years.

A more recent literature review [98] added new reports including the large UK study of 62 patients [99] and accounted for patients overlap between the studies. The authors used a variety of analytic methods to adjust for repeat measures and different follow-up time. The updated review covers 438 LOPD patients, who were monitored for 3 to 48 months. The major conclusions of this study are as follows: the mortality rate is fivefold lower in treated compared to untreated patients (hazard ratio, 0.21; 95% credible interval (CrI): 0.11, 0.41]; at the group level, in treated patients, FVC improved rapidly within the first few months of treatment (an average increase of 1.4%) and then gradually returned to baseline, followed by a slight decline after 2 to 3 years, whereas in untreated patients, a continuous decline is observed. The improvement in 6MWT was most pronounced over the first 20 months of treatment and was sustained over time. ERT was also shown to reduce fatigue [100]. The positive effect of ERT on survival was first demonstrated in a prospective international observational study of 283 adult patients [hazard ratio, 0.41; 95% confidence interval (CI), 0.19 to 0.87] [101]; an estimated hazard ratio, as mentioned above, was even lower when the survival results were estimated across all selected studies [98].

Thus, the development of enzyme replacement therapy was unquestionably a major scientific and commercial achievement in the history of Pompe disease. The introduction of ERT dramatically changed the natural course of the disease in infants and resulted in much longer survival. The most reliable effect of ERT has been on cardiac pathology and function regardless of disease severity. In contrast, the skeletal muscle response is variable and less impressive despite a high dose of the drug compared to those in other lysosomal storage diseases.

## Experimental Therapies Designed to Enhance the Effect of ERT

Effective ERT depends on the M6P content of the recombinant enzyme and on the abundance of CI-MPR on the target tissue. M6P groups are critical for efficient uptake and lysosomal delivery of the recombinant enzyme. The limited effect of ERT in Pompe skeletal muscle has been mainly attributed to both low number of M6P groups on the rhGAA and the low expression of the receptor on the cell surface of muscle cells [102]. Several approaches designed to improve traditional ERT are currently under investigation.

One of these is aimed at enhancing the enzyme delivery by increasing the number of M6P residues on the recombinant enzyme. Neo-GAA (Sanofi Genzyme) is a second-generation alglucosidase alfa that has an increased affinity for the CI-MPR, and is in a phase 3 randomized, multicenter, multinational, double-blinded study (NCT02782741). In preclinical studies, this modified enzyme showed greater efficacy compared to alglucosidase alfa and reduced glycogen to similar levels at a much lower dose [103]. Another experimental drug, ATB200, is a novel rhGAA with a high content of M6P- and bis-M6P glycan (Amicus Therapeutics, Inc.). The effect of ATB200 was tested in KO mice, and it caused much improved glycogen clearance compared to the current drug (our unpublished data).

Since the CI-MPR also binds insulin-like growth factor II (IGFII) [104], glycosylation-independent lysosomal targeting (GILT) technology has been developed for the improved uptake of IGFII-tagged proteins *via* CI-MPR without the need for M6P residues [105]. A novel fusion protein between rhGAA and IGFII has been produced and successfully tested in Pompe mice [106]; however, the drug was withdrawn from phase 3 clinical trials due to safety concerns (NCT01924845).

An attractive experimental approach involves the grafting of a synthetic analogue of M6P onto rhGAA leading to a significant increase in the affinity of the recombinant enzyme to the M6PR without changes in the catalytic activity. This glyco-engineered enzyme greatly improved muscle pathology and function even in hard-to-treat old KO mice, whereas rhGAA was inactive [107]. Another glyco-engineered rhGAA with a high content of M6P glycan has been recently successfully tested in fibroblasts from Pompe patients [108].

Upregulation of CI-MPR receptor by the β2-agonist clenbuterol or albuterol was shown to increase efficacy of ERT in a mouse model [109, 110]. A pilot open-label study of adjunctive albuterol therapy in LOPD patients with no improvements on ERT (following initial stabilization) revealed safety and potential efficacy of this strategy [111].

An emerging strategy for the treatment of Pompe disease as well as other LSDs is chaperone therapy which relies on the ability of small molecule pharmacological chaperones to promote folding, stability, and lysosomal trafficking of chaperone-responsive mutant enzymes. In addition, the chaperones have been shown to have a stabilizing effect on the recombinant enzymes leading to their improved pharmacokinetics and pharmacodynamics [112]. Indeed, improved stability of alglucosidase alfa in blood was observed in Pompe disease patients receiving ERT in combination with iminosugar *N*-butyldeoxynojirimycin [113]. A similar approach has been used by combining the iminosugar miglustat (also known as AT2221) with ATB200. The effect of coadministered AT2221 on ATB200 is being investigated in a phase 2 clinical trial in LOPD (NCT02675465).

## Experimental Therapies in Preclinical Studies

*Substrate reduction therapy (SRT)*, by definition, offers a new approach designed to diminish or even prevent accumulation of glycogen. Inhibition of glycogenin or glycogen synthase, the two major enzymes involved in glycogen synthesis, reduced lysosomal glycogen accumulation and lysosomal size in GAA-deficient myoblasts. Inhibition of glycogen synthase *in vivo* in KO mice reversed cardiac abnormalities, reduced glycogen storage and autophagic build-up, and improved exercise capacity [114, 115].

*The genetic suppression of autophagy* in KO mice by selective inactivation of a critical autophagic gene in skeletal muscles resulted in a significant decrease in the amount of stored lysosomal glycogen, supporting the idea that the autophagic pathway is, at least partially, responsible for the delivery of glycogen to the lysosomes. Once the autophagic build-up was eliminated, ERT worked remarkably well as was shown by a nearly complete clearance of lysosomal glycogen [116].

*Stimulation of lysosomal exocytosis* is an exciting recently proposed approach to therapy for LSDs. This approach takes advantage of the intrinsic ability of lysosomes to undergo exocytosis, a calcium-dependent process of lysosomal docking to the plasma membrane, followed by fusion with the membrane and discharge of the lysosomal content outside the cell [117, 118]. Inducing lysosomal exocytosis became possible with the discovery of the function of the transcription factors, TFEB and TFE3, in regulating lysosomal and autophagosomal biogenesis [119–121]. Overexpression of TFEB or TFE3 in Pompe muscle cells induced lysosomal exocytosis and promoted glycogen clearance in KO mice, thus circumventing the major obstacle of the current therapy—inefficient enzyme delivery to skeletal muscle [55, 121]. However, systemic AAV-mediated delivery of TFEB to skeletal muscle of KO mice yielded somewhat disappointing results, although an improvement of muscle pathology and function was observed [122].

## Gene Therapy Strategies

A potential alternative to ERT is gene therapy. Gene therapy is the delivery of a functional copy of a gene, deemed the transgene, into a patient's tissue without replacing or removing the mutated copy of the gene harbored within the patient's own genome. Gene therapy is currently being developed for treatment of Pompe disease, as well as other genetic disorders, and relies on delivery of the transgene within a viral vector.

Initial studies using adeno- (Ad), adeno-associated viruses (AAV), and retroviruses demonstrated the feasibility of gene therapy for Pompe disease [123–126]. Systemic correction of muscle pathology in KO mice was achieved by hepatic targeting of a modified Ad-virus encoding human GAA [125]. This study was the first to demonstrate that liver can be a source of secreted GAA for cross-correction of skeletal muscle. Retroviral vectors, such as lentiviruses, have been successfully used *in vitro* in GAA-deficient cell lines and *in vivo* in KO mice [127–129]. However, concerns about the safety of retroviral vectors in clinical studies remain, as they can integrate into the genome at random sites and cause unintended mutations in or knockout of bystander genes. Additionally, long terminal repeats (LTR) at the ends of the viral genome can promote expression of nearby oncogenes present in the patient's own genome. AAV vectors are now preferred because they are nonpathogenic, can infect both replicating and nonreplicating cells, require a helper virus for infection, have low immunogenicity when compared to other vectors, and are available in multiple serotypes, with each serotype having a specific tissue tropism, allowing for more specific targeting of the desired tissue [130]. Although wild-type AAVs integrate into the genome, engineered recombinant AAVs (rAAVs) that lack the rep protein do not, thus mitigating some clinical safety risks. Thus, AAV vectors have become the accepted delivery mechanism for Pompe disease gene therapies under investigation in KO mice and in clinical trials.

One strategy for Pompe disease patients is to target muscles with direct injections of rAAV expressing GAA protein. This approach resulted in increased expression of GAA protein in KO mice [126, 131], but glycogen reduction was restricted to the injected muscle, without significant improvement in other muscles. Furthermore, recent studies have demonstrated that targeting muscle alone may not be enough to fully restore muscle function. Preclinical studies and autopsy reports on ERT-treated children confirmed the accumulation of glycogen in motoneurons [132–134]. Neurological deficits caused by the excess glycogen accumulation in the central nervous system (CNS) and peripheral nervous system contribute to muscle dysfunction. In a series of studies using spinal, intrathecal,

or intracerebroventricular delivery of AAV-GAA, neuromuscular improvement was observed, although muscle glycogen storage was not affected by the treatments [135–137]. Better outcomes were achieved by systemic delivery of AAV vectors of different serotypes. AAV serotypes have been identified in dozens of preclinical studies that improve efficacy and reduce immunogenicity. These studies demonstrated advantages of gene therapy compared to ERT, but the immune response remained an obstacle, particularly because high vector doses are required to achieve therapeutic efficiency.

In general, immune reactivity to the viral capsid and the transgene product has remained a major challenge to translating advances in AAV-mediated gene therapy to the clinic. Immune response may occur if a patient has previously been infected with AAV of the same serotype earlier in life or as a cause of previous ERT. Patients with pre-existing immunity to the wild-type AAV virus are less likely to benefit from AAV vector-based therapies. An immune response against the AAV-encoded transgene products may develop in the course of gene therapy. However, it is important to note that such reactions have been controlled during clinical trial using B-cell depletion by the drug rituximab to reduce reactivity to both the AAV capsid and to the GAA transgene [138]. The numerous studies on immune reactions during Pompe gene therapy have been reviewed elsewhere [139–141]. Here, we will focus on preclinical studies that have led to clinical trials.

Systemic and intradiaphragmal delivery of rAAV1-hGAA was shown to improve respiratory function in KO mice [142, 143]. Subsequent studies demonstrated the capacity of AAV for retrograde movement and transduction of phrenic motoneurons: intralingual delivery of AAV produced temporary correction of motoneuron pathology in KO mice [144]. Based on these preclinical studies, the first-in-human trial of diaphragmatic gene therapy (AAV1-CMV-*GAA*) was conducted in five children with IOPD who required assisted ventilation prior to the study. This trial has been recently completed [145–148]. The study demonstrated the safety of the AAV treatment, but the clinical outcome was minimal: no improvements in muscle function or dissemination of the *GAA* transgene were detected outside of the injected tissue. However, patients did exhibit an increase in tidal volume and the period of time that they could tolerate unassisted breathing. Additional clinical trial is planned (ClinicalTrials.gov ID: NCT02240407). A recombinant AAV vector carrying the codon-optimized acid alpha-glucosidase gene under control of a human desmin promoter will be used (rAAV2/9-DES-hGAA). Because desmin is highly expressed in muscle, this improves expression levels of the *GAA* transgene when compared to other AAV vectors for Pompe disease. Neural and cardiorespiratory function improved following systemic or intrapleural delivery of this vector in KO mice [149, 150]. The proposed clinical trial will evaluate the toxicology, biodistribution, and potential for readministration of rAAV9-DES-hGAA injected intramuscularly into the tibialis anterior muscle using an immune modulation strategy [151].

Another approach is liver-targeted gene therapy. To enhance systemic delivery and expression of GAA protein, investigators have harnessed the high metabolic capacity of the liver to produce and secrete the GAA protein. This strategy relies on infection with AAV8, which has a tropism for hepatic cells. Researchers at Duke University developed such a strategy to enhance and potentially replace ERT. Systemic injection of a modified AAV8 vector containing liver-specific promoter (AAV2/8-LSPhGAA) induced immune tolerance to rhGAA and improved the efficacy of ERT in KO mice when administered at a low dosage of viral particles [152]. The appeal of this approach, termed “immunomodulatory gene therapy” [152], is twofold: it induces immune tolerance to GAA by activating regulatory T cells and can provide a stable expression of *GAA* in liver, thus converting liver into a depot for continuous secretion of GAA and cross-correction in distant organs. Preclinical studies have demonstrated that the secreted GAA is taken up by cardiac and skeletal muscles leading to glycogen reduction and improved muscle function [141, 153]. A clinical trial which is scheduled to begin in the fall 2018 will explore the safety of liver-targeted gene therapy.

A more recent preclinical study sought to optimize the liver-directed strategy by testing genetically engineered *GAA* transgenes (that were codon optimized and contained small deletions within the progene and modified secretion signals) for their ability to be expressed by hepatocytes and produce the highly secretable GAA protein [154]. The authors showed in further *in vivo* experiments that these transgenes, delivered by liver-tropic rAAV, resulted in high levels of secreted GAA, low immunogenicity, and metabolic cross-correction in muscle, central nervous system, and spinal cord. Experiments in nonhuman primates demonstrated the safety of this approach and confirmed that the liver-secreted GAA is efficiently taken up by peripheral tissues [154].

In comparison to ERT, gene therapy could offer several benefits to Pompe patients. Gene therapy could be more convenient and cheaper because it would potentially require as few as one treatment for the entire lifespan of the patient. In contrast, the currently available ERT must be delivered bi-weekly *via* IV drip over the course of 6 to 7 h per treatment, causing discomfort and inconvenience to the patient. Importantly, gene therapy has the potential to be more effective than ERT, particularly if administered early during disease development. This increased efficacy includes the ability of certain AAV serotypes to cross the blood-brain barrier as has been demonstrated in the correction of neurological symptoms associated with mucopolysaccharidosis IIIB in mice [155].

While gene therapy for Pompe disease is promising, it is likely that a cure will eventually arrive with genome editing *via* the CRISPR/Cas system. Current versions of this system rely on delivery of the Cas9 protein and an RNA guide

sequence to target and edit mutations in the genome. The gene may be edited by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) [156]. NHEJ leads to random mutations in the targeted portion of the gene, with the intention that a stop codon will arise causing the protein to no longer be expressed. NHEJ may be similarly applied at splice sites to edit out mutated exons for treatment of other diseases that do not require expression of the full-length protein for functionality. Modification of a splice site using NHEJ has been done successfully to correct disease pathology in *mdx* mice, the animal model for Duchenne's muscular dystrophy [157–160] and in $dy^{2J}/dy^{2J}$ mice, the animal model for congenital muscular dystrophy type 1A [161].

However, CRISPR strategies using NHEJ would not correct the site-specific mutations found in the majority of Pompe cases, in which restoring a functional full-length GAA protein would be desired. Instead, site-specific corrections *via* HDR or other methods, such as base editors, would be necessary. HDR-mediated CRISPR strategies are currently somewhat inefficient in muscle cells because DNA repair proteins that are required for HDR strategies are not highly expressed [162]. However, advancements in the targeting and efficiency of genome editing in muscle would facilitate a cure for many other muscle-wasting diseases, representing a focused strategy for the potential cure of many diseases with a single approach.

## Newborn Screening

The first nationwide newborn screening program for Pompe disease was established in Taiwan over a decade ago [163]. The screening (conducted between 2005 and 2007) covered close to half of all the newborns in the country and measured GAA activity in dried blood spots (DBS). The number of diagnosed IOPD cases was similar to the number of infants diagnosed clinically among the unscreened control population. Although the classic infantile form of Pompe disease in theory does not present major diagnostic challenge, delays in clinical diagnosis are unavoidable. Indeed, NBS resulted in earlier diagnosis (less than 1 month of age) compared to 3 to 6 months in the control group. The studies that followed clearly demonstrated the long-term benefits of early diagnosis and early initiation of ERT in classic infantile disease [28, 164, 165]. Any delay negatively affects the treatment outcome and even a few days can make a difference as shown in a group of patients identified through NBS conducted in Taiwan between 2008 and 2012 [166]. High frequency of pseudodeficiency in the Taiwanese population (p.G576S; 14.5%) [27] complicates the screen and could increase false-positive results. Therefore, to promote early treatment for IOPD, Yang et al. developed a diagnostic protocol which included a combination of low GAA activity in DBS ($\leq 0.5$ μmol/L/h) with hypotonia, elevated CK ($\geq 250$ U/L), and high left ventricular mass index (LVMI $\geq 80$ g/m$^2$). The authors also argue that the benefits of early ERT for patients with highly suspected IOPD outweigh the low risk of adverse effects associated with the administration of the drug [166]. Thus, the importance of NBS for early diagnosis and treatment of IOPD is unquestionable.

However, screening will identify newborns with all forms of the disease, and most cases will be LOPD since this form is more prevalent [167, 168]. These cases require decisions regarding the frequency of monitoring, the methods of follow-up assessments, and the timing of initiation of life-long treatment of individuals with unpredictable age of onset, not to mention psychological harm associated with the diagnosis and uncertainty. Because of these challenges, it took a while to convince policy makers to add Pompe disease to newborn screening panels. Again, Taiwanese experience showed that the screening and the subsequent follow-up of patients with LOPD allowed identification of the earliest manifestations of the disease and an early start of therapy leading to better outcomes [169, 170].

It is important to remember that because of the range of clinical presentations and a variable age at onset of the symptoms, patients with LOPD can remain undiagnosed for years [171]. For those who have been through a diagnostic "odyssey," the treatment may come too late. A recent prospective study by Rairikar et al. underscores the point: the authors followed up and evaluated the phenotype of infants identified through NBS, whose genetic makeup predicted late-onset disease based on the presence of a common "mild" leaky splice site mutation in the gene (c.-32-13T>G). When properly evaluated, compound heterozygotes had elevated CK and other biochemical parameters and exhibited symptoms, such as swallowing difficulties, limb-girdle weakness, and delayed motor milestones as neonates. Even when the mutation was present in homozygosity, infants had subtle signs of Pompe disease [172].

The recommendation by the Advisory Committee on Heritable Disorders in Newborns and Children (ACHDNC) to add Pompe disease to RUSP (Recommended Uniform Screening Panel) was finally approved in March 2015 and implemented in several states in the USA: NY state, Illinois, Kentucky, Pennsylvania, Missouri, Ohio, Tennessee, and Washington state (pilot). Enzyme activity is measured in DBS using a fluorometric method [173], tandem mass spectrometry, or microfluidics combined with fluorometry [174]. The Pompe Disease Newborn Screening Working Group, an international group of experts in both NBS and Pompe disease, developed recommendations for confirmatory testing after positive NBS result and guidelines regarding monitoring and management of patients before and during ERT [40, 89]. One of the unexpected findings from these studies is a much higher prevalence of the disease than previously recognized. The estimate is 1:27,800 (University of Washington), 1:8657 (Missouri), and 1:15,133 (Illinois) for all forms of the disease.

## Conclusion

The development and introduction of enzyme replacement therapy for Pompe disease have changed the natural history of the disease, significantly extended the lifespan of patients, and improved morbidity. However, the results have not fully met expectations, and many patients continue to be burdened by the disease. The limitations of therapy have led to re-examination of the pathogenesis of muscle damage, stimulated efforts to enhance the efficacy of the current therapy, and to develop new approaches including gene therapy. The advent of newborn screening will allow for early diagnosis and initiation of therapy before irreversible changes have occurred. Finally, newborn screening revealed that this rare genetic disorder is not so rare after all.

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### Compliance with Ethical Standards

**Required Author Forms** Disclosure forms provided by the authors are available with the version of this article.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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