Molecular Approaches for the Treatment of Pompe Disease

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Abstract

Glycogen storage disease type II (GSDII, Pompe disease) is a rare metabolic disorder caused by a deficiency of acid alpha-glucosidase (GAA), an enzyme localized within lysosomes that is solely responsible for glycogen degradation in this compartment. The manifestations of GSDII are heterogeneous but are classified as early or late onset. The natural course of early-onset Pompe disease (EOPD) is severe and rapidly fatal if left untreated. Currently, one therapeutic approach, namely, enzyme replacement therapy, is available, but advances in molecular medicine approaches hold promise for even more effective therapeutic strategies. These approaches, which we review here, comprise splicing modification by antisense oligonucleotides, chaperone therapy, stop codon readthrough therapy, and the use of viral vectors to introduce wild-type genes. Considering the high rate at which innovations are translated from bench to bedside, it is reasonable to expect substantial improvements in the treatment of this illness in the foreseeable future.

Keywords GSDII · Pompe disease · Alpha-glucosidase (GAA) · Therapy · Gene therapy · Molecular therapy · Antisense oligonucleotides

Introduction

Pompe Disease

Glycogenosis type II (GSDII), or Pompe disease, is a rare autosomal recessive disease caused by a deficiency of the enzyme solely responsible for glycogen degradation within lysosomes: acid maltase or acid alpha-glucosidase (GAA). Over time, the progressive accumulation of glycogen alters cellular architecture, causing a loss of function and eventually necrosis. Although it has long been considered a disease that mainly affects striated muscular tissue with a disproportionate involvement of respiratory muscles, GSDII is multisystemic: glycogen accumulates in all tissues and organs, particularly in the skeletal muscle, central nervous system, heart and brain (the latter are almost exclusively affected by the early-onset form of the disease), causing not only a reduction in motor function and important respiratory deficits, the main cause of death in patients with Pompe disease, but also arrhythmias, dysphagia, incontinence, gastrointestinal symptoms, and several other problems [1–3].

Pompe disease has been documented in most ethnicities, with an incidence ranging from 1:14,000 (in African populations and African-Americans) [4] to 1:238,000 in Europe [5]. The advent of newborn screening (NBS) and the increasing availability of genetic testing for at-risk patients contribute to more precocious diagnosis and are uncovering the real incidence of the disease; for example, in Taiwan, the global incidence of GSDII based on NBS programs is estimated to be 1:17,000 [6].

Patients are divided into two main groups based on the age at disease onset: early-onset Pompe disease (EOPD) and late-onset Pompe disease (LOPD); however, presentation is extremely varied and correlates only partially with residual enzyme activity levels and the mutations carried by patients [7]. Early-onset Pompe disease (EOPD) is defined as GSDII arising before 12 months of age and more typically manifests during the first two months of life; patients present with severe muscular hypotonia and hypertrophic cardiomyopathy, which

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lead to exitus in the span of a few months [8, 9]. Late-onset Pompe disease (LOPD) is defined as GSDII arising after the first year of life, but it has been diagnosed in patients of all ages, with an age at diagnosis ranging from 27 to 41.1 years [10, 11], often due to significant diagnostic delay; however, NBS programs allow early diagnosis of these patients and potentially early treatment [6, 12]. LOPD patients present with heterogeneous clinical manifestations of different degrees of severity; cardiac involvement is generally absent, and the progression rate is variable [1, 3, 13].

Current Therapeutic Approaches

In addition to support therapy, which is based on artificial ventilation, physical therapy, and nutritional support [1], Pompe patients can receive one targeted treatment: enzyme replacement therapy (ERT). Approved by the FDA in 2006, ERT radically changed the prognosis of hundreds of patients [14]. Patients receive intravenous biweekly administration of 20 mg/kg recombinant human GAA enzyme (rhGAA), a 110-kDa precursor of GAA containing mannose-6-phosphate residues that allow it to bind cell surface receptors, ensuring uptake; the precursor is subsequently cleaved into the mature forms (76 kDa and 70 kDa) and reaches lysosomes through the same pathway followed by the endogenous enzyme [15].

ERT is both safe and effective [14, 16]. The only adverse reactions reported are not life-threatening and are usually controllable by reducing the infusion rate. These side effects are of little relevance when compared with the improvements experienced by treated patients: specifically, in EOPD, prolonged survival (usually more than 18 months of age and more often well into childhood); later introduction of invasive ventilation; and improvements in cardiac hypertrophy, growth, and motor and cognitive development [1, 14, 17]. The benefits of ERT are less pronounced in LOPD patients but are still relevant, as documented in several studies [16, 18]. Treatment ameliorates skeletal muscle pathology, partially reverses markers of muscular damage [16], increases strength, improves motor function, and preserves respiratory function. In 2017, Schoser et al. documented significant, albeit not persistent, effects in a series of 43 patients, reporting that forced vital capacity (FVC) improves after 2 months of treatment, returns to basal levels after 36 months, and then slightly decreases and that 6MWT improvement peaks at 20 months and is maintained over time [18]. Patients also report an improvement in quality of life.

Response to ERT varies depending on several factors, including disease severity and age at the start of therapy [15]; it is generally accepted that EOPD patients should be started on ERT as soon as possible [15], while a consensus on the optimal time to start treatment in LOPD patients has yet to be reached. Some evidence indicates that there is a better chance of improvement if the treatment is started when the patient is asymptomatic and the muscle tissue architecture is not excessively compromised, but this evidence is not strong enough to support treatment of asymptomatic patients [15, 19]. A European consensus released in 2017 states that only symptomatic patients whose diagnosis is confirmed should be treated but also suggests that asymptomatic patients who present abnormal muscle biopsies might benefit from early ERT [19].

Although life-changing, ERT is not ideal: it is a lifelong treatment requiring regular infusions at a high dosage, making it onerous from both a monetary (1.4 million euros per year of life gained) and a personal point of view [20]. Moreover, ERT is not very effective in treating muscle and is thus not an ideal approach for LOPD patients or older EOPD patients. Furthermore, the effectiveness of ERT varies from patient to patient and tissue to tissue, and enzyme delivery can be impaired by neutralizing anti-rhGAA IgG antibodies, the production of which is more common in CRIM-negative patients (that is, patients who are entirely unable to synthesize GAA and are therefore cross-reactive immunologic material negative) [14, 21]. Long-term follow-up studies have shown that as many as 50% of ERT-treated EOPD patients go on to develop respiratory failure and motor deficiency during childhood, ultimately requiring artificial ventilation [22]. Due to its size, rhGAA is unable to cross the blood-brain barrier (BBB) and is therefore ineffective in central nervous system pathology [21]. Even more accessible tissues do not improve homogenously: cardiac muscle responds better than skeletal muscle, and in mice (but not in humans), type 1 fibers show more improvement than type 2 fibers. As demonstrated by Lim and colleagues [15, 23], the contribution of autophagy defects to ERT resistance might explain this difference. Interestingly, EOPD patients treated with ERT develop an autophagic buildup over time that is similar to that of LOPD patients, possibly indicating that the drug induces only a partial regression of the pathological alterations [15].

Given the limits of ERT, researchers have continued to work to find better approaches to the treatment of GSDII, both by improving ERT and by researching avenues to restore GAA production in the affected tissue. ERT enhancement has been attempted by improving enzyme delivery and trafficking and by inhibiting the immune response. Attempts to restore GAA synthesis include chaperone therapy, stop codon readthrough therapy, antisense oligonucleotides, and gene therapy [15]. It has been speculated that in many lysosomal storage diseases, including Pompe disease, substrate accumulates only if enzyme activity falls below a certain threshold, above which the accumulation of the substrate will not become clinically relevant during an individual's lifespan [24]. If we accept this assumption, then we can conclude that a modest increase in enzyme activity would be sufficient to alleviate symptom severity and slow disease progression if implemented early [15, 25]. The applicability of this hypothesis to Pompe disease is supported both by population studies

and experimental work; the difference in enzyme activity levels between EOPD and LOPD patients is small, yet the phenotypes of the diseases are dramatically different; and individuals who carry pseudodeficiency alleles have enzyme activity levels that border pathological ones, yet they never manifest the disease [26, 27]. In 2017, Hordeaux and colleagues showed that 10% GAA activity (compared with normal) can ameliorate lysosomal pathology in neurons and glial cells in a mouse model [28].

ERT Evolution

As mentioned above, the effectiveness of ERTs varies in different tissues. While the reasons why are not entirely clear, some hypotheses have been made: (1) Different tissues may require different levels of GAA activity to prevent (or reverse) glycogen accumulation and autophagic buildup; this assumption is substantiated by the fact that cardiac muscle is involved only when GAA activity is null in EOPD patients. (2) Recombinant enzyme uptake is influenced by the expression levels of CIM6P/IGFII, the cell surface receptor that mediates the uptake of the recombinant enzyme and targets it to the lysosomal compartment.

Based on this second theory, researchers hypothesized that increasing mannose-6-phosphate (M6P) levels on the exogenous enzyme may facilitate tissue uptake. This hypothesis led to the creation of avaglucosidase alfa (neoGAA), a new ERT molecule that differs from its predecessor due to the higher quantity of bis-M6P on the molecule [29, 30]. NeoGAA has a 1000-fold higher binding affinity to M6P receptors and has proved more efficient in transducing muscle and reducing glycogen content in the murine model than its predecessor. The first results from experimentation in humans were published in March 2019 by Pena and colleagues [31]: LOPD patients, both previously treated with ERT and ERT-naïve, received a 24-week course of biweekly administrations of 5, then 10, then 20 mg/kg of neoGAA, which were well tolerated and safe, with only two reported SAEs considered to be drug-related. Although evaluating effectiveness was not in the scope of this clinical trial, patients seemed to have remained stable or even improved in various functional tests; no improvement was observed in biopsies, consistent with other data on LOPD patients. Currently, an ongoing phase III study [32] is underway to compare avalglucosidase efficacy with currently available ERT; the primary endpoint of this study is upright FVC, and the secondary endpoints are 6MWT, MIP, lower extremity muscle strength, motor function, and health-related QOL.

A further attempt to enhance ERT efficacy consisted of combining a glycoengineered M6P-enriched rhGAA (ATB200) with a chaperone molecule (AT2221, NB-DNJ, miglustat) to improve its enzymatic activity through stabilization. ATB200 alone was first tested in vitro and in vivo on a murine model, showing substantial superiority over rhGAA at equivalent doses, as seen by greater glycogen content reduction [33]. Subsequently, the ATB200/AT2221(AT-GAA) combination drug has also demonstrated a greater efficacy in a mouse model than rhGAA, being able to reduce glycogen accumulation and to abate autophagic buildup, as seen by the clear reduction in autophagic vacuoles and the partial reconstitution of the muscle architecture [34]. These results were mirrored by improved muscle function and opened the way to human clinical trials that have thus far yielded promising results. ATB200-02, a phase I/II trial, evaluated the safety of the ERT/chaperone regimen in LOPD adult patients, ERT-experienced or ERT-naïve, and detected a low number of adverse effects and only three events of infusion-associated reactions. Although not powered to assess efficacy, this study showed marked improvements in pulmonary function, motor function and patient-reported outcomes in both cohorts of patients [35–37]. At present, the PROPEL study [38] is in recruiting phase: it is a phase III double-blind, randomized, multicenter study designed to evaluate 12-month AT-GAA treatment in LOPD adult patients, ERT-naïve or not, aiming to assess its efficacy by 6MWT (primary endpoint) and motor function tests, muscle strength, pulmonary function tests, and patient-reported outcomes (secondary endpoints).

Another method to improve GAA delivery and uptake in different tissues considers the generation of chimeric proteins that use glycosylation-independent pathways to enter target cells. Notably, VAL-1221 is a chimeric protein obtained by the fusion of an rhGAA with the Fab fragment of a murine lupus anti-ds-DNA antibody (3E10). In addition to M6P-directed lysosomal targeting, the fusion protein reaches the cytoplasm by binding the nucleoside transporter ENT2, where it catabolizes extralysosomal glycogen. Owing to the scarcity of cytoplasmic glycogen in diseased mice compared with human patients, studies on murine models only assessed cell penetration and efficacy in lysosomal clearance, which was promising [39]. A phase I/II dose-escalation trial is currently ongoing to assess the safety and tolerability of VAL-1221 in adult LOPD patients; preliminary data reported no serious adverse events, three infusion-associated reactions and dose-dependent improvements in efficacy outcome parameters [40].

Emerging Therapeutic Approaches

Chaperone Therapy

Chaperone therapy relies on small enzyme inhibitors that, when administered at suboptimal concentrations, interact with the mutated protein, promoting proper folding and increasing the stability and transit of the protein through the Golgi to the lysosomes [25, 41]. Chaperone therapy offers certain

advantages: the drugs generally have high bioavailability, can cross the BBB and are easy to take as they are administered per os.

The effects of chaperone therapy have been studied in fibroblasts and HeK cells derived from EOPD and LOPD patients carrying different mutations [25]. Deoxynojirimycin (DNJ, AT2220, duvoglustat) is an alpha-glucosidase inhibitor that has yielded positive effects in a mouse model of another lysosomal storage disease, Fabry disease, and it has been proven effective in reinstating the correct trafficking, maturation, and activity of GAA in an enzyme-specific manner, in both the short and long term, in a murine model that carries certain mutations known to cause GSDII (such as L552P and G549R) but not others [25, 42]. A phase I study demonstrated the safety and tolerability of DNJ, but a subsequent phase II study conducted on adult Pompe patients was interrupted after two patients experienced severe adverse events, which were attributed to the overdosage of the drug [25]. Data collected from cellular studies, murine studies, and the phase II study indicate that the combination of pharmacological chaperones with ERT induces an increase in the enzyme level, improving the efficacy of ERT [25]. The effect of molecular chaperones on ERT transcends the previously observed mutation specificity and might be beneficial to all ERT-treated Pompe patients; furthermore, less frequent administrations could decrease the risk of adverse events.

A study conducted by Kishnani and colleagues on 25 LOPD patients to evaluate the effects of the administration of variable doses (from 50 to 600 mg) of duvoglustat in association with rhGAA confirmed the safety and tolerability of the drug and demonstrated its beneficial effects on ERT; all doses increased the AUC[1] of rhGAA, mainly due to half-life prolongation, and GAA plasma activity was doubled compared with that induced by ERT alone [43]. GAA muscle activity was likewise improved; at 3 days posttreatment, the active GAA level measured from biopsied muscle tissue was twice as high in the duvoglustat + ERT group than in the controls, although the difference decreased at 7 days posttreatment [43].

A similar molecule, N-butyldeoxynojirimycin (NB-DNJ, miglustat), shares some of the properties of DNJ [25]. D'Alonzo and colleagues have shown that its enantiomer I-NB-DNJ is capable of increasing GAA levels in the lysosomes of fibroblasts without inhibiting enzyme function (an improvement compared with the original molecule) when coadministered with rGAA [44].

Although their effectiveness as independently administered agents has not yet been established in Pompe disease, these molecular chaperones might increase the effectiveness of ERT (and possibly of other treatments as well); furthermore, the combination of several types of treatments might increase their safety and tolerability.

[1] Area under the curve

Stop Codon Readthrough Therapy

The recognition of premature termination codons (PTCs) generated by nonsense mutations by the ribosome triggers nonsense-mediated decay (NMD), an evolutionarily preserved mechanism of cellular surveillance and control [45, 46], resulting in mRNA degradation. Seldomly (in less than 1% of cases), a partially complementary tRNA binds the premature stop codon, causing the insertion of a random amino acid to allow translation to continue and producing a potentially stable, albeit abnormal, protein [47]. This mechanism, known as suppression of termination, or stop codon readthrough [46], can be enhanced and exploited by nonsense suppression therapy, a group of approaches aimed at suppressing translation termination by employing various substances [46]. The rate at which read-through occurs depends on the amount of mRNA available and on the type of stop codon arising from the mutation, with UAA being the least amenable, UGA the most amenable, and UAG an intermediate [47].

Aminoglycosides are a class of antibiotics that are effective against gram-negative bacteria and interfere with protein synthesis by reducing translation accuracy [48]; by reducing the discrimination between complementary and quasi-complementary tRNA, they allow the introduction of an amino acid instead of stopping translation and have therefore been employed as stop codon readthrough agents. Certain aminoglycosides, such as gentamycin, also downregulate NMD, thus increasing mRNA stability and transcript levels [49]. This approach has been explored to treat certain cystic fibrosis class 1 mutations, and it has been demonstrated that G418 is capable of suppressing a nonsense mutation of the CFTR gene and reinstating significant levels and the function of the protein it encodes in treated cells [50]. As of now, only one molecule, Ataluren (PTC124), a non-aminoglycoside used to treat Duchenne muscular dystrophy caused by nonsense mutations (nmDMD), has been approved [41, 51]. However, clinical studies investigating the possibility of applying this drug in cystic fibrosis caused by nonsense mutations have been stopped. Aminoglycoside derivatives have been tested in a cellular model of another glycogenosis (McArdle's disease). Birch and colleagues treated cells derived from four patients carrying the R50X mutant allele with G418 and observed the inhibition of NMD and read-through [49]. As of now, there is no evidence showing the potential effectiveness of this therapeutic approach in GSDII, although some causative mutations, such as c.2560C>T (p.Arg854x), are fairly common and are nonsense mutations [13, 52].

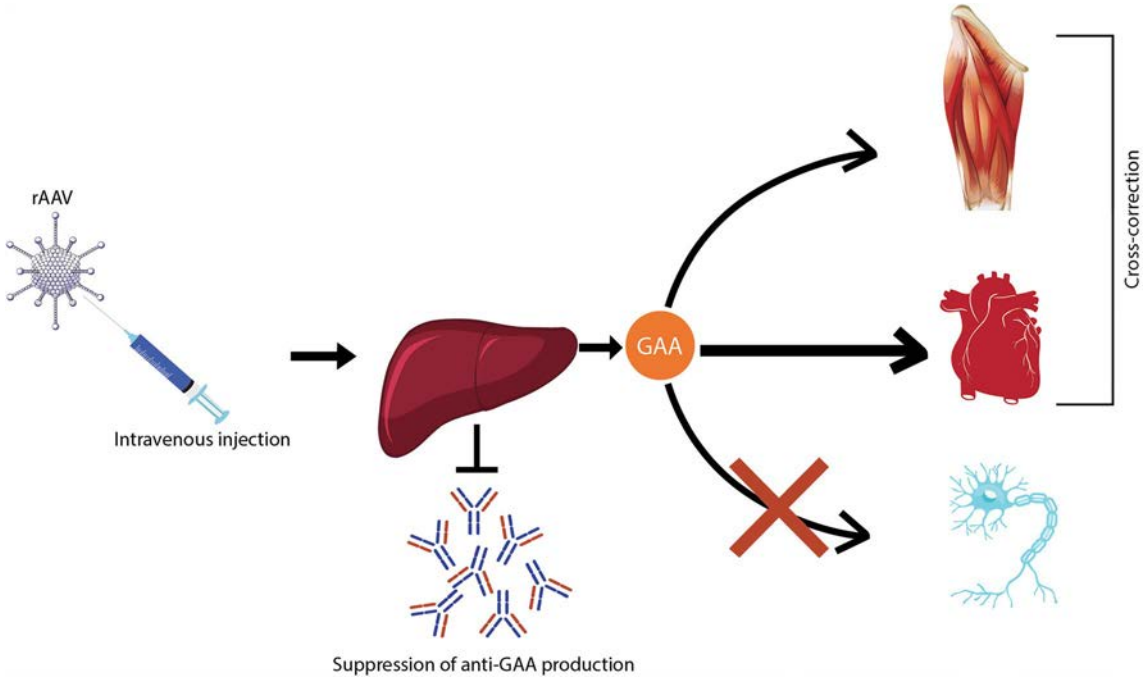

Fig. 1 Gene therapy for the treatment of Pompe disease. The therapeutic gene is inserted into the viral adeno-associated vector (AAV). The AAV can be administered through intravenous systemic routes targeting both skeletal muscles and the central nervous system

Gene Therapy

This type of experimental treatment consists of introducing genetic material to affected cells using several strategies, specifically, the substitution of mutated genes with nonmutated ones, the inactivation of a mutated gene that functions inappropriately, or the introduction of a new gene to contrast the effects of a certain pathology [53]. Genetic material can be introduced into cells using different vectors, often genetically engineered viruses such as retroviruses, adenoviruses, and adeno-associated viruses (AAVs). Pompe disease, which is caused by the mutation of a single gene, is a viable candidate for gene therapy [15] (Fig. 1). Since the pathological mutations are numerous—more than 500 have been documented [54][2]—and often private (meaning that nonrelated individuals usually carry different mutations), the most common approach has been to introduce a functional gene into affected cells. Gene delivery has been attempted using both lentiviral and adenovirus-associated vectors.

AAVs are appealing vectors for several reasons: they yield persistent gene expression without inducing an immune response in transduced cells, despite controversial evidence linking AAV infection to hepatocellular cancer [55]; they are not known to cause diseases in humans [56]; and AAVs are polytropic and can transduce the peripheral and central nervous systems by retrograde transduction [57, 58]. It is possible, though not yet confirmed, that the cellular stress observed in Pompe disease might positively influence the life cycle of AAVs, a characteristic that has been exploited in several cellular studies using cellular stress–inducing compounds such as arsenic trioxide [59]. Several AAV serotypes (AAV1, AAV2, AAV5, AAV7, AAV8, and AAV9) as well as several hybrid serotypes have been employed in an effort to improve transfection levels, and many administration routes have been explored, including systemic, intraperitoneal, intramuscular, intrathecal, and systemic targeted delivery (such as to the liver). Lentiviral vectors also seem promising, and their safety and efficacy have been improved through the development of self-inactivating vectors [60].

Various studies have been conducted on cellular and animal (mouse, quail, and nonhuman primate) models of the disease and even on a small number of patients, with encouraging results; various cell types derived from Pompe patients consistently express viral-delivered hGAA, and the enzyme localizes to lysosomes [61, 62] (Table 1). By introducing a functioning *GAA* gene to the cells, it is possible to increase GAA enzymatic activity in various tissues (typically skeletal muscle, heart, and liver) and to maintain higher GAA expression levels for months; glycogen content decreases as well, and the histology reverts to a more physiological phenotype [1, 56, 61–69]. It has also been shown that successfully transfected fibroblasts and myoblasts are able to synthesize and secrete the enzyme, allowing its uptake by neighboring cells via mannose-6-phosphate receptors [64]; this phenomenon, called cross-correction, has been documented in animal models as well. However, the humoral immune response influences the

[2] www.pompevariantdatabase.nl

Table 1 Results from various studies conducted

Study	Year	Model	Delivery	Vector and promoter	Endpoints
Elmallah MK, Falk DJ, Nayak S, et al. [58]	2014	*Gaa*$^{-/-}$ mice (treated at 8 weeks old, sacrificed at 4 months old)	Single intramuscular delivery (tongue)	ssAAV1 and ssAAV9 vectors hGAA driven by CMV promoter Vector dose: 1×10^{11} vg[a]/ml	(1) GAA activity and glycogen accumulation in tongue myofibers and XII motoneurons (2) Comparison of the effectiveness of AAV1 and AAV9 for motoneuron transduction via retrograde movement (3) Weight gain (4) Plethysmography[b]
Hordeaux J, Dubreil L, Robveille C, et al. [28]	2017	GAA-KO 6neo/6neo mice treated at 1 month old, assessed for 1 year	Single intrathecal administration (in the cisterna magna)	AAVrh10-CAG-hGAA and AAV9-CAG-hGAA Vector dose: 10^{11} vg (5×10^{12} vg/kg)	(1) rAAV distribution from the CSF to the blood (using GFP-marked vectors) (2) GAA expression, activity, and glycogen storage quantification in the CNS, heart, liver and skeletal muscle (3) Functional testing of neurological function, neuromuscular function, and cardiac function (4) CNS, muscular, and cardiac histopathology (5) Detection of GAA antibodies
Sun B, Young SP, Li P, et al. [74]	2008	GAA-KO mice	Single systemic delivery	AAV2/8-CK1-hGAApA 2 and AAV2/8-MHCK7hGAApA 1×10^{11} or 10×10^{12} vp[c]	(1) Transduction efficiency and vector distribution (2) GAA activity and glycogen content in the heart and skeletal muscle; Glc4 levels (3) Striated muscle function (at 18 weeks) (4) Lymphocytic infiltrates in skeletal muscle
Mah CS, Falk DJ, Germain SA, et al. [76]	2010	GAA-KO mice treated at 3, 9 and 21 months old	Gel-mediated delivery to the diaphragm	rAAV2/1-CMV-hGAA 1×10^{11} vg	(1) GAA enzyme activity (2) Diaphragm contractile strength (3) Ventilatory function (4) Phrenic nerve activity in one treated animal at 2 years of age (anecdotical)
Qiu K, Falk DJ, Reier PJ, Byrne BJ, Fuller DD [85].	2012	*Gaa*$^{-/-}$ adult mice evaluated at 1 or 4 months posttreatment	Gene therapy–AAV–spinal delivery (C3-C4)	AAV5-GAA or AAV5-GFP	(1) GAA expression, enzyme activity and glycogen accumulation in the cervical segment of the spine (2) Ventilatory function (plethysmography)
Conlon TJ, Mah CS, Pacak CA, et al. [92]	2016	Rhesus monkey	In utero administration at 50 days of gestation and evaluation at 3 months old (7 months post transfer)	rAAV1-CMV-hGAA $4{,}5 \times 10^{12}$ vp	(1) Transgene expression levels in striated muscle (diaphragm, heart, skeletal muscle), peritoneum, liver and cerebral hemispheres (2) Immune responses to AAV1 and GAA transgene
Keeler AM, Zieger M, Todeasa SH, et al. [94]	2018	Pompe mice (B6;129-GaaTm1Rabn/J) at 3 months old	Systemic injection	AAVB1 or AAV9	(1) Survival (2) Weight gain (3) Vector genome levels, GAA enzyme activity, glycogen content and histological analysis in the heart, diaphragm, skeletal muscle, and CNS (4) Respiratory function
Han S, Li S, Brooks ED, et al. [105]	2015	GAA-KO mice (6 months old)	Systemic injection Systemic injection on day − 1, + 6, and + 13	AAV2/9-CBhGAApA 1.0×10^{11} vp Anti-CD4 mAb (YTS177 50 mg/kg)	(1) Antibody quantification for anti-GAA IgG1, IgG2b and IgG2c (2) Survival

Table 1 (continued)

Study	Year	Model	Delivery	Vector and promoter	Endpoints
					(3) GAA activity and glycogen content in the heart, diaphragm, other skeletal muscle and liver
Puzzo F, Colella P, Biferi MG, et al. [114]	2017	GAA-KO mice (treated at 4 months old and followed for 3 or 10 months)	Systemic injection	AAV8-hAAT-coGAA, AV8-hAAT-sp2-8-coGAA, AAV8-hAAT-sp7-8-coGAA Vector doses: 2×10^{12} vg/kg 5×10^{11} vg/kg	(1) Circulating GAA activity in plasma (2) GAA activity and glycogen accumulation in the heart, diaphragm, skeletal muscle, and CNS (3) Skeletal muscle and CNS histology and autophagy impairment (p62 protein quantification) (4) Anti-GAA IgG1 quantification (5) Neuromuscular function (grip test, wire hang test) (6) Ventilatory function (plethysmography)
		NHP (*Macaca fascicularis*)	Systemic injection	AAV8-hAAT-sp7-8-coGAA 2×10^{12} vg/kg	(1) Circulating GAA activity in plasma (2) GAA activity and glycogen accumulation in the heart, diaphragm, skeletal muscle, and CNS
Han S, Ronzitti G, Arnson B, et al. [116]	2017	GAA-KO mice	Systemic injection (ERT and AAV)	AAV2/8-LSPhGAA 8×10^{11} vg/kg	(1) GAA enzyme activity and glycogen content in the heart, diaphragm, liver, and skeletal muscle (2) Antibody formation (anti-GAA IgG1)
Falk DJ, Soustek MS, Todd AG, et al. [117]	2015	*Gaa*$^{-/-}$ mice (3 months old)	Bimonthly systemic administration of ERT vs. single systemic administration of AAV9-DES	rAAV9-DES-hGAA-1 $\times 10^{11}$ vg ERT 20 mg/kg	(1) Weight gain and mortality (2) Cardiac function and morphology (3) Respiratory function (4) Anti-GAA antibody formation (5) Vector genome copy number, GAA activity and glycogen deposition in heart, diaphragm, and skeletal muscle
Smith BK, Collins SW, Conlon TJ, et al. [119]	2013	Clinical trial (Pompe disease affected children, 2-15 years old, full-time invasive ventilation treated with ERT)	Intradiaphragmatic delivery	rAAV1-CMV-hGAA	(1) Vector biodistribution (2) Immune response: anti-AAV1 and anti-hGAA antibody levels and T-cell-mediated response (3) Ventilatory function
Smith BK, Martin AD, Lawson LA, et al. [120]	2017	Clinical trial	Intradiaphragmatic delivery	rAAV1-CMV-hGA	(1) MIP (2) Respiratory responses to inspiratory threshold loads (3) Diaphragm activity (MRI, EMG) (4) Hours of daily mechanical ventilation

[a] Vector genome (vg)

[b] Points (3) and (4) are not declared endopoints, but are observations collected during the study and reported

[c] Vector particles (vp)

extent of correction attained by gene therapy, as it does that of ERT [70]. In this section, we report on the different administration routes that have been explored (systemic, intradiaphragmatic, spinal, intrathecal, and fetal); we also discuss the differences between AAV serotypes and gene promoters and describe how the immune reaction influences the effectiveness of gene therapy and the ways in which this obstacle has been approached. Finally, we compared gene therapy with ERT and detailed clinical trials involving AAVs. While the majority of studies have used AAV, we include a small paragraph regarding the use of lentiviral vectors in GSDII.

Systemic Delivery Systemic delivery of human *GAA* by AAV in murine models of Pompe disease results in robust transgene expression in the heart and diaphragm that persists over time [67] and yields significant improvements both in cardiac pathology and in respiratory and motor function [67, 71].

Studies conducted on newborn animals have consistently shown a reduction in glycogen heart content (down to 30% after 1 year in mice treated as newborns with the systemic administration of AAV1/2-CMV-hGAA [72]); the reversal of the electrical alterations observed in affected mice, specifically the lengthening of the shortened PR interval [73]; the reduction in cardiac mass to that of wild-type mice [67]; and functional restoration [72, 73]. Skeletal muscle pathology also benefits from treatment. As shown by Mah and colleagues, both soleus and diaphragm force mechanics were improved 1 year after the treatment of mice at birth, with diaphragm peak contractile strength reaching approximately 90% of that registered in wild-type mice and ventilatory capabilities exhibiting similar improvement [67]. In neonate rhesus macaques, treatment with a single injection of rAAV2/9-CMV-GAA resulted in a 4-fold increase over basal levels of GAA in heart tissue, and this increase was maintained for 6 months after treatment [73].

AAV transfection has been shown to be beneficial in adult mice as well. Sun and colleagues showed that 18 weeks after the systemic delivery of muscle-restricted rAAV2/7, rAAV2/8, or rAAV2/9 vectors in adult $Gaa^{-/-}$ mice, glycogen content was significantly reduced in striated muscle and the diaphragm, with rAAV2/9-MHCK7hGAApA being the most effective vector in terms of transduction efficiency and GAA enzyme activity levels [74]. The systemic delivery of rAAV2/9 in $Gaa^{-/-}$ mice increased the contractile force of the diaphragm (measured ex vivo in excised sections of the muscle) coupled with a reduction in spinal kyphosis [73]. However, early treatment seemingly yields the best results: $Gaa^{-/-}$ mice to which rAAV1/2-CMV-hGAA was delivered systemically as newborns showed a significant reduction in the amount of accumulated glycogen in the diaphragm and, more importantly, higher diaphragmatic contractile force compared with untreated animals of the same age and even younger animals. Respiratory function was likewise improved, with a more functional response to hypercapnia at both 6 and 12 months of age [67].

Intradiaphragmatic Delivery The systemic delivery of *GAA*-encoding AAV vectors, similar to ERT, yields the best results in cardiac tissue, and achieving high levels of *GAA* expression in the diaphragm has proven challenging [75]. To overcome this obstacle, researchers have developed diaphragm-targeted transfection methods, among which direct intradiaphragmatic rAAV administration has yielded the best results [72].

Mah and colleagues have developed an effective technique—a gel-based delivery method in which the virus is mixed with a vehicle, which is then applied directly on the surface of the diaphragm during open surgery. Gel-mediated delivery of rAAV2/1 to the diaphragm resulted in full-thickness muscle transduction, high levels of GAA expression (120% of normal levels), and glycogen clearance [75]. In 2010, the group showed that delivery of rAAV2/1-CMV-hGAA by this method significantly improved diaphragm contractile strength (peak force, 21.59 ± 1.59 vs. 13.94 ± 1.15 N/cm^2 for 1-year-old mice treated at 3 months vs. untreated) to near wild-type levels (24.43 ± 0.29 in 1-year-old mice). GAA activity increased regardless of age at treatment, but greater functional improvements were obtained with early treatment [76]; however, regardless of age at treatment, all animals showed improved responses to hypercapnic respiratory challenges [76]. The authors suggested that the blunted effectiveness of correction that accompanied the aging of the animals and that characterized the effects of treatment later in life might have occurred because, while transgene expression remained stable, not all fibers were corrected by transfection, and differences in the immune response were disregarded as an unlikely explanation for this phenomenon. Regardless, even the oldest cohort of animals showed significant biochemical as well as functional improvements, which suggests that both newborn patients and those with long-standing disease could benefit from this type of treatment [75, 76].

Approaches to CNS Pathology The presence of neural involvement in Pompe disease is supported by various lines of evidence; the autoptic examination of EOPD and LOPD patients, as well as animal models (mouse and Japanese quail), shows glycogen accumulation in the central and peripheral nervous system [77, 78]. The anterior horn of the spinal cord, especially the motoneurons, the motor nuclei of the brainstem, and the spinal ganglia are particularly affected [79–81]. In EOPD patients, glycogen storage diffusely affects sensory neurons of the brainstem and sensory neurons, motoneurons, and interneurons throughout the spinal cord [28].

In Pompe disease, neuropathology is involved in muscular dysfunction, particularly diaphragmatic, laryngeal, and pharyngeal dysfunction, thus contributing to ventilatory

deficiency [79]. The pathogenesis of respiratory insufficiency in Pompe disease is connected not only to the progressive weakening of respiratory muscle groups, particularly the diaphragm, but also to neuropathology as well as airway muscular dysfunction [3, 79, 81]. DeRuisseau and colleagues have shown in a murine model that spinal motoneuron pathology, and in particular phrenic motoneuron pathology, substantially contributes to deficits in the diaphragm [79]. In 2013, Fuller and colleagues confirmed the conclusions of DeRuisseau, showing that in $Gaa^{-/-}$ mice that selectively express GAA in the diaphragm (MTP mice), ventilatory deficits persist, although they are less severe compared with those of KO mice; the remaining defects were partially corrected by transducing the spinal cord with GAA using an AAV vector [79].

It has also been theorized that the respiratory control centers in the brainstem have different intrinsic activity in Pompe patients, explaining the lower respiratory frequency that correlates to lower levels of PaO_2 [81]. CNS lesions could explain the frequent persistence of bulbar muscular weakness. There are also some reports on the emergent neurological phenotype in some patients [82, 83]. The ineffectiveness of ERT for neuropathology makes the possibility of correcting defects in the central nervous system (CNS) an enticing therapeutic objective [81].

The delivery of therapeutic agents to the CNS has been approached by several routes, namely, spinal delivery, intrathecal delivery, and even intramuscular delivery, as AAV vectors can achieve retrograde transduction of the motoneurons innervating the treated muscles [57]. Some studies have even reported an association between glycogen reduction in the brain and the presence of vector DNA and RNA following the systematic delivery of AAV2/8-hGAA in $GAA^{-/-}$ mice [84].

In 2012, Qiu and colleagues evaluated the spinal delivery of AAV vectors, demonstrating their effectiveness in delivering the encoded gene as well as their therapeutic potential. Transducing the spinal cord with AAV5-GFP at C3-C4 yielded positive immunostaining throughout the cervical ventral horn, and GAA-AAV5 injection into $Gaa^{-/-}$ mice resulted in the restoration of GAA expression and activity, a reduction in glycogen accumulation (although it was not quantified), and ventilatory improvements; minute ventilation[3] was greater in the GAA-AAV5-treated group than in the mock treatment group at 1 through 4 months post injection [85].

Intrathecal Infusion of AAV In 2017, Hordeaux and colleagues published a study in which they evaluated the effects of a single intrathecal administration of AAV9 or AAVrh10 for the AAV-mediated gene transfer of human *GAA* in $Gaa^{-/-}$ mice [28]. The animals were treated at 1 month of age, when they were not yet symptomatic but presented well-developed neural lesions. In addition to the obvious fact that the less advanced the disease is, the easier it might be to reverse its consequences, studies have shown that the age of the animals at the time of the intrathecal delivery of AAV influences the transduction profile in terms of both distribution amplitude and cellular tropism [86]. In their study, Hordeaux and colleagues observed significant functional neurological correction beginning 4 months post treatment, neuromuscular improvements from 9 months, and cardiomyopathy correction at 12 months [28]. The brainstem, the spinal cord and the myocardium of the left ventricle exhibited higher enzymatic activity, reduced glycogen, and a reversal of histological abnormalities compared with those of other tissues. After 4 months, glycogen storage was entirely (AAV9) or mostly (AAVrh10) absent from the entire CNS of all treated animals; in particular, motoneurons demonstrated normal cell organization (AAV9 treatment) or at least partial reorganization (AAVrh10), glycogen storage correction was noted in glial cells 11 months after treatment, myelin composition was normalized, and AAV9 prompted a reduction in vacuolated cells located within the cervical and lumbar root ganglia. CNS function was preserved in the treated mice, nerve conduction within the brainstem was normalized, and motor coordination was improved [28]. The detection of the GAA protein in distal spinal segments, in which the vector genome was undetectable, suggested cross-correction [28].

In AAV9-treated mice, cardiac glycogen accumulation was corrected, and mature 76 kDa GAA was detected in the cardiac fibers. The vector genome was not recovered from hearts, suggesting that cardiac GAA was taken up from the circulation [28]. Global strength vastly improved in both treatment groups, nearing wild-type levels, and was correlated with motor coordination improvement. This finding, and the fact that glycogen accumulation, vacuolization, and the atrophy of muscle fibers persisted in the treated mice, indicates that the correction of CNS pathology was directly responsible for muscle function restoration, which could not be explained by the cross-correction of muscular pathology by circulating GAA [28]. This experiment complements a 2009 study conducted by DeRuisseau and colleagues that showed that the MTP mouse model, a $Gaa^{-/-}$ mouse variant that expresses GAA only in skeletal muscle, exhibits improved ventilatory function compared with that of the $Gaa^{-/-}$ mouse model but is not equal to that of the wild-type mouse [81]. This finding shows that neuropathology has a role in the pathogenesis of respiratory insufficiency in Pompe disease [79]. While promising, the approaches proposed by Hordeaux and DeRuisseau only partially corrected the causes of ventilatory dysfunction; instead, an ideal therapeutic approach would target both the neural and muscular components.

[3] Minute ventilation, or respiratory minute volume, is defined as the volume of gas inhaled or exhaled from one's lungs in a minute. This parameter is closely related to blood carbon dioxide levels.

However, studies showing a reduction in glycogen content with muscle strength preservation attained by inducing high levels of GAA expression and secretion in the liver without CNS correction suggest that neural transduction is not essential to strength improvement [56, 74, 87]. This point is debatable, as other groups, such as Byrne and colleagues, have proposed that therapies targeting both skeletal muscle and the CNS are necessary to obtain a complete reversal of pathology [72].

Correction of Motoneuron Histopathology via Intramuscular Delivery of GAA The intramuscular delivery of AAV-GAA can be beneficial to motoneuron pathology; these vectors are in fact able to spread from the point of injection along axons until they reach neuron bodies. AAV9 vectors seem to be particularly efficient in doing so [57], as demonstrated by ElMallah and colleagues. In 2014, they showed that a single intralingual injection of single-stranded AAV-GAA (1×10^{11} v.g.) yielded persistent GAA expression and a marked glycogen content reduction in tongue myofibers and in the innervating motoneurons. Furthermore, characteristic muscle and nervous tissue histopathological features were reversed, both by reversing already existing glycogen accumulation and preventing new accumulation [58]; however, treatment did not influence overall ventilation or breathing patterns. The authors used two different vectors: AAV9 and AAV1. The former seemed superior to AAV1 in many aspects: it yielded higher GAA activity (407 μmol/l/h*μg vs. 294 μmol/l/h*μg 4 months after treatment), a higher number of GAA-positive XII cranial nerve motoneurons (three times as many), and lower antibody titers at 6 and 16 weeks post-injection [58]. However, both vectors were determined to be significantly increased compared with those of control and mock-treated mice (averaging 59 μmol/l/h*μg and 3 μmol/l/h*μg, respectively) [58]. Furthermore, the GAA-positive motoneurons did not show glycogen accumulation by PAS staining. The restriction of GAA expression to the hypoglossal nucleus excluded the possibility of this effect being due to the liver secretion of GAA. Given the lack of evidence of the retrograde axonal transport of the GAA enzyme, the authors concluded that GAA expression in neural bodies was due to the retrograde transport of the viral vector [58].

Studies have shown that the intrapleural delivery of rAAV vectors results in the transduction of the diaphragm, intercostal muscles, and myocardium, both in a mouse model as well as in nonhuman primates [88, 89]. Falk and colleagues treated $Gaa^{-/-}$ mice with rAAV9 vectors encoding human GAA coupled with a CMV or DES promoter (the latter restricts transduction to the myocardium, diaphragm, and CNS). Six months after injection, both PCR and immunolabeling showed the presence of vector genome copies in the cervical and thoracic spinal cord, diaphragm, and myocardium, suggesting the retrograde transport of the rAAV9 vector [90]. GAA activity consequently improved in the heart, diaphragm, and intercostal muscles, while liver GAA activity remained unchanged. PAS staining showed a reduction in glycogen accumulation. These changes resulted in significant improvements in cardiac function compared with untreated $Gaa^{-/-}$ animals [90]. There was no impact on inspiratory frequency, but it is possible that respiratory motor drive increased, as suggested by the greater inspiratory phrenic bursts recorded in treated animals than in nontreated animals. This theory is further supported by the observation that in *Gaa*$^{-/-}$ mice, phrenic nerve output and diaphragm activity are not correlated, whereas in treated mice, phrenic nerve activity is significantly related to diaphragmatic EMG output, which might be due to the restoration or enhancement of transmission between the phrenic nerve and the diaphragm [90].

A novel addition to our knowledge of Pompe disease histopathology is the characterization of neuromuscular junction (NMJ) architecture. Falk and colleagues showed evidence of NMJ pathology in $Gaa^{-/-}$ mice and its association with widespread neuropathology. They documented a significant increase in the area occupied by acetylcholine receptors (AChRs) on the motor endplate area, which is suggestive of a neuromuscular phenotype, in $Gaa^{-/-}$ mice 9 months of age and speculated that NMJ abnormalities contribute to the genesis of muscle weakness in Pompe disease [91]. Todd and colleagues explored the possibility of treating NMJ pathology with AAV9-hGAA to improve cellular pathology and physiological endpoints, although treatment effectiveness was dependent on the severity of muscular alteration [59] [4]. A single injection of AAV9-hGAA achieved *GAA* expression in the affected tissue, especially in mice treated at 1 month of age, thus modifying AChR mRNA expression and resulting in increased strength; the effect was attenuated in mice treated at 6 months of age. Five months post treatment, in mice treated at 1 month of age, the endplate area was smaller than that of age-matched untreated Gaa−/− mice but larger than that of WT animals. Treatment at any stage (1, 6, and 15 months old) resulted in glycogen clearance from myofibers; however, treatment at an advanced stage did not restore motor endplate gene expression or force production, normalize endplate area, or improve innervation [59].

Fetal Delivery The abovementioned study by Todd and colleagues, in agreement with observations of other muscular dystrophies, indicates that the ability of scAAV9 to restore motoneuron survival is limited to a narrow therapeutic time window [59]. This evidence, coupled with the fact that glycogen accumulation in early-onset Pompe disease begins in utero [9] (as shown by the fact that patients are often born with marked cardiomegaly [8]), makes fetal gene delivery an

[4] Stages: early, mid- and advanced, expressed by age at treatment (1 month, 6 months, and 15 months of age)

attractive therapeutic objective for this disease, as well as many others, as it would prevent irreversible damage.

The delivery of *GAA*-encoding AAVs to the peritoneal cavity of the fetuses of Gaa$^{-/-}$ mice resulted in high-level transduction in the diaphragm, and muscle function at 6 months of age was near normal in the treated animals [68]. This technique was then translated to nonhuman primates. In 2016, Conlon and colleagues administered an rAAV1 vector expressing human *GAA* under the control of a CMV promoter (as well as two other rAAV1 vectors expressing hAAT and miniDMD) to rhesus macaque (*Macaca mulatta*) fetuses in the first trimester of gestation, and this resulted in the stable transduction of the muscular component of the diaphragm without evidence of adverse effects [92]. All six animals subjected to the procedure remained healthy until 3 months of age, when they were sacrificed to collect tissue samples [92]. Both the diaphragm and peritoneum of vector-treated animals showed a widespread and even distribution of GAA gene expression by IHC and high GAA protein expression [92]. Low-level, unevenly distributed GAA expression was also observed in other muscles and in the heart and liver [92]. The immune response ranged from absent to briefly present but self-limiting [92]. Five neonates out of 6 tested positive for anti-rAAV1 antibodies that decreased gradually over time and were likely pre-existing maternal antibodies [92].

The results of these studies indicate that the muscle is a target area for the transduction of not only *GAA* but also other proteins, such as DMD and AAT [92], and that transduced cells maintain transgene expression as they proliferate and the diaphragm grows and matures; therefore, fetal transduction might represent a viable therapeutic approach for Pompe disease. Fetal intraperitoneal delivery is an already established technique both in animal testing and in prenatal medicine, and this approach might be extended to patients who are diagnosed with various myopathies in utero.

On Promoters and AAV Serotypes Adeno-associated viruses (AAVs) are parvoviruses that are not known to cause diseases in humans and are often used as a means to deliver genomic material to cells; the removal of the rep and cap ORFs from the viral genome means it remains mainly episomal, making them preferable compared with lentiviruses. Another useful feature of AAVs is their ability to infect both dividing and nondividing cells. There are several AAV serotypes, the most commonly used of which are AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9; furthermore, hybrid serotypes have been generated by mixing the capsids and inverted terminal repeats (ITRs) from different serotypes. The development of more efficient viral vectors allows the administration of lower vector doses, with two important consequences: first, less virus needs to be produced, reducing costs; and second, overall safety increases because there is less capsid protein to elicit an immune response, and a lower dose of viral genome equals a lower chance of genome integration (which is already a rare occurrence).

Several hybrids, such as rAAV1/2, rAAV2/5, rAAV2/7, rAAV2/8, and rAAV2/9, have been used in Pompe disease gene therapy studies. Among these, rAAV2/9 is perhaps the most promising for the treatment of muscular dystrophies, as it transduces striated muscle with an efficiency equal or superior to that of other vectors, and it transduces a higher number of myofibers [73, 74, 93]. Notably, as shown by Sun and colleagues [74], both rAAV2/9 and rAAV2/7 transduce both type I and type IIb fibers in mice, a feat not attained by ERT, likely due to autophagic build-up [15]. Although these findings are not entirely reproducible in humans, it is possible that gene therapy might represent an effective tool to correct autophagic abnormalities, which are further discussed in a dedicated chapter. rAAV2/9 is effective in both neonate and adult mice and in nonhuman primates [73], with similar distributions of GAA expression that demonstrate a marked preference for cardiac tissue over skeletal muscle (like rAAV1/2). Transfection with rAAV2/9-CMV-hGAA is also effective for correcting conduction abnormalities [73]. A new, promising vector is AAVB1: it transduces the heart, diaphragm and CNS with an efficiency comparable with that of AAV9, prolonging survival. Keeler and colleagues compared the two vectors in 3-month-old Gaa$^{-/-}$ mice, showing that AAVB1-treated animals had higher *GAA* activity levels and greater glycogen clearance, gained more weight, and showed improved respiratory function, nearing that of healthy mice [94].

Given the relevance of neuropathology in the pathogenesis of Pompe disease, the ideal vector would efficiently deliver transgenes to the CNS. AAV9 seems to be apt for this purpose [95, 96], as it effectively crosses the blood-brain barrier [95] and extensively and persistently transduces neurons in several species, including primates [96]. In 2014, ElMallah and colleagues were able to obtain a high rate of retrograde transport from muscle to the nucleus of the innervating motoneuron that exceeded the 1–15% transduction rates obtained by other groups using different serotypes [97–99]. Gransee and colleagues reported AAV7 to be very effective in transducing motoneurons, even more so than AAV9. Using AAV7, they obtained a transduction rate of at least 11% following intrapleural injection; however, this result is not specific to the Pompe mouse model and might therefore be only partially applicable [99]. With regard to immune tolerance to treatment, in 2008, Sun and colleagues transfected *Gaa*$^{-/-}$ mice with three different AAV-GAA vectors and found significantly elevated levels of anti-hGAA antibodies 6 weeks after the administration of both high and low doses of AAV2/8 vector. Despite this, rotarod times were significantly increased for animals treated with the AAV2/7, 2/8 and 2/9 serotypes, and Glc4 levels were significantly reduced at 18 weeks for the high-dose group (all vectors) [74].

Although viral vectors cannot transfect every cell, the final results can be improved via cross-correction, in which nontransduced cells take up the defective enzyme that is secreted by transduced neighboring cells. This process explains why the liver-directed delivery of rAAV2/5 and rAAV2/8 results in significant increases in GAA activity and a reduction in glycogen content in the diaphragm and hindlimb musculature in mice [56, 70].

Promoters help direct gene transduction to target tissues. In general, cardiac tissue is more amenable than skeletal muscle to transduction with rAAV vectors [73], but a possible solution to this is directing AAV vectors specifically to this tissue. MHCK7 and CK1 are two of the most commonly used promoters; both can drive highly efficacious hGAA expression in striated muscle tissue in the Pompe mouse model, regardless of the viral vectors encoding them (AAV2/6, AAV2/7, AAV2/8, AAV2/9). In a comparative study, Sun and colleagues showed that at equivalent vector doses, MHCK7 yielded higher GAA levels in all tissues of treated mice compared with nontreated mice; this difference was detected only at higher doses, while at lower doses, it was not significant. Compared with CKI-encoding vectors, MHCK7-containing vectors were also very effective at reducing glycogen content in all muscles except the gastrocnemius, which seemed more amenable to CK1-driven transduction, at both high and low doses [74]. On the other hand, the CK1 cassette better restricted transgene expression to the muscle, as shown by the lower yield of liver GAA activity levels compared with that induced by the MHCK7 cassette [74]. Compared with CKI, the MHCK7 cassette yielded higher GAA activity levels in the heart, quadriceps, gastrocnemius, and diaphragm [74], while AAV-CK1hGAA administration resulted in significantly higher levels of vector DNA in the liver [74]. Another commonly used vector is CMV, and it has an ideal length, can transduce striated muscle and may be less promiscuous than other vectors, such as the CBA promoter (chicken-β-actin). Conlon and colleagues used CMV to transfect fetal rhesus macaques with three different transgenes with satisfying results [92].

On Immunotolerance One of the challenges presented by gene therapy approaches to muscular dystrophies is the frequency of humoral and cytotoxic immune responses against the introduced proteins: there is an inverse correlation between the efficacy of gene therapy and the presence of the immune response [74]. This response is directed against both the viral capsid, particularly in the presence of pre-existing immunity, and the gene product [86]. Several measures can be taken to prevent and lessen this response. The use of muscle-specific regulatory cassettes decreases the T CD8+ lymphocyte response against gene therapy vectors compared with that induced by ubiquitously active promoters [100], allowing more persistent expression of the transduced gene, although sometimes at the cost of reduced transduction efficiency [101]. On the other hand, gene therapy itself could be a possible approach to reducing the immune response against ERT or transfected hGAA by inducing tolerance to the enzyme [102, 103]; in fact, the immune response to GAA is an obstacle to ERT effectiveness [14, 21, 104], reduces the extent of cross-correction, and might also shorten the efficacy of viral transfection [74]. *Gaa*$^{-/-}$ mice are a good model for developing solutions to this problem, as they respond to rGAA in a manner similar to CRIM-negative patients [105].

Gaa$^{-/-}$ mice mount a humoral and cellular immune response to ubiquitously expressed GAA following AAV-GAA transfection, exhibiting CD4+ and CD8+ T cell activation and evidence of lymphocytic infiltrates in the injected muscle as well as in the liver following systemic administration [74]. However, it has been observed that certain promoters are less immunogenic than others; for example, muscle-restricted promoters (such as the MHCK7 cassette) can be less immunogenic than ubiquitously expressed promoters (such as CMVor CB). Furthermore, vectors containing muscle-specific cassettes tend to not produce lymphocytic infiltrates in transduced muscles and yield longer-lasting hGAA expression (over 18 weeks) [74, 100]. The persistence of hGAA in the bloodstream in the presence of a humoral, but not cellular, immune response demonstrates that muscle-specific hGAA is impervious to circulating antibodies, possibly allowing the circumvention of the obstacle of the humoral response to ERT [74, 106].

A novel approach to modulating the immunological response to GAA gene therapy comes from the addition of an anti-CD4 monoclonal antibody (mAb) to gene therapy, which allows the control of humoral immune responses directed against both the transgene and its vector. The rationale behind this approach is that the rhGAA immune response depends on high-affinity antibodies, the production of which hinges on T helper cells so much so that *Gaa*$^{-/-}$ mice, which are depleted of T-C CD4+ cells, fail to initiate antibody production against the proteins encoded by the delivered genes. Preclinical studies conducted in rodents and nonhuman primates demonstrated that a 1- to 2-week course of a nondepleting anti-CD4 mAb administered with the desired antigen induces long-term im- mune tolerance to the antigen [103, 107–110]. In a study pub- lished in 2015, Han and colleagues showed that the administra- tion of a nondepleting mAb prior to treatment with a GAA-encoding AAV2/9 vector to *Gaa*$^{-/-}$ mice leads to a substantial reduction in anti-GAA immunoglobulins, including those whose production (IgG1, IgG2a, IgG2b, IgG2c, and IgG3) reflects T helper involvement, associated with increased GAA activity in the heart, diaphragm, and liver; the authors also reported a reduction in glycogen content that did not achieve significance. The coadministration of an anti-CD4 mAb with AAV2/9-CBhGAApA resulted in a significant improvement in GAA activity and glycogen accumulation reduction in heart

and skeletal muscle [105]. Controlling the immune response to the vector from the beginning improved the response to subsequent treatments as well; the transduction of the liver of AAV2/9-treated mice with a second AAV2/8 vector was vastly improved when anti-CD4 mAb had been administered with the initial vector [105]. The authors noted some differences between male and female mice: GAA activity in the liver was higher and glycogen content in the heart was lower (but without a difference in GAA expression that could explain this finding) in males. The authors propose that the effect of the coreceptor blocker was greater in males [105]. The use of such monoclonal antibodies might be beneficial in ERT-treated patients as well. Sun and colleagues were able to successfully induce ERT tolerance in *Gaa*$^{-/-}$ mice by treating them with a short course of YTS177, a nondepleting anti-CD4 mAb [111].

Exploiting the Liver The liver has been targeted for gene transfer in several inherited and acquired diseases. Experimentation in humans is underway for the treatment of hemophilia, generating considerable interest towards this approach. This technique has also been applied to the experimental treatment of Pompe disease with two main goals: to induce tolerance to the infused recombinant enzyme, thus increasing ERT efficiency, and to create a site of secretable GAA synthesis to correct muscular pathology.

Several groups have succeeded in reducing glycogen content and preserving the strength of striated muscle by transfecting the liver; however, liver-produced GAA did not achieve CNS correction [56, 74, 87]. Kiang and colleagues showed that in mouse and quail models, vectors that can transduce the liver with the *GAA* gene yield long-term secretion of GAA, which is taken up by various tissues [87]. They also showed that a GSDII mouse model tolerant to human GAA (h*GAA*tol/*Gaa*$^{-/-}$) treated with a viral vector carrying h*GAA* (FDAdh*GAA*) exhibited GAA secretion from the liver for up to 300 days and improved muscle strength and endurance without eliciting anti-hGAA antibody production. When *Gaa*$^{-/-}$ mice were treated in the same manner, hGAA levels decreased after a certain period of time, likely due to the humoral response [87]. In a study published in 2005 by Sun and colleagues [56], a single intramuscular administration of an hGAA-encoding AAV2/8 vector to immunodeficient *Gaa*$^{-/-}$ mice yielded normal GAA activity in the heart, diaphragm, and skeletal muscle; glycogen reduction was observed only in the heart in female GSDII mice. Since the heart and skeletal muscle contained only a low number of copies of vector DNA, the authors hypothesized that the liver secreted a 110-kDa hGAA precursor, which was then taken up by the heart and skeletal muscle. A similar result was obtained by Xu and colleagues, who were able to normalize muscle strength and coordination that persisted for at least months in a *Gaa*$^{-/-}$/SCID mouse model only 2 weeks after the injection of an AdhGAA vector [112].

One of the main limitations of these studies is that the mice needed to be immunosuppressed for the treatments to have a lasting effect; in all studies, the level of correction was at least partly dependent on the presence of an antibody immune response, or lack thereof, against GAA. Other factors were the age of the animals and mannose-6-phosphate receptor expression in the affected tissue [76, 102]. More recently, various groups have been able to overcome this limitation to improve the pathological phenotype of immunocompetent mouse models by exploiting the ability of the liver to induce immune tolerance by coupling gene therapy with the suppression of immune response against the introduced gene [113]. In 2017, Puzzo and colleagues experimented with an innovative approach based on the use of transgenic viral vectors to transform the liver into a biofactory of the mutated enzyme, thus correcting the deficit while simultaneously minimizing the immune response. This was accomplished by transducing the liver of a transgenic Gaa$^{-/-}$ mouse with a gene coding for a secretable GAA protein using an AAV vector; the enzyme was secreted into the bloodstream and taken up by other tissues. The results were encouraging in terms of glycogen clearance, muscular function, and survival. Furthermore, the fact that the enzyme was directly synthesized by the recipient's liver instead of being delivered by an exogenous source induced immunological tolerance, increasing efficacy. The researchers were also able to define the lowest bloodstream enzyme levels necessary to clear glycogen; unsurprisingly, they were lower for the heart and higher for the skeletal muscle and central nervous system than for other tissues. This difference might explain the correlation observed by the authors between vector dosage and response to treatment. At lower dosages, cardiac glycogen was entirely cleared but muscular glycogen was only partially cleared. At higher doses, the diaphragm, quadriceps, and triceps were also free of glycogen accumulation three months after treatment. The benefits observed in the central nervous system were less pronounced, likely due to the filtering effect of the blood-brain barrier. It is likely that the continuity of exposure to the enzyme, in addition to the dosage, contributes to determining the degree of histopathological improvement observed [114]. However, liver-targeted gene therapy has the potential to become a one-time treatment if stable transgene expression can be achieved [113].

Liver transfection has also been used as a tool to induce immune tolerance to ERT. For example, Sun and colleagues were able to prevent the formation of anti-rhGAA antibodies, thus increasing ERT efficiency, by transfecting the liver of *Gaa*$^{-/-}$ mice with the human *GAA* gene using an AAV vector [115].

Starting from the assumption that GAA expression in the liver might suppress the antibody response to rGAA, Han and colleagues conducted a study in 2017 in which they transduced mice with GAA under the transcriptional control of a

liver-specific promoter. A low dose of AAV8 vector (8×10^{11} v.g./kg body weight) was shown to be as effective as ERT, yielding significant glycogen content reductions in both the heart and liver. Both therapies reduced left ventricular mass, but the viral vector therapy did not elicit an immune response, and GAA activity in the blood was continuously elevated, as opposed to falling below the threshold of detectability after 7 days [116]. When ERT was administered following rAAV8, it determined glycogen reduction in the quadriceps muscle (a feat unachieved by ERT alone); furthermore, the immune response was less frequent when ERT followed vector administration and even more so at a higher vector dose (2×10^{11} v.g./kg) [116]. Finally, the vector was able to suppress or eradicate neutralizing antibodies even after the beginning of ERT, which is of great relevance as it suggests that this approach could be applicable to already immunized patients as well [116]. Furthermore, the liver would continuously secrete GAA into the bloodstream, improving ERT effectiveness [116].

Puzzo and colleagues conducted a series of three similar independent studies in which they used AAV8 vectors to compare the effectiveness of a series of GAA transgenes in Pompe mice [114]. They showed that a secretable GAA-encoding AAV8 vector is effective in clearing glycogen from the heart, diaphragm, quadriceps, and triceps muscles in a markedly dose-dependent, time-dependent manner, showing that continuous exposure to GAA is effective in correcting the pathological phenotype; treatment was also associated with improved function and increased survival. While the animals in this study likely had a milder phenotype than other *Gaa*$^{-/-}$ specimens that did not survive as long, they all showed glycogen accumulation in all tissues and signs of cardiac hypertrophy and mild muscle weakness and therefore were a satisfactory model for the disease [114]. The authors identified a threshold level for circulating GAA activity necessary to obtain glycogen clearance; consistent with other findings, this level was lower for the heart and higher for skeletal muscle. CNS pathology benefited from treatment as well; both native and secretable GAA-encoding vectors increased the survival of spinal motor neurons and reduced neuroinflammation, but only the latter treatment yielded sufficient levels of circulating GAA to normalize astrogliosis [114].

Comparing ERT with AAV-Mediated Delivery of GAA In 2015, Falk and colleagues compared the efficacy of gene therapy with that of ERT in a murine model of Pompe disease (the Gaa$^{-/-}$ mouse). They treated mice with either a single injection of AAV-DES-GAA or bimonthly ERT infusions; both treatments significantly improved body mass, significantly reduced cardiac mass and increased the ejection fraction after 3 months, while only AAV-DES-treated animals showed improvements in cardiac conduction (PR elongation) at this time point. Both treatments yielded similar improvements in diaphragmatic contractile strength measured ex vivo after 3 months, but only AAV-DES–treated mice showed respiratory functional improvements, such as increased respiratory frequency under normoxic conditions, decreased expiratory time and shortening of the respiratory cycle. This finding, coupled with the detection of vector genome copies in the spinal cord, suggests that the action of AAVs on both muscles and neurons is more effective in improving global respiratory function. AAV9 also yielded a significant increase in GAA activity in the heart, diaphragm, and intercostal muscle. Both ERT- and AAV9-treated animals developed higher titers of anti-GAA antibodies compared with those of nontreated mice, and these levels were higher in the ERT group, which also experienced higher mortality due to anaphylactic shock following the third ERT injection (this problem was overcome by diphenhydramine administration prior to ERT infusion), a problem not observed in the AAV9 group. In conclusion, gene therapy proved to be at least as effective as ERT while also presenting certain advantages, such as motoneuron transduction, a greater improvement of the respiratory phenotype and higher immunological tolerability [117].

Translation to Humans Gene therapy studies conducted in nonhuman primates supported the development of the first trial in humans, that is, an open-label, single-center, sequential two-arm, phase I/II clinical study that evaluated the safety and potential therapeutic benefit of a single administration of rAAV1-CMV-hGAA injected into the diaphragm [118–120].

A phase I trial involving 5 ventilator-dependent children was completed in 2013 [119]. The patients, who were chronically treated with ERT, underwent muscle conditioning and received one intradiaphragmatic delivery of AAV-GAA. The treatment was deemed safe, with only one serious, procedure-related adverse event being reported (without long-lasting consequences). All subjects also reported grade II toxicity and transient pain related to the study procedure. Muscle conditioning alone proved ineffective in improving ventilatory function in these patients; however, some improvement was noted following gene therapy, as reported below. One hundred and eighty days after treatment, the best-effort unassisted tidal volume was significantly improved (28.8% change), while the maximal voluntary ventilation (MVV) and maximal inspiratory pressure (MIP) did not show significant changes. This might have been because MIP varies mostly in mild to moderate respiratory muscle weakness, while it is less sensitive to changes in severe disease [119]. Nevertheless, patients more than tripled their endurance to unassisted breathing, and most children reduced ventilator pressure and rate settings during daytime hours, possibly containing chronic ventilation-associated damage [119].

A phase I study conducted on 10 patients that was published in 2014 showed that the intraparenchymal infusion of an AAV1 vector coding for a *GAA* transgene in the diaphragm

is a well-tolerated procedure and that the benefits are limited to the treated muscle; this shows that reaching significant levels of plasmatic secretions of the enzyme coded by the transgene is still a challenge [121]. Further results regarding the safety of the diaphragmatic infusion of *GAA* using a viral vector were published in 2017; the authors reported no primary adverse events connected to the agent, although the invasive nature of the procedure resulted in expected adverse events such as pneumothorax and lung contusion [118]. These events support the transition from local to systemic vector delivery, the safety of which has been recently confirmed in a phase I/II study of the use of AAV9 systemic delivery to treat spinal muscular atrophy (SMA) [122].

A phase I study involving nine LOPD patients with the aim of evaluating certain pharmacological characteristics and the effects of the intramuscular administration of rAAV9-DEShGAA is currently ongoing [123][5].

Exposure to adenovirus is common; therefore, patients have often already developed antibodies against it, and this may influence its use as a gene therapy vector [124, 125]. Furthermore, it is possible that patients, especially those treated early, might require the readministration of gene therapy to achieve a sustained response. However, there are some strategies that prevent the development of neutralizing antibodies or counter their action. Treatment with rituximab (a CD20+ B-cell depleting monoclonal antibody) combined with sirolimus (an mTOR-targeted immunomodulatory drug that acts on both T and B lymphocytes) is effective in preventing the immune response against AAV-GAA gene therapy [118]. In 2015, Corti and colleagues proposed a clinical trial aimed at developing a strategy to manage immune responses against AAV vectors to safely achieve the long-term expression of therapeutic genes [126]. They tested the safety, biodistribution, and immunogenicity of a single or double administration of AAV-hGAA in mice and rhesus macaques [126].

Lentiviral Vectors Although lentiviral vectors have been less frequently employed, they also show promise. In a mouse model, lentiviral HSCs achieved glycogen reduction and ameliorated cardiac, respiratory, and skeletal muscle pathology [60]. In 2015, Sato and colleagues improved GAA enzyme activity and glycogen accumulation in cardiomyocytes differentiated from late-onset Pompe disease-specific iPSCs by means of lentiviral rescue [127]. A GAA-expressing third-generation lentiviral vector was generated and used to infect iPSCs at various multiplicities (0, 10, 50, and 100), yielding a dose-dependent increase in GAA activity and a significant reduction in glycogen contents at the highest dose. After cardiomyocyte differentiation, GAA expression persisted; cellular pathology, as observed by electron microscopy, improved; and glycogen accumulation and lysosomal enlargement decreased [127]. A lentiviral vector was administered in newborn $Gaa^{-/-}$ mice as well, resulting in hGAA expression in the serum up to 24 weeks posttreatment and the clearance of glycogen storage in the heart and skeletal muscle 16 and 24 weeks after treatment without the development of a significant immune response against the vector or the gene [128]. The problem of insertional mutagenesis, which is specific to these vectors, remains unresolved, but the use of self-inactivating vectors with more random integration patterns is effective in reducing such occurrences [60].

Antisense Oligonucleotides

Antisense oligonucleotides are small single- or double-stranded DNA or RNA molecules that are chemically modified to increase stability. They target specific mRNA sequences that they recognize and bind according to base complementarity. They are a potential tool to treat several genetic diseases in a targeted and effective manner by acting upstream of the pathological cascade. After they bind a complementary mRNA molecule, they can induce its degradation, modulate splicing, stop translation, or sequester endogenous miRNA molecules [129].

Potential applications of this technology have elicited great interest from the scientific community, and several studies on various types of neoplasms, autoimmune diseases, and congenital syndromes such as Duchenne muscular dystrophy have been conducted [129, 130]. Splicing defects are the cause of many diseases, and the role played by this process in many others is being progressively elucidated; antisense oligonucleotides can modulate splicing in several ways, for example, by blocking a splicing junction, which induces exon skipping and allows the elimination of a pathological mutation from a mature transcript, thereby allowing the production of a truncated but at least partially functioning protein, as in Duchenne muscular dystrophy [130, 131]. Another possible method is through acting on exonic or intronic splicing silencer elements (ESS or ISS, respectively) to promote exon inclusion. The synthesis of antisense sequences that are able to mask an exon 7 exclusion-inducing sequence in the SMN2 gene has led to the creation of the first approved treatment for spinal muscular atrophy (SMA5q) [130, 131]. In the wake of this recent success, this approach has been applied by independent investigators to Pompe disease. The in vitro modulation of the abnormal splicing of GAA mRNA induced by the ISV1-32-13T>G mutation has been attained [130–134].

[5] That is, the *GAA* gene expressed under the control of the promoter of the gene coding for muscular desmin packaged in an AAV9 vector.

The IVS1-32-13T>G mutation is highly prevalent among LOPD patients of Caucasian descent [24, 135, 136]. It is localized in the polypyrimidine tract of exon 2, a pre-mRNA uracil-rich region of 15-20 bases located 5-40 bases upstream of the 3′ border of the intron that promotes spliceosome assembly. The IVS1-32-13T>G mutation weakens the recognition of the exon 2 splicing acceptor site, inducing its partial or complete exclusion from mature mRNA; however, 10–15% of splicing events occur correctly even in mutated transcripts, a phenomenon known as leaky splicing [133, 134]. In the presence of the IVS1 mutation, three splicing variants can be produced: a mature mRNA, which contains exon 2 and is achieved using regular splicing sites; and two pathological variants, namely, SV2, which excludes exon 2 entirely, and SV3, which includes the 3′ extremity of exon 2 and uses the splicing acceptor site contained within exon 2 and the physiological donor site [134]. Van der Wal and colleagues hypothesized the existence of *cis*-active splicing motifs that favor exon 2 inclusion in the final transcript. They were able to identify two sequences that, when simultaneously blocked with morpholino antisense oligonucleotides, were able to increase GAA expression in a concentration-dependent manner (with peak effect at 5–20 μM concentrations), allowing the attainment of GAA expression levels greater than 20% of those measured in healthy individuals. The sequences identified by van der Wal's group correspond to three splicing regulatory elements of intron 1 located approximately 280 nt upstream of the physiological splicing acceptor site of exon 2; the polypyrimidine tract of a cryptic splicing acceptor site together with an intronic cryptic donor site delimits a pseudoexon. In the presence of the IVS132-13T>G mutation, which weakens the recognition of the canonical splicing acceptor site of exon 2, these cryptic sites are recognized by the splicing machinery, determining pseudoexon inclusion and exon 2 exclusion in the transcript [133]. By blocking these cryptic splicing sites and impairing their recognition, it is possible to reinstate canonical splicing [133, 134]. In an independent study, using a different approach, Goina and colleagues identified three sequences inside intron 1 that, when targeted with antisense morpholino oligonucleotides, induced a 2.5-fold increase in normal mRNA expression of the GAA gene. To verify oligonucleotide effectiveness in reinstating exon 2 inclusion, the Padua group used cells that expressed a *GAA* mini-gene that reproduced the region between exon 1 and exon 3. Then, they demonstrated that the treatment of patient-derived myotubes, which exhibited moderate glycogen accumulation, with the three identified molecules induced a reduction in glycogen content [132].

In the cited studies, AONs, the effects of which are short-lived, were administered in a naked form, as AONs are stable for a few months at most. Cloning these small molecules into AAVs could result in permanent expression, reducing the number of necessary administrations, a technique employed by Garanto and colleagues to restore the aberrant splicing of the *CEP* gene in patient fibroblasts as well as in a murine model [137].

Autophagy

Decades of evidence collected in the murine model as well as from the analysis of human tissues show deep dysregulation of the autophagic process in late-onset Pompe disease; interestingly, EOPD patients who receive ERT and survive infancy develop similar alterations over time. Autophagic buildup alters tissue architecture, compromises function, and impairs recombinant enzyme delivery to the lysosome [15, 23]. ERT is only partially effective in resolving autophagic build- up [16]: autophagosome clearance occurs mainly at the beginning of treatment and is maintained inconsistently during the following years [16]. Studies in the animal model have highlighted a correlation between autophagosome accumulation and ERT ineffectiveness.

Given its crucial role in cellular homeostasis, autophagy represents an enticing therapeutic target in Pompe disease as well as in other lysosome storage diseases. Among the expected benefits from autophagy suppression in GSDII are the reduction of glycogen transport to the lysosomes, and therefore the reduction or elimination of glycogen accumulation and the reduction of ERT resistance. Autophagic suppression has been attempted in cellular and murine models of GSDII by suppressing various molecules involved: Atg5 inactivation worsens the clinical manifestation in $Gaa^{-/-}$ mice, while Atg7 silencing was associated with a marked reduction of glycogen accumulation and the elimination of autophagic buildup. Unfortunately, these effects were countered by an increase in debris accumulation: dysfunctional mitochondria, increased atrophy and decreased strength. In the end, the conditions of the mice remained substantially unaltered. Both Atg5 and Atg7 suppression were associated with a very good response to ERT, which stimulated research in this direction [15, 23, 138, 139].

Autophagy inhibition by mTORC1 activation yielded better results; in the treated fibers, an increase in autophagosome-lysosome fusion was noted, the number of vesicles decreased, and proteasome activity was slightly elevated [23]. mTOR dysfunction in Pompe disease is being progressively characterized in greater detail, providing novel potential therapeutic targets. In a recent article, Lim and colleagues showed that enlarged lysosomes in $Gaa^{-/-}$ mice are still able to recruit mTOR but that its activity is inhibited by TSC2. Additionally, lysosomes are unable to release mTOR upon starvation. Lysosomal acidification,

amino acid (especially leucine) supplementation and the targeting of the AMPK-TSC2 pathway might reverse aberrant mTOR signaling in Pompe disease [140].

Conclusions

ERT, while effective, remains unsatisfactory when used alone to treat patients affected by either early- and late-onset GSDII. Several independently produced pieces of data suggest that a therapy targeting global cardiac, muscular and CNS tissues is needed to fully reverse the phenotype of EOPD, and the autophagy defects dominating muscle pathology in LOPD are largely refractory to the recombinant enzyme. Several routes are currently being explored both as a means to improve the effectiveness and tolerability of ERT and as independent and revolutionary treatment approaches for several genetically determined illnesses. Promising results have been obtained in both mice and nonhuman primates, and AAV vectors have been safely used for intradiaphragmatic delivery in a clinical trial. Ongoing studies on the effects of the readministration of AAV-GAA might increase our confidence in this practice and its ability to improve the pathological phenotype. The ability to attain tissue-targeted expression with increasing accuracy opens the possibility of targeting several aspects of this complex disease and obtaining better results, particularly in terms of ventilatory function. Finally, progress in regulating the immune response to the recombinant enzyme will increase the efficacy of both ERT and gene therapy. Of note, the potential of the liver to become an enzyme biofactory, thus bypassing immune surveillance, provides an enticing perspective for the treatment of this and many other diseases.

While the main focus of researchers seems to be AAV-driven gene therapy, lentiviral vectors and chaperone therapy also show promise, the latter in particular as a means of increasing the effectiveness of ERT. Finally, oligonucleotide therapy, the most recent addition to GSDII therapy research, is effective and represents a revolutionary approach to single gene disorders; although in the earlier stages of experimentation, this novel technique might represent a safe, targeted approach to correcting cellular alterations, particularly in LOPD patients.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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