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Lysosomal Enzyme Replacement Therapies: Historical Development, Clinical Outcomes, and Future Perspectives

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Abstract

Lysosomes and lysosomal enzymes play a central role in numerous cellular processes, including cellular nutrition, recycling, signaling, defense, and cell death. Genetic deficiencies of lysosomal components, most commonly enzymes, are known as “lysosomal storage disorders” or “lysosomal diseases” (LDs) and lead to lysosomal dysfunction. LDs broadly affect peripheral organs and the central nervous system (CNS), debilitating patients and frequently causing fatality. Among other approaches, enzyme replacement therapy (ERT) has advanced to the clinic and represents a beneficial strategy for 8 out of the 50–60 known LDs. However, despite its value, current ERT suffers from several shortcomings, including various side effects, development of “resistance”, and suboptimal delivery throughout the body, particularly to the CNS, lowering the therapeutic outcome and precluding the use of this strategy for a majority of LDs. This review offers an overview of the biomedical causes of LDs, their socio-medical relevance, treatment modalities and caveats, experimental alternatives, and future treatment perspectives.

Keywords

Lysosomal enzyme deficiency; lysosomal storage disorders; lysosomal diseases; multi-organ dysfunction; neurodegeneration; enzyme replacement therapy; side effects; enzyme targeting and delivery; blood-brain barrier delivery; enzyme carriers; ICAM-1 mediated enzyme delivery

1. INTRODUCTION

Lysosomes are acidic, membrane-bound intracellular compartments ubiquitously located in cells throughout the body, which occupy approximately 5% of the intracellular volume [1]. They are intricately linked to numerous cellular homeostatic processes, where degradation of biological macromolecules (endogenous or exogenous to the body) is paramount [1]. Said degradative role is achieved by concerted action of acidic hydrolytic enzymes and small activator molecules within the lysosome lumen, and enabled by transport proteins and ion pumps in the lysosomal membrane [1]. Hence, any disturbances in this organelle can disrupt normal functioning of cells in several organs, leading to multi-system diseases. While

lysosomal dysfunctions have been described for several neurodegenerative diseases such as Alzheimer's disease [2], Parkinson's disease [3], and Huntington's disease [4], the most well studied class of diseases directly caused by abnormal lysosomal function are the lysosomal diseases (LDs).

LDs are a group of 50–60 distinct metabolic disorders that exhibit an abnormal, progressive storage of non-degraded substances in affected cells, which can be severely debilitating and, in many cases, fatal [5]. The combined prevalence of LDs is thought to be 1 in every 2,000–5,000 live births (this may be underestimated due to under-diagnosis or mis-diagnosis), with some LDs occurring at a much higher frequency in certain populations, such as the prevalence of Gaucher disease in the Ashkenazi Jewish population (1 in 855 births) [6]. The following sections present the reader with an overview on various important topics. Section 1 reviews the biology of lysosomes as important cellular organelles, offering information of the composition, structural and functional characteristics of this organelle, as well as a classification and depiction of LDs and their pathological effects, advantages and hurdles of translational development of treatments for these rare diseases, and finally a description of said different treatment modalities. Section 2 then offers an extended review on a particular treatment strategy, enzyme replacement therapy (ERT), including the biochemical bases for its inception, historical development of this approach, and current clinical development and implementation. Section 3 describes some of the most important caveats encountered by current ERT, namely hypersensitivity reactions and immune effects leading to “resistance”, as well as problems pertaining to the suboptimal ability of recombinant enzymes to reach their targets in the body because of particular glycosylation requirements, altered cellular pathways, and/or impermeability of certain body barriers, such as the one preventing access to the central nervous system. Section 4 discusses strategies under development aimed to cope with these shortcomings, e.g., by bypassing glycosylation-dependent delivery of lysosomal enzymes, exploiting alternative means to deliver them into the central nervous system, improving their pharmacokinetics/pharmacodynamics and ultimately their bioavailability through the use of nanomedicine and drug delivery approaches, of which the particular case of ICAM-1-mediated ERT is further described.

The article aims to offer both fundamental and translational information about LDs and their treatment by ERT, covering historical, current, and exploratory strategies, as well as discussing under a critical perspective the achievements, limitations, and future prospects in this field.

2. LYSOSOMES AND LYSOSOMAL STORAGE DISORDERS

2.1. Lysosome Structure and Function

Lysosomes, often referred to as “suicide-bags” in cells, were discovered quite serendipitously by de Duve *et al.* in 1955 while performing ultracentrifugation steps on rat liver homogenates [7, 8]. They were initially described as intracellular membrane-bound compartments that mainly house degradative enzymes and had heterogeneous morphology as shown by electron microscopy, with an electron dense appearance and membranous whorls [1, 7, 8]. Lysosomes consist of a phospholipid bilayer membrane enclosing a lumen wherein the pH is maintained at 4.5–5.0 to facilitate the action of acid hydrolases (Figure

1A) [9, 10]. In addition, the lysosomal membranes consist of integral proteins that are heavily glycosylated to prevent their own degradation by the hydrolytic enzymes in the lumen. The major proteins, lysosome-associated membrane proteins LAMP-1, LAMP-2, LAMP-3 or tetraspanin CD63, and lysosome ‘integral’ membrane protein LIMP-2, assist in maintaining the structural integrity of the lysosome and are involved in biogenesis, enzyme targeting, autophagy and fission-fusion events [11, 12]. Other less abundant proteins in the lysosomal membrane include (a) vacuolar H⁺-ATPases that utilize the energy from ATP to pump protons from the cytosol into the lysosomal lumen, thus maintaining its acidic pH [10], (b) membrane transporters such as cystinosin, sialin, NPC1, and CLN-3 that regulate the transport of specific metabolites [12], (c) membrane-bound enzymes such as acetyl-CoA:α-glucosaminide N-acetyltransferase, that transfers acetyl groups from acetyl-CoA in the cytosol to heparan sulfate molecules in the lysosomal lumen [12], (d) lysosomal apyrase-like protein LALP70, a UDPase involved in nucleotide metabolism [13], and (e) mucolipin-1, a transient receptor potential (TRP) channel related to the regulation of lysosomal calcium involved in trafficking, autophagy and signaling mechanisms [14, 15].

Over the years, more than 50 acid hydrolases have been identified and described which reside within the lysosomal lumen [5, 10]. Lysosomal hydrolases are synthesized in the rough endoplasmic reticulum (ER) together with other proteins intended for secretion [16] (Figure 1B). The asparagine residues on the nascent polypeptide are post-translationally processed to bear N-acetylglucosamine moieties modified with a (glucose)₃-(mannose)₉ oligosaccharide chain [17] (Figure 1B₁). Following their proper folding, these enzymes are directed to the *cis* Golgi network, where the mannose residues on the oligosaccharide subunits are phosphorylated at position 6, yielding mannose-6-phosphate (M6P)-N-acetylglucosamine bearing enzymes [17] (Figure 1B₂). In the *trans* Golgi network, the N-acetylglucosamine residues are removed by a phosphodiesterase enzyme, thus exposing the M6P residues, by which enzymes can now bind to the M6P receptor (M6PR) in the *trans* Golgi network [18, 19] (Figure 1B₃). The enzyme bound to M6PR is directed to a pre-lysosomal compartment called the endosome (Figure 1B₄). This endosome undergoes fission and fusion events with lysosomes, whereby the enzymes detach from the M6PR in the acidic environment of the lysosome (Figure 1B₅), while the M6PR is recycled back to the *trans* Golgi network or to the plasma membrane via endosomes [18, 19] (Figure 1B₆). Some M6P independent pathways are also involved in the trafficking of enzymes to the lysosome. For instance, the lysosomal membrane protein LIMP-2 binds glucocerebrosidase enzyme in the ER and shuttles it into the lysosome as a membrane-bound enzyme, releasing it at the lysosomal acidic pH [20]. Similarly, sortilin can bind and sort lysosome proteins such as acid sphingomyelinase, cathepsins and sphingolipid activator proteins [21–23]. Between 5–20% of each lysosomal enzyme synthesized escapes the lysosomal trafficking pathway and is directed to the secretory pathway (Figure 1C) for extracellular secretion [18, 19, 24, 25] (Figure 1C₁). The secreted enzyme can then bind to plasmalemma receptors, such as the M6PR (Figure 1C₂), resulting in enzyme endocytosis (Figure 1C₃). Enzyme-containing endosomes eventually fuse with lysosomes, thus delivering the contents from the extracellular milieu to lysosomes [17–19] (Figure 1C₄). Owing to this mechanism, some of the lysosomal enzymes that are secreted extracellularly are “fished” by M6PR-mediated endocytosis on the plasma membrane and directed to lysosomes [18, 19].

These lysosomal hydrolases are responsible for the degradation of a variety of substrates, including complex carbohydrates, proteins, lipids and nucleic acids [11, 26]. Hence, lysosomes are central organelles to the process of organelle autophagy and recycling of membrane lipids and proteins, wherein intracellular components that are no longer needed are hydrolyzed by enzymes and the monomers recycled to the cytosol to form new macromolecules [1, 5, 11] (Figure 1D). In most cases, lysosomes degrade and inactivate foreign invaders, toxins, and pathogens that are taken into the cell by endocytic mechanisms, thus protecting cells [1, 5, 11]. Lysosomes are also involved in plasma membrane repair processes, wherein targeted fusion events triggered by calcium influx into the lysosome following injury, can repair damaged or compromised areas of the plasma membrane [27, 28] (Figure 1D).

Due to their role in biosynthesis, autophagy and secretion, mammalian lysosomes closely resemble other organelles involved in similar processes. Probably owing to a common evolutionary origin, they bear a strong semblance to yeast vacuoles, plant and fungi vacuoles, and to mammalian endosomes and autophagosomes [29, 30]. However, lysosomes are generally smaller, have a higher buoyant density, and a higher proton and calcium content [31]. Furthermore, lysosomes differ from these other compartments by the presence of specific integral proteins on their membranes, lack of M6PRs, and a higher content of mature hydrolases in their lumen [30]. Lysosome-related organelles such as melanosomes, lytic granules, major histocompatibility complex (MHC) class II compartments, platelet-dense granules, basophil granules, and neutrophil azurophil granules, serve a storage and secretion type of role rather than degradative [30]. Hence, lysosomes and lysosome-related organelles exhibit a range of structures and functions depending on the type of cells to which they belong and the eventual function they will perform. Owing to the myriad of functions performed by lysosomes, dysfunctions in these organelles lead to a multitude of diseases and disorders [5, 30], which are discussed below.

2.2. Lysosomal Diseases

The LDs are a group of about 50–60 distinct, genetically inherited disorders that affect humans and several animal species [5, 26]. They are caused by the inability of the lysosome to degrade one or more of the complex substrates in its lumen or membrane, and are characterized by a progressive accumulation of undigested substrates and cell death [5]. All LDs are monogenic diseases inherited as autosomal recessive traits, except Fabry disease and mucopolysaccharidosis (MPS) type II (also called Hunter disease), which are both inherited as X-linked recessive traits, and Danon disease inherited in an X-linked dominant pattern [26].

At the cellular level, LD-causing mutations can affect enzyme activity, post-translational modifications or trafficking, as well as lysosomal shuttling of substrates, or the biogenesis of the lysosome itself [31] (Table 1). In most LDs, mutations in the genes that encode for these lysosomal enzymes render them inactive, for which their corresponding substrates cannot be degraded [31]. Alternatively, mutations in regions other than the active site generate active enzymes that may fail to fold correctly and, consequently, are directed to the ER-associated degradation pathway [32]. In some cases, although only one gene is defective, the enzyme it

encodes may modify the activity of several other enzymes, leading to multiple enzyme defects such as the case of multiple sulfatase deficiency [33, 34]. In certain LDs the primary defect is in enzymes that sort lysosomal cargo in the Golgi apparatus, such as in the case of Mucopolysaccharidosis II (also called I-cell disease) and Mucopolysaccharidosis III (pseudo-Hurler polydystrophy) [24]. These diseases occur due to the lack of phosphotransferase enzymes that phosphorylate mannose residues, as a result of which all lysosomal enzymes are secreted extracellularly and none of the substrates in lysosomes are degraded [24]. Furthermore, in some LDs, defective membrane transporters in lysosomes do not effectively shuttle metabolites out of lysosomes, which leads to abnormal storage. This has been seen for Type C Niemann-Pick disease (NPC), cystinosis and Salla disease, which are due to defects in the NPC-1 transporter [35], cystinosis transporter [36] and sialin transporter [36], respectively, all located in the lysosomal membrane. Also, mutations in the mucopolysaccharin-1 channel in lysosomal membranes can de-regulate lysosomal calcium, as seen for Mucopolysaccharidosis IV [37]. Defects in the integral lysosomal membrane protein, LAMP2A can also lead to abnormal lysosomal function, as in the case of Danon disease [38]. In some LDs, lysosomal biogenesis is affected when the motility of endosomes is altered. This is the case for NPC and has been correlated to reduced Rab7 and Rab9 cytosolic expression, which disrupts normal endosome transport [39, 40].

The substances that accumulate in the lysosome depend on the particular enzyme or catabolic pathways affected and include sphingolipids, cholesterol, carbohydrates, glycoproteins, or mucopolysaccharides [5, 26]. Lysosomal storage of non-degraded substrates impact regular cellular processes, such as cell signaling and communication through disruption of lipid rafts, as well as downstream autophagy processes through metabolic imbalances [41]. In addition, altered calcium homeostasis from ER stores triggers the unfolded protein response, which in turn prompts the cell to undergo apoptosis [42, 43]. This has been reported in the case of animal models of Gaucher disease, Sandhoff disease, and GM1 gangliosidosis, and confirmed in several other neuropathies [44]. Impaired clearance of the apoptosed cells by macrophages, neuroglial cells, etc. (which are also unable to degrade the substrates due to defective enzymes) worsens the pathology by inducing inflammation, oxidative stress and leukocyte activation [44]. Adding to these downstream effects, abnormal substrate accumulation can cause secondary effects that impact cell homeostasis [5, 31, 45]. This is especially true in the case of endocytosis processes that proceed with final fusion to lysosomes, wherein excessive lysosomal storage creates a “traffic-jam” situation and hinders molecules from entering the cell [46, 47]. Cytoplasmic crowding due to enlargement of lysosomes alters vesicular transport, which can severely affect processes of axonal transport and synaptic transmission [48, 49]. Interestingly, some LDs also associate with a less acidic lysosomal pH [50, 51] although vacuolar H⁺-ATPases are not defective [36], which in turn affects hydrolase activity, vesicle maturation, intracellular trafficking, M6PR recycling, secretion, signaling, and other functions [51–53]. Abnormal substrate accumulation can also cause lysosomal membrane permeabilization, release of cathepsins into the cytosol and cell death [54]. Furthermore, the accumulation of some substrates such as sphingosine leads to a decrease in lysosomal calcium, which further affects trafficking, recycling, and vesicular transport events [55]. Altogether, this series of pathological events leads to cellular dysfunction [5, 31].

Depending on the metabolic pathway directly affected by the genetic deficiency, particular cell types within the body can be impacted to different extents in each LD [47]. There is generally multi-system involvement, including peripheral organs and the CNS, since lysosomes are housekeeping organelles [56, 57]. LDs exhibit a wide-spectrum of symptoms and disease severity depends on several parameters, including the particular pathway and main tissue(s) affected, the extent of the genetic deficiency leading to residual versus no enzyme activity, and epigenetic causes [47, 56]. In fact the prevalence and location of gene mutations frequently differ in patients with the same LD, and the same mutation can lead to varying degrees of severity in different patients [56]. Based on the age of onset of clinical symptoms, LDs are classified as infantile (early-onset), juvenile, or adult forms (late-onset) [47]. The more acute infantile forms exhibit severe dysfunction of the CNS and early infant mortality [44, 57]. Progressive neurological symptoms are also seen in juvenile and adult forms, and overall more than half of all LDs are associated with severe CNS disorders (seizures, dementia, ataxia, developmental delay, vision loss, movement disorders, etc.), which result in early death [44, 57]. Less severe LDs manifest in infancy and progress slowly into adulthood, mainly causing peripheral disease, including damage to the liver, spleen, heart, striated muscle, kidneys, lungs, bone, connective tissue, immune system, etc. [47, 56]. Importantly, all LDs are associated with morbidity and early mortality [56, 57].

As mentioned in the Introduction, the combined prevalence of LDs is estimated to be 1 in every 2,000–5,000 live births [6]. However, this incidence may be underestimated since LDs are multisystem diseases and can be mis-diagnosed as other conditions affecting the same organs, and also acute cases may lead to patient death prior to diagnosis in countries/regions without an established screening system or with low medical awareness [58]. These reasons may also leave mild cases undiagnosed for decades, at which point the damage may be irreversible for treatment [58]. Therefore, several efforts are geared towards early diagnosis of LDs through implementation of population screening measures, such as newborn screening to detect the underlying genetic defect [58, 59]. Because of their cost, these measures are often met with criticism regarding the utility of such programs when an effective therapeutic intervention does not exist [60]. Furthermore, since the manifestation of the underlying genetic defect in LDs is variable, a positive screening test often leads to a lifelong monitoring of the patient even when they do not exhibit severe symptoms [58]. However, with the development of high-throughput analytical technologies and discovery of newer therapies that have demonstrated a benefit when started early, newborn screening programs for LDs (such as the case for Krabbe, MPS I, Pompe and Fabry diseases) are now implemented in several states in the U.S. [59, 60]. Another approach that has been implemented quite successfully is that of screening potential parent-carriers in families with disease history or among high prevalence subpopulations, which can help to prevent the spread of these diseases [58]. In fact, carrier screening approaches have reduced the incidence of Tay-Sach's disease in the Ashkenazi Jewish population by 90% [58]. With the advances in whole genome sequencing technology, both in terms of throughput and cost, such measures may be successfully adapted in the future.

2.3. Drug Development for Lysosomal Diseases

Historically, the development of therapies for LDs has been limited due to the low prevalence of these disorders, which hindered patient recruitment for trials and offered insufficient financial incentive for drug developers. These factors, compounded by a scant understanding of the biological complexities of these pathologies resulted in clinical translation of very few therapeutics. In order to spur drug development for rare diseases, including LDs, legislation was introduced in several countries to offer incentives for ‘orphan drug’ development [61]. The U.S. Orphan Drug Act of 1983 offered incentives such as a 7-year marketing exclusivity, 50% tax credit on expenses incurred during clinical testing, waiver of the application fees (~\$700,000), and availability of grant funding (~\$400,000/year for four years) for rare diseases, which were defined as those affecting less than 200,000 individuals in the U.S.) [62]. As of 2012, the Food and Drug Administration Safety and Innovation Act (FDASIA) was signed into law, whereby special provisions were put into place to enable rare disease drug development. For instance, the Fast-Track Designation (FTD) was a measure intended for serious illnesses with an unmet need, which enabled Priority Review (PR) of drugs rendering significant advance in therapy of these illnesses, and an Accelerated Approval Program (AAP) for drugs demonstrating significant clinical benefit, as assessed by reasonable surrogate outcomes for these conditions [62].

In the European Union (E.U.), since the year 2000, orphan designation of a drug for a rare disease (defined there by an incidence of not more than 5 in 10,000 individuals) confers a 10-year marketing exclusivity, tax benefits and application fee waivers, in addition to accelerating the approvals of rare disease drugs through Conditional Approvals (CAs) and approvals under Exceptional Circumstances (ECs) [63]. In fact, six LD drugs were approved in Europe under the EC category, which meant that the developer would not have to demonstrate the same stringent standard of efficacy and safety for these drugs compared to conventional therapies, owing to their rarity [62]. However, registries of such patients would have to be maintained and monitored over a longer time period, to detect adverse effects and assess long-term outcomes [62]. Similar orphan drug legislations were passed in several countries, including Japan (1993), Singapore (1997), and Australia (1988), to encourage development of therapies for rare diseases [63].

The “orphan drug” status is designated for drugs for which “there is no reasonable expectation that the cost of developing and making the drug available in the U.S. for this rare disease or condition will be recovered from U.S. sales of the drug”. Despite these financial and regulatory advantages, in the current scenario, drug manufacturers have priced LD therapy products as some of the most expensive drugs on the market [61]. The demand for these products is retained even on patent expiration due to lack of competition [61]. Hence, even with a few number of patients who avail of such products, the market is extremely profitable for rare disease drugs [61]. Unfortunately, due to the high cost of such therapeutics, they are oftentimes not accessible by countries which do not have sufficient funds [61]. Such practices are increasingly calling for the need to revisit some of these incentives [61, 62]. In spite of the constant debate surrounding this issue, the success of such legislation in driving drug development for LDs must not be undermined. However, even with these incentives, LDs with a very low incidence or those that display primarily

neurological symptoms, which are currently untreatable, have been faced with a low “development pressure”, probably due to a lower chance of success [64].

One major drawback with regard to drug development for LDs is that patient recruitment for clinical trials is limited due to their rarity in the population, despite multicenter efforts [62]. Recognizing these limitations, clinical trials for lysosomal therapeutics are often conducted with a much smaller sample size compared to conventional therapies, and with different trial designs to provide sufficient statistical power [62]. It is interesting to note that the clinical studies for some of the currently approved therapeutics for LDs were performed with a sample size of 12–18 patients, without including placebo controls [62]. The small sample size meant that the efficacy would have to be almost 100% to achieve statistical significance. However, since LDs display a broad spectrum of manifestations and different extents of symptoms, the clinical outcomes of therapies was also variable, for which they often do not meet the set statistical significance even though they may be effective [62]. While this is a disadvantage, many of these products have been approved due to the flexibility afforded by regulators in examining data from these trials [62].

2.4. Current Treatments for Lysosomal Diseases

The management of LDs can be broadly categorized into (a) symptomatic treatments that consist of palliative approaches to treat the symptoms and (b) disease specific treatment to correct or ameliorate the underlying biochemical defect. A number of strategies have been proposed to treat the biochemical defect in LDs including correction of the causative genetic defect, enhancement of lysosomal enzyme transport to the lysosome, supplementation of the defective enzymes, reduction of accumulated substrates, or modification of the lysosomal environment to reduce excess storage of macromolecules. These include gene therapy, small molecule therapies, organ/cell transplantation, enzyme replacement therapy (ERT), overexpression of cytosolic molecules and lysosome exocytosis (Table 2).

Gene therapy is a promising therapeutic option for LDs since these are monogenic disorders, for which correction is theoretically possible [65]. This technique involves somatic gene transfer of wild-type (Wt) cDNA sequences using viral vectors such as adenovirus, adeno-associated virus, herpes virus, retrovirus or lentivirus [65–67]. One of the challenges in gene therapy stems from the inefficient transformation of all the cells in a tissue to correct the defect [67]. However in LDs, even small improvements in the residual enzyme activities can lead to a dramatic clinical outcome, and even if a small proportion of cells are transduced efficiently, they can restore normal lysosomal functioning of surrounding cells through cross-correction [67] (described in Section 3.1 and Figure 2). Some gene therapy strategies utilize an *ex vivo* approach, wherein the cells of the patient are “corrected” and re-infused or implanted into the patient to elicit a systemic cross-correction effect. This has been seen in the case of metachromatic leukodystrophy by transducing autologous hematopoietic stem cells with the arylsulfatase B gene, followed by reinfusion into patients [68]. This approach has also shown promise for treatment of MPS I, II, III, and VII [69–72]. Another gene therapy strategy involves systemic administration of the vector carrying the correct gene, which can integrate into liver cells and transform the liver into an enzyme-producing “factory”, in order to supply enzyme throughout the body [67]. This has shown promise in

animal models of Fabry disease, Pompe disease, Krabbe disease and several types of MPS [58, 73–78]. While gene therapy is efficacious for peripheral organs, especially those of the reticuloendothelial system, it has not been successful for the CNS possibly due to the impenetrability of the blood-brain-barrier (BBB; described in Section 4.2) [52]. To circumvent this, intracranial administration of AAV2 vectors has been attempted in clinical trials of Infantile Neuronal Lipid Fuscinosi (also called Batten disease) [79] and MPS III (Sanfilippo syndrome) [80]. In addition, vectors that have CNS tropism, such as AAV9, have been administered systemically and observed to cross the BBB in animal models of MPS IIIB and VII [81, 82]. Another approach relies on generating neural stem cells derived from induced pluripotent stem cells of patients, which can be subjected to gene therapy and re-infused into the patient. These cells being CNS-tropic would restore the enzyme activity in the CNS, as has been seen for metachromatic leukodystrophy, MPS VII and Batten disease [83–85]. However, a major roadblock to such therapies is the overall safety of gene therapy regarding immunogenicity, random insertion in the host genome, and mutagenicity, which are still being investigated [52, 67]. In recent years, genome-editing techniques have been successfully applied to several genetic diseases and could be a potential strategy for treating LDs [86]. This viral vector independent strategy utilizes zinc finger nucleases to induce double strand breaks in the DNA to “remove” the faulty sequences, followed by homologous recombination to “add” the correct sequence [86]. This technology has been explored for treatment of Fabry disease, MPS I and II, and Gaucher disease [87]. CRISPR-Cas9 is another genome editing technology that has been patented for application in LDs [88]. This technology is based on the prokaryotic cell defense system utilizing Clustered Regularly Interspaced Short Palindromic Repeats (or CRISPR), wherein guide RNA sequences can be customized to target specific DNA sequences. Once these target sequences are located, the Cas9 enzyme produced by the system cuts the DNA at the specific location, thus allowing precise control on genome editing [88]. This type of strategy has been proposed for the treatment of Gaucher, Fabry, MPS I, MPS II, and NPC, where integration of the transgene of interest at a safe-harbor locus (e.g., albumin gene), a disease-associated gene, or a gene overexpressed in certain cell types (e.g., globin gene in erythrocytes) could be used to produce a Wt enzyme in addition to the mutated enzyme or to replace the mutated gene by a wildtype gene, therefore producing only the Wt enzyme. In theory, this could be done in an *ex vivo* setting by integrating the gene of interest into circulating blood cells or hematopoietic stem cells, which would then supply the enzyme through the circulation. It could also be applied in an *in vivo* scenario by administering the nucleases and vectors intravenously, for integration in specific tissues (e.g., liver, muscle, brain), where they would produce the deficient enzyme [88].

Organ transplantation, especially of the liver and kidneys, has been clinically implemented in cases of severe organ failure to alleviate phenotypic symptoms in several LDs, such as Gaucher, Pompe, and Krabbe diseases, MPS I, and others [65]. Similarly, cell therapy involves transplantation of bone marrow or mesenchymal stem cells obtained from a healthy donor [89]. Healthy transplanted cells can secrete normal or Wt lysosomal enzymes, which can be endocytosed via the M6PR on the plasma membrane in neighboring diseased cells, and transported to lysosomes for repair of the defective enzyme activity (cross-correction, described in more detail in Section 3.1) [89]. This approach has found utility in the treatment

of MPS I and II, Krabbe disease, and cystinosis [90–93]. These options alleviate LD symptoms in organs of the reticuloendothelial system, but are limited in scope due to lack of suitable donors and graft versus host disease [89]. Alternatively, exogenous enzymes can be intravenously infused into LD patients for uptake by cells of different organs and transport to their lysosomes. This approach, called enzyme replacement therapy (ERT), will be discussed in more detail in Section 3.

Small molecule therapies, including substrate inhibitors, chemical chaperones and cytosolic molecule modulators, can offer some benefit in treating LDs since they are BBB permeable, less toxic, less immunogenic, and less mutagenic compared to gene therapy and enzyme therapy approaches [52]. A class of small molecules reduce the accumulation of substrates by inhibiting their biosynthesis; hence they are known as substrate reduction therapy [94]. One class of such molecules inhibit the enzyme glucocereamide synthase, the first step in the glycosphingolipid biosynthesis pathway, for which these molecules are useful treatment options for sphingolipidoses [95]. A few examples include ZavescaTM (Miglustat; *N*-butyldeoxynojirimycin) and CerdelgaTM (Eliglustat tartrate), both clinically approved for Gaucher disease, and Ibiglustat (GZ/SAR402671) in clinical trials for Gaucher and Fabry diseases [95]. Another molecule, genistein (an inhibitor of glycosaminoglycans) is in clinical trials for MPS treatment [95]. This approach has also shown promise in the treatment of Tay-Sach's and NPC diseases [94, 95]. Similarly, cysteamine, another substrate inhibitor has been used successfully for treatment of cystinosis [96]. However, a limitation of this strategy is the lack of specificity, which can have long-lasting implications on other anabolic routes. Cyclodextrins, small sugar molecules consisting of ring-like structures, are another class of small molecules that can sequester cholesterol and reduce their accumulation in the lysosome for diseases where this substrate is aberrantly stored [97]. One such molecule, hydroxypropyl- β -cyclodextrin is in clinical trials for NPC treatment, both by intrathecal and intravenous routes [98, 99]. A major limiting factor for cyclodextrins, however, is that high doses are required to elicit a CNS response [100].

Other approaches focus on correcting the trafficking defects of enzymes to lysosomes. In those LDs for which the mutations in non-catalytic sites lead to improper folding and triggers their degradation, enzyme enhancement therapy is a valuable approach [101, 102]. This strategy uses pharmacological or molecular chaperones that assist enzymes to fold correctly, thus preventing their degradation, and redirecting them to the lysosome [101, 102]. Examples of pharmacological chaperones include GalafoldTM (Migalastat; 1-deoxygalactonojirimycin) clinically approved for Fabry disease in the E.U. and others in clinical trials, such as *N*-nonyldeoxynojirimycin for Gaucher disease, and *N*-octyl-4-epi- β -valienamine for GM1 gangliosidosis [103–105]. Recently, molecules that modulate the function of the molecular chaperone, heat-shock protein 70 (Hsp70), have shown promise for treatment of sphingolipidoses [106, 107]. However, identification of such molecules takes large-scale screening efforts and each molecule is generally specific for just that mutation [102]. Another approach of modulating lysosome trafficking utilizes overexpression of the Rab9 cytosolic molecule, which is linked to endosome maturation and lysosome biogenesis, through gene therapy or protein transduction approaches. This strategy has shown promise in reducing cholesterol and sphingolipid storage in NPC [108], although it is bound by the limitations of gene therapy.

Other approaches focus on modifying lysosomes in LDs rather than correcting for the enzymatic defect. One such approach includes modulating the trafficking of lysosomes. Lysosomes bearing excess non-degraded materials can be induced to undergo exocytosis in order to release their contents extracellularly [109]. One way to induce lysosomal exocytosis is via overexpression of transcription factor EB, which has been documented in Batten disease, MPS IIIA, Pompe disease, and others [110, 111]. Another approach using δ -tocopherol and its derivatives has been shown to reduce lipid accumulation in patient fibroblasts of diseases such as NPC, Wolman, Batten, Fabry, Farber, MPS IIIB, Tay-Sachs, and Type A Niemann-Pick disease (NPD-A) [112]. In NPC cells, cyclodextrin treatment reduced cholesterol levels by triggering calcium-dependent lysosome exocytosis [113, 114]. Similarly, agonists of the mucolipin-1 channel can enhance calcium-dependent lysosomal exocytosis, as seen for NPC fibroblasts [115]. A potential concern of these approaches is that the undigested contents “dumped” outside the cell may not get cleared, given that alterations also affect clearance cells, and this may further contribute to inflammation and other unwanted side effects [109]. Recent approaches have also focused on restoring the pH in lysosomes to normal acidic values, thus facilitating enzyme function and reducing lysosomal impairments. This has been demonstrated using small molecules for Krabbe disease [116] or using acidic nanoparticles, as discussed in Section 5.3 [117].

Among these approaches, the treatment strategy that is translated most often and clinically effective is ERT. Of all the new drug approvals for LDs, 13 ERT (Table 3), and 4 small molecules have been approved to this date.

3. LYSOSOMAL ENZYME REPLACEMENT THERAPY

3.1. Lysosomal Enzyme Trafficking and the Cross-Correction Effect

With the discovery of lysosomes and the identification of lysosomal diseases due to enzyme deficiencies, the administration of normal exogenous enzymes to correct the defects was proposed by de Duve and Brady in 1964 and 1966, respectively [118, 119]. However, it took almost 3 decades for the first ERT to become clinically available, which required significant understanding of the biosynthesis, trafficking, and complexity of lysosomal enzymes.

A natural cross-correction phenomenon in LDs was serendipitously discovered in 1968 by Frattoloni *et al.* while performing cell complementation studies with fibroblasts from different MPS patients [120] (Figure 2). Co-culturing genetically distinct fibroblasts from MPS I and II patients, as well as of each one of these with healthy fibroblasts, it was observed that the metabolism of the respective glycosaminoglycan (the storage material in MPS) was restored to normal levels in both LD fibroblasts [120]. Further studies indicated that the fibroblasts did not need to be in contact for this correction, instead the conditioned medium from one “cross-corrected” the storage defect in the other leading to the speculation that these corrective factors may be lysosomal enzymes [121]. Later, the corrective factors were indeed identified as lysosomal enzymes, iduronate-2-sulfatase and α -L-iduronidase, respectively [122, 123].

Concomitantly, a disorder with multiple inclusion bodies in fibroblasts, termed I-cell disease was identified, wherein multiple lysosomal enzymes were present in the medium

surrounding cells [124]. The medium from I-cell fibroblasts were not corrective in other LD fibroblasts leading to the speculation that these enzymes lacked a signal that directed their uptake into cells [125]. Interestingly, α -L-iduronidase showed multiple elution patterns when separated on a sepharose column and a fraction of the enzyme had a component not needed for activity but required for uptake [126]. This uptake signal was identified as a post-translational modification, specifically a carbohydrate sensitive to periodation [127]. This was corroborated by other studies that showed surface carbohydrates as recognition and uptake signals [128]. It was hypothesized that the uptake signal should bind in a saturable manner to the cell surface and could be competitively inhibited. Several compounds were tested for their inhibitory effects and M6P was identified as an inhibitor of β -glucuronidase and α -L-iduronidase uptake [129, 130]. Structural analysis confirmed M6P as the specific recognition signal that facilitated exogenous enzyme uptake into cells [131]. Indeed M6P was also shown to be the signal that directs lysosomal trafficking of nascent enzymes synthesized in the ER (Figure 2A) [25]. Possibly due to saturation of the M6PRs in the Golgi or due to insufficient phosphorylation of the glycosylated enzyme, about 10% of lysosomal enzymes escape the lysosome pathway and are instead secreted extracellularly [25] (Figure 2B, C).

Identification of the cross-correction phenomenon meant that if LD patients were to be treated with exogenous enzymes displaying the necessary glycosylation patterns, their cells should be able to internalize said exogenous enzyme (Figure 2D) and direct it to the lysosome (Figure 2E, F) to correct the biochemical defect. Importantly, very low amounts of the 'correcting' enzyme (10–20% of the endogenous levels) are needed within lysosomes for therapeutic effect, similar to the case of heterozygous individuals with one mutated allele who still present a healthy phenotype [132]. This formed the basis for development of ERT for LDs.

3.2 Historical Development of Lysosomal Enzyme Replacement Therapy

The possibility that active lysosomal enzymes could be obtained from tissue sources was demonstrated in 1967 with the isolation of ceramide-trihexosidase (now called α -galactosidase) from rat small intestine, which resulted in a modest enzyme enrichment of 220-fold [133]. However, for clinical translation, these enzymes would have to meet certain criteria. They had to be of human-origin, active once isolated, purified adequately, and be available in large-enough quantities. Initial efforts to obtain enzymes were carried out using human placenta. Glucocerebrosidase and α -galactosidase purified from human placenta were effective in reducing circulating substrate levels following intravenous injection in patients of Gaucher and Fabry disease, respectively [134, 135]. The purification procedure enriched these enzymes 4100-fold but only recovered ~5% of the initial amount, yielding only 330 μ g/1.7 Kg placenta [136]. A large-scale purification method was then developed following a cholate extraction method, which yielded similar enrichment but higher recovery (30%) [137]. In studies conducted *in vivo* in rats, intravenously administered enzymes (β -glucuronidase and N-acetyl- β -D-glucosaminidase) were rapidly cleared from the circulation but persisted longer on periodate treatment [138]. Sequential deglycosylation of glucocerebrosidase and β -glucuronidase exposed mannose residues, and these enzymes preferentially accumulated in the macrophages (5-fold higher uptake) [139–141]. This effect

was exploited for targeting purified glucocerebrosidase to this main disease target [142]. The enzyme was administered as weekly infusions of 167 units in a patient of Gaucher disease for 105 weeks and the patient showed hematologic and skeletal improvement, which began to reverse after stopping treatment [143]. Finally, a trial of 12 Gaucher disease patients was conducted using 60 U/Kg glucocerebrosidase infusions every 2 weeks for 9–12 months, which showed improved hematologic and skeletal response, as well as a decrease in hepatic and splenic volume [144]. This was the first ERT approved by the U.S. Food and Drug Administration (FDA) for the treatment of Type-1 (non-neurological) Gaucher disease. Acid sphingomyelinase is another example of an enzyme isolated from placenta [145], and other tissues have also been used to isolate this and other lysosomal enzymes, including the spleen, plasma, urine, kidneys, lungs, and liver [146–156]. However, a main challenge was maintaining a supply to meet the demand. For instance, annual treatment of one adult patient of Gaucher disease required 50,000 individual placentae and, at the time, one-third of the world's placentae were being used for enzyme isolation [157]. Hence, alternative sources were investigated that could produce active recombinant human enzymes while maintaining the glycosylation patterns for cellular uptake.

Chinese hamster ovary (CHO) were tested for this purpose, as they grow easily, overexpress products, render post-translational modifications similar to native enzymes, and secrete the enzyme extracellularly, making this system suitable for large-scale production. This approach was shown to be efficient for producing glucocerebrosidase, which was then treated with exoglycosidases to expose the mannose residues on the enzyme [157, 158]. A comparison of mannose terminated glucocerebrosidase from placenta versus the recombinant source revealed two orders of magnitude higher macrophage targeting for the recombinant counterpart, with increased activity over endogenous levels [158]. Many other recombinant enzymes were purified from CHO cells [159–172], yet the additional deglycosylation steps following purification were cumbersome and prompted the development of methods to obtain mannose-terminated enzymes directly from cell supernatants. Glycosylation mutants of the CHO cell system are an attractive choice since the carbohydrate remodeling in these cells is genetically blocked by inhibiting the activity of N-acetylglucosaminyltransferase [173, 174]. Hence, these enzymes are mannose terminated and can be taken up via mannose receptors on cells. Using this cell system, arylsulfatase B, α -N-acetylglucosaminidase, and other enzymes have been produced [175–177].

Another attractive approach utilized gene activation technology, or targeted recombination with a promoter that activates endogenous enzyme expression in host cells [178]. This has been used to produce glucocerebrosidase from human fibrosarcoma cells grown in a medium containing kifunensine, an α -mannosidase inhibitor, leading to secretion of high-mannose glycans modified enzyme (bearing 9 mannose residues) [179, 180]. This glucocerebrosidase was structurally similar to the one from CHO cells but its internalization by macrophages was ~3-fold increased [180]. Other mammalian cell systems that have been used to produce recombinant enzymes for ERT include murine fibroblasts cells, murine myoblasts, and myotubes [181–183]. Also large-scale production by molecular pharming in transgenic animals has been attempted, e.g., for production of α -glucosidase in milk of transgenic rabbits [184, 185], and for lysosomal acid lipase and α -N-acetylglucosaminidase in egg whites of transgenic hens [186, 187]. Transgenic silk-worms producing active cathepsin-A,

able to bind to and be internalized into murine macrophages, have been developed [188]. Insect cells are another expression system that involve Baculovirus-mediated infection of the host cell line. They can produce large amounts of the recombinant proteins and their post-translational modifications generate proteins terminated with 2 mannose residues [189]. Glucocerebrosidase produced in insect cells showed similar macrophage binding and uptake as the CHO cell-derived enzyme [179, 189–191]. Cathepsin A, neuraminidase, and β -glucosidase produced in insect cells were effective in clearing lysosomal storage in several organs in galactosialidosis mice [192], while α -galactosidase produced in this system was taken up efficiently into murine monocytes [193]. However, α -glucosidase and β -hexosaminidase expressed in this system were active but differently glycosylated than the human form, affecting their uptake [194, 195]. Other enzymes have been expressed using this system, although their uptake has not been studied yet [196–198].

With the advances in plant biotechnology, transgenic plants have also been explored as sources for recombinant lysosomal enzymes due to the lower cost, less contamination risk with human pathogens, and large-scale supply [199]. The major drawback of this system is that post-translational modifications in the Golgi add residues that are potentially immunogenic to humans [200]. Hence, strategies to reduce plant-specific maturation have been attempted [199]. One approach tried to retain proteins in the ER, preventing their transit to Golgi compartments, attempted for production of α -L-iduronidase in maize seeds [201]. Another approach deals with trafficking proteins from the ER directly to vacuolar compartments bypassing the Golgi, as for glucocerebrosidase produced from carrot cells [202, 203]. Alternatively, mutant plants that expose the M6P residues on secreted enzymes are an attractive expression system [204]. Mutant *Arabidopsis thaliana* seeds so generated have been used for production of α -L-iduronidase, β -glucuronidase, glucocerebrosidase, and others [204–207]. The benefit of these systems is that enzymes can remain relatively stable in seed form, can yield high amounts of enzyme (1 mg glucocerebrosidase from 25–30 g seeds), and are as active as the glucocerebrosidase from CHO cells [204]. Similarly, mutant moss plants have been used to produce α -galactosidase [208], and transgenic tobacco plants have been used to expression large amounts of glucocerebrosidase and α -galactosidase [209–211]. In addition, the yeast expression system has also been used to produce recombinant lysosomal enzymes. Yeast cultures are more economical, can be scaled up, secrete proteins into the medium, post-translate proteins and can be metabolically engineered to tailor N-glycosylation patterns [212]. Since the N-glycosylation in yeast produces proteins with an additional capping mannose residue on the M6P, approaches to uncap terminal mannose have been used to generate humanized glycosylation patterns [212]. The methylotrophic yeast *Pichia pastoris* has been most widely used, e.g., for production of lysosomal acid lipase, α -galactosidase, glucocerebrosidase, and several other enzymes [213–217]. *Ogataea minuta*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* have also been used [218–226]. Bacteria, in particular strains of *Escherichia coli*, have also been employed for this purpose, yet formation of protein aggregates as inclusion bodies and lack of post-translational modifications severely limit their applicability in producing recombinant enzymes for lysosomal ERT [227–231]. Alternative bacterial systems such as *Pseudoalteromonas haloplanktis* have been used to

generate active α -galactosidase, but their uptake and effect have not been demonstrated yet [232].

Finally, an important consideration is that major progress in the advance of ERT should be attributed to the animal models that have been developed for the various LDs. The first few ERT to be developed were directly tested in clinical trials since there were no animal models for pre-clinical testing. In the last 50 years, many animal models have been generated and others identified to occur naturally, such as the case for murine, rat, feline, canine and quail models for many LDs [233–258]. These models are generally correlative to the human form of the disease, although in some cases they can be very different from the human clinical scenario. This is the case for Tay-Sach's and Sandhoff mice, which display drastically different phenotypes, although the human clinical manifestations of the two diseases are similar [259, 260]. In some cases, knockout mice are not viable for long after birth as has been seen for Gaucher disease mice [261]. However, the importance of performing pre-clinical testing in animal models is well warranted. For instance, during pre-clinical testing of the enzyme, acid sphingomyelinase in Type B Niemann Pick Disease (NPD) mice, toxicity was seen with higher doses possibly due to the rapid metabolism of sphingomyelin generating excess amounts of ceramide [166]. Interestingly, during dose escalation studies in Phase I trials, patients experienced adverse effects when the doses were increased, leading to the conclusion that an individualized dose escalation must be adopted for this ERT [166]. Hence, the pre-clinical animal models can be a good representative of the clinical effects that can be expected from the ERT regimen.

3.3. Lysosomal Enzyme Replacement Therapies Clinically Available or Under Development

Since the first ERT was clinically approved for LDs in 1991, which pertained to Gaucher disease, several other enzymes have been approved for clinical use and/or tested for their efficacy and safety in human clinical trials (Table 3).

The first generation glucocerebrosidase ERT, called Aglucerase (CeredaseTM), was obtained from human placenta and deglycosylated to expose the mannose residues, and was approved in 1991 for ERT in non-neurological Gaucher disease Type 1. This enzyme has now been withdrawn and replaced with Imiglucerase (CerezymeTM), which is a recombinant glucocerebrosidase produced in CHO cells, approved in 1994. It is administered as intravenous infusions of 60 U/Kg every two weeks. The cost of this therapy is very high: about \$200,000/year. Some studies have shown that lower doses of Imiglucerase at 2.5 U/Kg three times a week is also effective to treat Type 1 Gaucher, while considerably reducing cost [262]. However, the high dose therapy is more effective than the low dose form and other approaches to reduce cost have been sought [263]. An alternative glucocerebrosidase produced by gene-activation of fibrosarcoma cells, Velaglucerase (VPRIVTM), was approved in 2010 [178]. This treatment is also administered as infusions of 60 U/Kg every two weeks, yet this enzyme formulation is about 15% cheaper than Imiglucerase [178]. Following the contamination of Imiglucerase-producing CHO cell stocks with vesivirus in 2010, the shortage of Imiglucerase prompted development of alternative sources for this enzyme [203]. As a result, recombinant glucocerebrosidase produced in carrot cells, Taliglucerase (ElelysoTM) was approved in 2012. This was the first plant-cell derived product to secure

FDA approval, is administered as 30–60 U/Kg infusions every two weeks, and is considerably cheaper than Imiglucerase [203].

For treatment of Fabry disease, two products have been approved to this date: α -galactosidase produced in CHO cells, called Agalsidase β (FabrazymeTM), was FDA and EMA (the European Medicines Agency, homolog of the U.S. FDA) approved in 2003 and is administered as infusions of 1 mg/Kg every two weeks [264]. Agalsidase α (RepagalTM) was approved by EMA in 2001 and is administered at 0.2 mg/kg every other week by intravenous infusion. A tobacco-cell derived recombinant α -galactosidase modified with polyethylene-glycol chains exhibited greater half-life, less immunogenicity, and similar activity as the CHO cell derived recombinant enzymes (Figure 3A) [210]. In addition, when compared to the CHO-produced enzyme, the tobacco-produced counterpart showed higher levels in the heart and kidneys (main disease targets) of the mouse model for Fabry disease for two weeks after intravenous administration (Figure 3A). This enzyme, called Pegunigalsidase (PRX-102), is now in Phase III trials (Table 3).

For Pompe disease, two ERT products have been approved for clinical use [264]: α -glucosidase produced in CHO cells in a 160 L bioreactor, Aglucosidase α (MyozymeTM) was approved in Europe, U.S., Canada, and Japan in 2006 for the treatment of infantile onset Pompe disease [264]. The same enzyme produced at a larger scale (4,000 L bioreactor), LumizymeTM was approved for adult-onset Pompe disease in 2010 [264] and was expanded to include the early-onset forms in 2014. Aglucosidase is administered at 20 mg/Kg every two weeks [264]. In addition, α -glucosidase from milk of transgenic rabbits is being investigated in Phase-II trials [184] (Table 3).

Another class of LD enzymes that have secured several FDA approvals are those used for several MPS: α -L-iduronidase produced in CHO cells, Laronidase (AldurazymeTM), was FDA approved for treatment of MPS I (Hurler syndrome) in 2003 [164, 264]. Laronidase is administered as intravenous infusions of 0.58 mg/Kg per week [264]. Galsulfase (NaglazymeTM), CHO cell produced arylsulfatase B, was approved by the FDA for treatment of MPS VI (Maroteaux-Lamy syndrome) in 2005 [264, 265]. It is to be administered as intravenous infusions of 1 mg/Kg per week [265]. Idursulfase (Elaprase[®]) was FDA approved in 2006 for the treatment of MPS II (Hunter syndrome) [264]. This enzyme is also produced in CHO cells and is administered as intravenous infusions at 0.5 mg/Kg per week [264]. Elosulfase Alfa (Vimzim[®]) was FDA approved for treatment of MPS IV A (Morquio A syndrome) in 2014 [266]. Elosulfase alfa consists of the N-acetylgalactosamine 6-sulfatase produced in CHO cells and is administered as 2 mg/Kg infusions per week [266]. In addition, β -glucuronidase produced in mouse L cells (UX-003) is in Phase III trials for treatment of MPS VII (Sly syndrome) and α -N-acetylglucosaminidase produced in egg whites of transgenic hens (SBC-103) is in Phase I/II trials for treatment of MPS-III B (Sanfillipo syndrome) (Table 3).

The recombinant form of the enzyme lysosomal acid lipase produced in egg whites of transgenic hens, Sebelipase α (Kanuma[®]) was FDA approved in 2015 for the treatment of lysosomal acid lipase deficiency, both the severe infantile form (Wolman disease) and adult-

onset form (cholesteryl ester storage disease) [186, 264, 267]. Sebelipase is administered as 1 mg/Kg to 3 mg/Kg intravenous infusions every week [264, 267].

Another enzyme in clinical testing is acid sphingomyelinase produced in CHO cells (Olipudase®), which was tested in Phase I clinical trials for treatment of Niemann-Pick disease Type B (non-neurological) where dose escalation studies were conducted [268]. Due to adverse drug reactions, the study was terminated, yet subsequent tolerance studies demonstrated potential and this enzyme is currently undergoing Phase II/III clinical trial [268].

Although the average cost of ERT ranges from \$50,000–200,000/year, it is the most effective therapy to this date. Hence, improvements to ERT are being continuously investigated to increase therapeutic efficacy, reduce dosage and adverse effects, and eventually increase ERT affordability.

4. OBSTACLES ENCOUNTERED IN THE CLINICAL APPLICATION OF LYSOSOMAL ERT

4.1. Immune Responses to Lysosomal Enzyme Replacement Therapy

A considerable complication of the use of ERT to treat LDs relates to the immune response exerted against these biologicals [269–271]. As described above, lysosomal ERT involves patient infusions with exogenous enzymes and this often results in immune responses, ultimately affecting patient safety and/or treatment efficacy [269, 272]. Pre-clinical and clinical trials, and approved treatments for various LDs have all shown the frequent occurrence and interventional relevance of these outcomes [269, 270, 273]. They have been long observed and documented [274–276] and, most recently, the very focus of a workshop organized by the FDA [270] and the National Organization of Rare Disorders (NORD). This meeting resulted in a publication that provided awareness and guidelines for the management of these reactions, recognized gaps in knowledge regarding causative mechanisms and predictive markers for these phenomena, and identified the need for future research on the development of monitoring assays, counteracting measures, and treatment optimization [270].

In particular, ERT associates with humoral immunity and/or hyperreactivity. With regard to humoral immunity, short and long-lived plasma cells, as well as memory B cells account for this reaction, which leads to generation of antibodies against the infused enzymes [270]. Most commonly, the generated antibodies belong to IgG types and can act as neutralizing or non-neutralizing counterparts [272, 277] (Figure 4). Although a priori it would seem only the first category of antibodies would represent a hurdle for ERT, a closer look offers a different picture. Neutralizing antibodies are those that bind to the administered enzyme in regions required for its function (Figure 4A), including both sites involved in binding to M6PR on target cells (Figure 4A₁) or sites involved in the catalytic activity (Figure 4A₂). Evidently, blocking either region results in a “neutralizing” effect, by impairing the enzyme ability to reach its lysosomal destination (Figure 4A₁) or degrading the accumulated substrate (Figure 4A₂). An extreme example is that of a young patient of MPS II, who did

not benefit from Elaprase® (Shire HGT) after years of treatment because of high titer neutralizing antibodies [270]. On the other hand, non-neutralizing antibodies bind to the infused enzyme in non-functional regions; hence, they do not affect its receptor-binding or catalytic activities. However, non-neutralizing antibodies still affect treatment as they can divert enzyme uptake from the intended M6PR to Fc receptors (FcR) (Figure 4B). This reduces the enzyme dose able to effectively reach lysosomes in the target tissues and enhances immune responses associated to FcR signaling and uptake by immune cells (Figure 4B). These mechanistic effects have been documented using animal models for several LDs, such as the case of MPS I and VI, and been shown to have an effect in patients [274, 278, 279].

As per hypersensitivity reactions, these are also immune-mediated and often the consequence of the presence of patient anti-enzyme antibodies [269]. They include rash, swelling, abscesses, fever, chills, tachycardia, respiratory symptoms, hypotension, etc. [280]. These outcomes tend to be more severe at the start of treatment and lower in intensity with time, perhaps due to an effect of immunotolerance [269]. Allergic reactions and anaphylactic episodes, although uncommon, have been observed in some cases and represent severe, life-threatening occurrences [269, 273]. They associate to generation of IgE antibodies, such as reported for some cases of ERT for patients of MPS II [281].

Importantly, there is a significant degree of variability on these immune responses, which ranges from mild to severe as observed both in animal models and patients [269, 270]. For instance, published data points to Cerezyme® (Genzyme Corporation treatment for Gaucher disease Type 1) as a low reactive ERT, with only 15% of patients developing anti-enzyme antibodies and 14% having infusion-related reactions [270]. Instead, Fabrazyme® or Replagal® (Fabry disease ERTs from Genzyme Corporation or Shire HGT, respectively) associate with almost 70% antibody generation and 60% hypersensitivity reactions [270]. Of note, the monocyte-macrophage system is a main target for Gaucher disease Type 1, and the dysfunctional status of these cells may lower their ability to induce immune reactions most commonly observed for other LD treatments. Also, these patients typically have residual endogenous enzyme (as described below) and may be “tolerized”. In other cases, only one type of reaction is observed in patients, e.g., VPRIV® (Shire HGT ERT for Gaucher disease Type 1) associates with 52% hypersensitivity rate but only 2% antibody generation, while Lumizyme® (Genzyme Corporation ERT for Pompe disease) associates with only 5% hypersensitivity but 100% antibody production [270]. ERTs undergoing clinical trials are also incurring similar diverse responses, e.g., in the case of Olipudase® (Genzyme ERT for Type B Niemann-Pick disease) [268].

The reason for such variability seems to depend on multiple factors, including both the actual recombinant enzyme used and individual characteristics pertaining to each disease and patient [270]. For instance, it is known that some genetic alterations causing LDs result in patients having no detectable levels of the particular enzyme affected, while on the other side of the spectrum, some mutations associate to normal enzyme levels despite an altered activity. Consequently, it is reasonable to expect that patients with no endogenous enzyme would readily recognize the infused counterpart as foreign, while the immune response of patients with a present endogenous enzyme would be diminished. This appears to be the

case; patients with measurable levels of endogenous enzyme seem to be more immunotolerant, such as reported for Pompe disease patients [282]. Patients with no detectable endogenous enzyme had both higher antibody titers and more sustained responses versus those who expressed a defective enzyme [282], which is similar to that observed in knockout mouse models for Fabry disease [283]. Nevertheless, even in this later case, one must realize that the recombinant enzymes used in ERT may considerably differ from the endogenous ones with regard to sequence, structure, post-translational modifications, and/or quantity, therefore, causing immune responses. The genetic background and overall pathophysiological state of the patient may also influence these outcomes.

In any instance, although the type and severity of the immune response toward the infused enzyme and the effect on the treatment outcome are diverse, they ultimately lower therapeutic efficacy and promote disease progression against the improvement otherwise achieved. Acute reactions are commonly managed using antihistamines, corticosteroids, and antipyretics, as well as reducing the infusion rate [280, 284]. In addition, induction of immunotolerance is arising as a valuable solution for this outcome and has been observed after long ERT or administration regimens intended to achieve this effect, such as the case of treatment for Gaucher disease, MPS I, and in clinical trials for Type B NPD, etc. [276, 285–288]. This avenue is being considered as a potential prophylactic measure for patients with undetectable levels of endogenous enzyme, at high risk for immune responses causing “resistance” to ERT and/or detrimental side effects.

Therefore, it is important to understand and monitor these reactions. Given the association between the presence of patient anti-enzyme antibodies and the response to treatment, monitoring circulating antibody levels has been proposed as an interesting predictive tool to estimate the occurrence of potential unwanted reactions against the infused enzyme [270]. These type of assays are typically implemented during the early stages of translation, e.g., Phase I or Phase I/II combined clinical trials [270]. Unfortunately, most animal models used for early development are knockout mice; hence administration of a human or humanized enzyme in mice entirely lacking the endogenous counterpart does not enable scientists to evaluate these side reactions. The existence of naturally occurring animal models for many LDs, described in Section 3.2, provides valuable means to consider the influence of the presence of endogenous enzyme, yet inter-species immunogenicity still applies. Determining patient levels of endogenous enzyme prior to treatment may also offer some predictive value as to potential side effects, yet this is difficult to ascertain for individual LDs because of their relatively low incidence and insufficient existing data. New therapeutic means, e.g., drug delivery systems discussed in Sections 5.3 and 5.4, may offer alternative or complementary avenues to minimize these reactions.

4.2. Poor Enzyme Accessibility to Main Targets for Intervention

As described in Section 3.3, lysosomal ERTs are clinically approved only for a very few number of LDs (8 out of more than 50), where the main targets for intervention are peripheral sites [289, 290]. However, accessing the CNS represents one of the major hurdles toward LD therapies, preventing effective treatment for the large majority of these conditions [44, 52]. This is the case for Types A or C Niemann-Pick disease, neuronal ceroid

lipofuscinosis, GM1 gangliosidosis, Sandhoff disease, and many other lysosomal-related neuropathies [52, 291]. The endothelial layer and associated structures that separate the bloodstream from the CNS pose an impenetrable obstacle for efficient transport of enzymes into this tissue [292, 293]. This is because endothelial cells at the blood-CNS interface form a continuous cellular lining, whereby the junctions between adjacent cells are sealed by intercellular protein plaques, i.e., those involved in the tight junctions and adherens junctions [294, 295]. In addition, subendothelial cells participate biochemically and/or physically in the formation and maintenance of this barrier, such as the case for astrocytes, pericytes, microglial cells, and neurons (the neurovascular unit) in the BBB [296]. This type of permeability control not only applies to the BBB but to all interfaces between the vascular endothelium and the CNS, including the blood-cerebrospinal fluid barrier and blood-meningeal barrier in the brain, and similar interfaces in the spinal cord, the retina, the labyrinth, etc. [297]. In these vascular barriers, the transport of metabolites and larger macromolecules is controlled by endothelial cells, mainly by active mechanisms. In particular, larger macromolecules are transported via transcytosis, a specialized type of endocytic transport encompassing binding of the cargo to specific endothelial membrane markers at the luminal (vascular) or basolateral (abluminal tissue) surface, formation of membrane-bound vesicles enveloping said cargo, and vesicular transport across the endothelial cell body with secretion at the opposite surface [52, 298, 299]. Importantly the M6PR, to which lysosomal enzymes bind to enter target cells, seems to be expressed on the BBB mainly during early developmental and neonatal stages, but it is downregulated at this interface soon after birth [300]. This has been shown experimentally in the case of MPS IIIA and VII, whereby some enzyme transport into the brain was observed in neonatal but not adult mouse models of these syndromes [300]. Increasing the frequency and dose administered, as attempted in a clinical trial for metachromatic leukodystrophy, does not relieve this hurdle [301]. Therefore, a major emphasis is being put into developing strategies to overcome this obstacle, which will be summarized in Section 5.2.

Conversely, LDs whose main targets are peripheral are better poised to benefit from ERT. This is the case for approved ERTs aimed at Gaucher, Fabry, and Pompe diseases, as well as MPS I, II, and VI, all of which are characterized by lack of major CNS involvement [289, 290]. Similarly, ongoing clinical trials for Type B NPD, lysosomal acid lipase deficiency, α -mannosidosis deficiency, and others, aim to treat mostly effects of these syndromes on peripheral sites [302]. This is because the infused enzymes have relatively easy access to the organs and tissues affected in these diseases. For instance, the monocyte-macrophage system is the major target for treatment of patients with Gaucher disease Type 1 [303], Fabry disease highly impacts endothelial and periendothelial (juxtaposed epithelia, smooth muscle, etc.) cells in the renal and cardiovascular system [304], Type B NPD associates with pulmonary and hepatosplenic alterations [305], etc. These organs, tissues, and cell types can be easily accessed from the bloodstream. For instance, the vascular wall in the liver and the spleen pose no barrier for most circulating materials, which simply diffuse through the discontinuous capillaries in these organs, reaching freely the subjacent tissues [306, 307]. Similarly, the kidneys are a prime site for clearance of circulating molecules, and the lungs receive the whole cardiac output after intravenous administration [306, 307]. At the cellular level, the monocyte-macrophage system is easily accessible, as these and other phagocytic-

like cells are abundantly located in reachable organs, they broadly reside in most body compartments, and they specialize in the uptake of extracellular materials [308, 309]. Likewise, the vascular endothelial cells are in direct contact with the circulation and are one of the first cell types encountered after intravenous administration [78, 308].

Nevertheless, even for cases where non-CNS delivery is intended, effective access of infused enzymes to their tissue, cellular, and/or subcellular (lysosomal) targets may not be a straightforward path. For instance, as discussed in Section 3.2., this is the case for enzymes which may be insufficiently or not properly glycosylated, impairing their ability to bind to the MR or M6PR on the surface of target cells, such as observed for some formulations of β -glucuronidase for MPS VII [138, 141], N-acetyl- β -hexosaminidase for Sandhoff disease [127] and α -L-iduronidase for MPS I [126]. The need for the presence of these post-translational modifications restricts production of enzymes for lysosomal ERT, e.g., to mammalian systems over other platforms for recombinant expression [309, 310]. However, as described in Section 3.2., some cases of poor glycosylation have been observed even when mammalian systems are used, e.g., for α -N-acetylglucosaminidase deficient in Sanfilippo syndrome [176]. In some instances, mannose residues are “masked” within the tertiary structure of enzymes and need secondary processing in order to expose them, as for early attempts to use glucocerebrosidase as treatment for Gaucher disease patients [139, 311]. Also, enhanced presence of mannose over M6P residues on recombinant enzymes tends to deviate their uptake toward the monocyte-macrophage system and reticuloendothelial organs, precluding other tissues from being targeted. This was observed for β -glucuronidase deficient in MPS VII, where the enzyme was rapidly cleared by Kupffer cells in the liver upon intravenous injection in mouse models [141].

In other cases, the obstacle to proper lysosomal ERT was not the presence or absence of these targeting residues on the therapeutic enzymes, but complications posed by their endogenous receptors. For instance, reduced levels of expression of MR on macrophages of Gaucher patients were observed to lower delivery of recombinant glucocerebrosidase [312]. Consequently, strategies aimed to increase receptor expression, such as the use of dexamethasone, had to be utilized to improve enzyme targeting and uptake [312]. In addition, decreased uptake function has also been associated to the M6PR. Several examples of this phenomenon have been published, including the case of reduced uptake of recombinant α -glucosidase by fibroblasts of Pompe disease patients, that of acid sphingomyelinase by fibroblasts of Type A NPD, or that of the same enzyme by alveolar macrophages from the mouse model for Types A and B NPD [313–315].

More generally, altered endocytosis has been documented for many LDs, cell types, receptors, and pathways [315–318]. Lowered clathrin endocytosis has been shown for the transferrin receptor using fibroblasts from patients of Types A and C Niemann Pick disease, Fabry, and Gaucher [315, 319]. Deficits in neurotransmitter recycling via clathrin-coated pits have been shown in Gaucher and Batten disease mice [320, 321]. These examples, along with decreased uptake via the M6PR which is also mediated by clathrin-coated pits, suggest that lysosomal storage in LDs may be a contributing factor. Clathrin is involved in the biosynthetic trafficking of lysosomal enzymes, and lysosomal storage disturbs the display and/or function of lysosomal-membrane proteins required for clathrin coating, signaling, and

fusion/fission events, altogether diminishing clathrin availability for endocytosis [5, 45, 322]. Caveoli are not involved in endogenous lysosomal enzyme trafficking and often bypass lysosomes [323]; hence, it may be less broadly affected in LDs. For instance, caveolar-mediated endocytosis was reported to be lower in Types A and C Niemann-Pick disease fibroblasts, which accumulate lipid-raft components such as cholesterol and sphingomyelin, versus Gaucher and Fabry cells which do not accumulate these membrane components [319, 324]. Yet, common caveolar alterations may arise in lipidoses that ultimately alter membrane lipid rafts, as reported for aberrant trafficking of lactosylceramide and ganglioside ligands, disruption of caveolar microdomains in Batten disease, and poor signaling molecule recruitment to lipid rafts in Krabbe disease [317, 321, 325, 326]. Even macropinocytosis- and phagocytosis-related uptake has been shown to be altered in some LDs, e.g., in cells from Type A NPD patients or pharmacological models, osteoclast uptake of bone matrix, pathogen uptake, and many other examples of alterations in endocytic uptake have been reported for these diseases [46, 49, 316, 319, 327–330]. Paradoxically, in some cases instead of a decrease, enhancement in endocytosis via certain pathways has been found, such as for increased enzyme uptake by renal tubular cells with metachromatic leukodystrophy, or enhanced macropinocytosis by NPC [319, 331]. Therefore, careful examination of these events is warranted in order to optimize current therapeutic means for LDs and to design new alternative or complementary approaches.

5. ADDITIONAL APPROACHES TOWARD THE OPTIMIZATION OF LYSOSOMAL ERT

5.1. Strategies to Bypass Glycosylation-Dependent Targeting and Uptake Mechanisms

As described above, the endogenous mechanism for lysosomal enzyme uptake into cells relies on these enzymes displaying particular post-translational modifications, e.g., M6P residues that provide binding to M6PR positive cells and elicit clathrin-mediated endocytosis with trafficking to lysosomes [19]. To avoid problems related to recombinant enzymes not possessing the required targeting residues, alternative approaches have been explored that invoke glycosylation-independent mechanisms (Table 4).

Most typically, this has been achieved by creating chimeric enzymes where the enzyme codifying sequence is fused to a sequence codifying a peptide that recognizes an independent cell surface marker [302, 332, 333]. An example is that of a recombinant form of β -glucuronidase, the enzyme deficient in MPS VII, which was fused to a peptide derived from insulin-like growth factor II (IGF-II) [334]. This growth factor is known to bind to the M6PR, although it recognizes a different epitope than that of M6P. As such, this design was aimed to target the same receptor and pathway as classical enzymes, yet without the need of the enzyme bearing M6P residues [334]. When this fusion enzyme was incubated with MPS VII patient fibroblasts in culture, it showed enhanced binding and uptake via clathrin-coated pits as compared to the enzyme in the absence of the targeting peptide [334]. A similar outcome was observed in vivo using a MPS VII mouse model [334]. Because the fusion enzyme had been expressed in a mammalian system, a possibility was that the fusion enzyme was also glycosylated. To demonstrate the mechanism of targeting and uptake, similar experiments were conducted in the presence of M6P or IGF-II competitors, which

demonstrated that both IGF-II peptide and also M6P present on the fusion enzyme were involved [334]. However, when this fusion enzyme was subjected to enzymatic hydrolysis to remove the polysaccharide chains, it was still active in targeting and inducing uptake by cells. This demonstrated that the IGF-II peptide was sufficient to bypass glycosylation-dependent delivery of the enzyme [334].

Other approaches have focused on the use of fusion peptides that target receptors other than those used by the endogenous enzymes. An illustrative example is that of targeting the low-density lipoprotein receptor (LDLR) family, whose cell uptake is also mediated by clathrin-coated pits as in the case of the M6PR. For instance, α -L-iduronidase or α -glucosidase, which are deficient in MPS I and Pompe disease, respectively, have been fused to receptor associated protein RAP [335]. RAP is a chaperone known to bind to LDLR to prevent it from binding its ligand while in transit through the secretory route. Hence, it was hypothesized that RAP-fused enzymes could similarly bind to LDLR on the surface of cells. This was shown experimentally by incubating RAP-fusion enzymes in several cell culture models, including patient fibroblasts, rat glioma cells, and mouse myoblasts, all of which showed efficient binding and uptake into lysosomes [335]. Excess RAP added in the culture medium out-competed the uptake of these fusion enzymes, and internalization was found to be mediated by LDLR-related protein 1 (LRP1), apoER2, VLDLR, and megalin, all of which belong to the LDLR family [335]. Therefore, this demonstrated that this strategy also provides glycosylation-independent delivery.

In addition to these strategies aiming to target receptors associated with endocytic transport, instances of recombinant enzymes tagged by protein transduction domains have also been attempted. An example is that of recombinant enzymes fused to the HIV-derived Tat peptide, as the case for β -glucuronidase, glucocerebrosidase, and galactocerebrosidase, which are the enzymes deficient in MPS VII, Gaucher disease, and Krabbe disease, respectively [336–339]. These fusion enzymes were internalized in epithelial and fibroblast cell culture models, where they reached lysosomes mediated by both M6PR-dependent and independent pathways [336, 338, 339]. This may be due to potential glycosylation of the fusion enzymes, as observed in the case described above for IGF-II enzyme fusions. Positive charges in the Tat sequence appear to provide this peptide the property of interacting with cellular membranes in a charge-dependent manner, enabling binding and uptake by cells without the need to target a specific receptor [336–339]. While this is unfavorable for some therapeutic applications, it is not a major concern for LD treatment since these diseases often affect multiple organs, tissues, and cell types within the body. However, the mechanism of Tat uptake remains controversial, as it seems to obey both to membrane-permeating properties and also adsorptive endocytosis, providing little control over the final lysosomal destination [306, 340].

All of these approaches represent valuable strategies which may further the efficacy of lysosomal ERTs. Other examples of fusion enzymes, as well as conjugates of recombinant enzymes and targeting ligands or antibodies, and targeted drug delivery systems for glycosylation-independent targeting and transport are discussed in the following sections.

5.2. Strategies to Overcome the Blood-CNS Barriers for Treatment of Neurological LDs

As described in Section 2.2, more than half of LDs associate with neurological dysfunction, such as the case for Types A and C Niemann-Pick disease, Batten disease, gangliosidosis, metachromatic leukodystrophy, Krabbe disease, Types 2 and 3 Gaucher disease, and many others [44, 52, 291]. They affect diverse types of neurons and glial cells in the CNS and cause neurodegenerative complications that affect cognitive and motor functions. Unfortunately, recombinant enzymes infused intravenously fail to access the CNS, for which lysosomal ERT has not advanced to the clinics for any of these applications [44, 52, 291]. Alternative strategies are being actively investigated, either involving local administration or the use of available transport routes between the circulation and the CNS, some of which are summarized below (Table 4).

For instance, intracranial administration into the brain parenchyma or brain ventricles, or between spinal cord vertebrae (e.g., epidural and intrathecal injections) enable direct access into the CNS [291, 292, 341]. As an example, local injection into the cerebrospinal fluid (CSF), including but not limited to intrathecal injections, has been performed in several animal models for LDs, and have also been tested in clinical trials, e.g., those for MPS I and III, and others [78, 291]. Although valuable, these studies have revealed that only a relatively small fraction of the injected enzymes enter and diffuse through the brain parenchyma [342, 343]. This is because of poor permeation of the administered therapeutics throughout the whole CNS: e.g., the rate of diffusion within the brain has been reported to be slower than that of clearance in the case of intraventricular injections, which results in overall transport into the circulation [341]. The use of implantable, convention-enhanced diffusion pumps helps with this obstacle, yet the level of resistance to fluid flow in the brain does not provide for deep tissue penetration even when using this approach [344]. However, importantly, because small amounts of enzyme activity are necessary to normalize lysosomal storage levels, these local administration routes appear to still render a therapeutic benefit [342, 343]. The same principle can be applied to the delivery of gene therapy vectors or the implantation of transduced cells [344–347].

Still, since LDs are chronic conditions and local administration into the CNS is relatively invasive and risky, avenues to access the CNS from the peripheral circulation are actively investigated. Several routes support blood-to-CNS transport, including the paracellular route between adjacent endothelial cells, or the transcellular route that includes diffusion, saturable transporters, and vesicular transcytosis [292, 293]. Unfortunately, the paracellular route between the blood and the CNS is rather restricted and does not allow passage of large, polar entities such as the recombinant enzymes used for lysosomal ERT [52, 292]. This is also the case with regard to diffusion across membranes in the blood-to-CNS barriers [292, 293]. As per saturable transporters at the endothelial plasma membrane, they shuttle small molecules such as glucose, vitamin B12, L-DOPA, amino acids, etc. [292]. Yet, transport of relatively large enzymes through this route is not favorable [292]. Nevertheless, it has been observed that administration of certain pharmacological agents, e.g., those that act as α -adrenergic agonists or inflammatory mediators (epinephrine, insulin, retinoic acid, lipopolysaccharide, etc.) transiently helps uptake of lysosomal enzymes across the BBB, such as shown in animal models in the case of β -glucuronidase, deficient in MPS VII [348].

Transport of most macromolecules between the circulation and the CNS is regulated by vesicular transcytosis across endothelial cells in capillaries and post-capillary venules irrigating this tissue [52, 292]. This is induced by said macromolecular ligands binding to their respective receptors on the endothelial plasmalemma, which renders endocytic engulfment within vesicles that pinch off and traffic across the endothelial cell body, and are finally exocytosed at the opposite side of the barrier [298, 299, 340]. Two main transcytosis routes exist, the clathrin- and caveolar-mediated routes, which are used by most endogenous macromolecules transported between the circulation and the subjacent tissues [298, 299, 340]. Caveoli are small flask-shaped invaginations (100 nm diameter) rich in caveolin proteins and lipid raft-like components (e.g., cholesterol, sphingolipid, etc.). They are abundant in certain organs, such as the case for the lungs, but appear minimally involved in the BBB [299, 349]. Most events of transcytosis at the BBB are mediated via clathrin-coated pits, slightly larger vesicles (100 - 200 nm diameter) surrounded by clathrin proteins and adaptors [298, 299, 340]. Receptors in charge of trans-BBB transport of transferrin, lipoproteins, insulin, insulin-like growth factors, and other macromolecules associated to this transcytosis pathway have been capitalized to improve the delivery of lysosomal ERTs into the CNS [298, 299, 340]. For instance, recombinant enzymes such as the case of iduronate-2-sulfatase (deficient in MPS II), arylsulfatase A (deficient in metachromatic leukodystrophy), and others have been fused to peptides derived from ligands, antibodies, phage display, etc., which can bind to the insulin receptor, the transferrin receptor, LRP-1 (e.g., via melanotransferrin, human Hsp-70, angiopeptide 2, receptor binding domains of apolipoproteins B and E, etc.), and others [348–361]. Figure 3B shows promising results by one of these examples, an anti-insulin-arylsulfatase A fusion protein, which efficiently reached lysosomes of deficient cells in culture and accumulates in the brain in Rhesus monkey [355]. Interestingly, it has been hypothesized that pinocytosis may also contribute to a certain level of lysosomal enzymes crossing the BBB when infused at high concentrations, although the degree of transport by this non-specific mechanism may not be sufficient for clinical intervention and conversely detrimental effects would arise from high doses being needed for such an event [362, 363].

Alternative transcytosis routes have been more recently identified, such as the case of the cell adhesion molecule (CAM)-mediated pathway, which may provide additional avenues for lysosomal ERT, as discussed in Section 5.4. Exosomes, 50–100 nm diameter vesicles secreted by some cells (e.g., dendritic cells, endothelial cells), are being actively explored as natural vehicles capable of transport across the BBB. They contain nucleic acids and proteins, and are involved in cell-to-cell communication [364]. Exosomes can be modified in different manners to modulate their cargo and/or targeting moieties that can be displayed on their membrane [364]. Functional readouts measured in the brain after systemic injection of therapeutic exosomes, e.g., shown for siRNA delivery, points to this avenue as a powerful one in order to delivery functional macromolecules into the CNS [364]. Interestingly, it has been observed that exosomes modified to contain the mRNA codifying sequence that is deficient in Pompe disease increased the enzyme activity in the brain of the mouse model for this disease [365]. Access into this tissue has also been shown via the intranasal route, as compounds that enter this path seem to be transported through perivascular channels or along the olfactory or trigeminal nerves [366, 367]. In particular, both α -L-iduronidase (the

enzyme deficient in MPS I) and viral vectors expressing this enzyme have been shown to access the brain when administered via the intranasal route, where they rendered measurable enzyme activity in various regions, including the cortex, striatum, hippocampus, thalamus, cerebellum, and brainstem [368]. In the context of MPS, guanidylated glycosides conjugated to lysosomal enzymes also seem to facilitate entry into the brain after intranasal administration [369].

5.3. ERT Improvement by Nanomedicine and Drug Delivery Strategies

Additional delivery approaches are being explored for lysosomal ERT, including those that encompass drug carriers aimed to enhance the efficacy and minimize side effects of treatment [52] (Table 4). For instance, current enzyme formulations do not offer an opportunity to control the circulation of the infused enzymes or their potential degradation, immunogenicity, or controlled release. Although fusion proteins and other enzyme-ligand conjugates facilitate glycosylation-independent targeting and uptake, they do not help with the above problems.

Encapsulation within drug carriers can protect recombinant enzymes from premature degradation, limit their release until reaching their lysosomal destination (therefore, minimizing side effects), and pace their release rate to achieve sustained effects [332]. Further modification of carriers with “stealth” moieties, e.g., polyethylene glycol (PEG), can minimize enzyme degradation and lengthen the circulation in the bloodstream while lowering immune responses [370, 371]. In fact, some drug carriers are being explored in experimental settings to induce immunotolerance [372]. Targeting moieties can be coated on the surface of drug carriers to provide glycosylation-independent binding, transcytosis, and/or uptake into lysosomes, whereby presentation of multiple copies of the targeting moiety on carriers enhances their overall avidity [373, 374]. Drug carriers can also be used for combination therapy, e.g., by co-loading recombinant enzymes and other compounds such as small molecule therapeutics which may help in treatment. These drug carriers can be fabricated from biological or synthetic materials, assembled into a variety of architectures (ranging from a few nanometers to a few micrometers in size, depending on the application), and can be functionalized to control their physicochemical properties and functions stated above [375–385]. Liposomes, polymersomes, dendrimers, nanoparticles, and many other configurations can be loaded with hydrophobic and/or hydrophilic drugs as well as biologicals, including proteins and enzymes [381, 382, 386–389].

The potential of drug carriers in the realm of lysosomal enzyme delivery was recognized early on, with the first studies in this direction being conducted using liposomes, back in the 1970s and 1980s. These works investigated liposomal delivery of enzymes isolated from human placenta or recombinantly produced, such as β -glucuronidase, β -glucosidase, β -galactosidase, β -fructofuranosidase, α -mannosidase, etc. [390–397]. As expected, it was observed that the biodistribution and effects in animal models changed after enzyme encapsulation within liposomes, which depended upon the combination and proportion of phospholipid used, synthesis protocol, surface charge and modification, etc. [391–395]. For instance, when β -glucuronidase (the enzyme deficient in MPS VII) was injected as a liposomal formulation, its hepatic delivery increased [395]. Also, different liposomes

rendered different lysosomal transport and activity, e.g., negatively-charged liposomes trafficked to lysosomes more readily, while positively-charged liposomes resulted in more prolonged enzyme activity [395]. Liposomes were also coated with IgG, IgM, or apoE with the intent to improve uptake by immune cells, for cases where these represented primary targets for intervention [396, 397]. PEG also provided improvement by increasing enzyme delivery and activity [398]. In the last decade, modification of liposomes with RGD peptides or lysosomotropic agents has also shown promise, such as the case for α -galactosidase delivery by RGD-functionalized liposomes, or VPRIV®-loaded liposomes targeted by octadecyl-rhodamine B for Gaucher disease [399–402].

Of particular interest are applications of poly(lactic-co-glycolic acid) (PLGA) nanoparticles because of the additional advantages of this polymer in the context of LDs. PLGA is an FDA-approved material which degrades into lactic acid and glycolic acid, both molecules endogenously present in the body and metabolizable by cells [377, 403, 404]. PLGA particles possess greater stability than liposomes and their degradation rate can be tuned to achieve control over their release rate [377, 404], which may be used for more sustained delivery in order to lower the administration frequency and associated side effects. PLGA nanoparticles that passively enter cells via pinocytic mechanisms and those that are targeted to specific cell surface markers, both traffic to lysosomes, which is required for LD treatment [374, 377]. PLGA degradation within lysosomes has been experimentally shown to promote an acidic environment both in cell culture and *in vivo* [117, 405]. This is ideal not only for proper activity of lysosomal enzymes but also to help restore lysosomal acidification, which is otherwise defective in LDs [117, 405]. PLGA nanoparticles targeted to intercellular adhesion molecule 1 (ICAM-1), discussed in Section 5.4, or by a small glycopeptide (g7), have been shown to significantly enhance transport of enzymes into the brain [406, 407]. In the latter case, albumin was used as a model protein and these nanoparticles were examined in animal models of MPS I and II [407]. As shown in Figure 3C, only g7-targeted PLGA nanoparticles, but not nanoparticles lacking g7 glycopeptide, were found in the brain of animals 2 h after intravenous administration [407]. As a protein which helps maintain the oncotic pressure in tissues, albumin is known by its ability to cross certain endothelial barriers, which may have contributed to this outcome, but this remains an encouraging result.

Several additional delivery systems have been tested on the grounds of improving lysosomal ERT, including enzyme coating on magnetic microparticles or encapsulation within alginate microspheres for sustained release upon implantation [408]. Enzyme coupling to metal (e.g., gold) nanoparticles, along with lysosomal sorting proteins and cell penetrating peptides has also shown good lysosomal trafficking in cell culture models, as has enzyme coupling to quantum dots [409, 410]. Trimethyl chitosan nanoparticles tested in cellular models showed enzyme dissociation from the nanoparticle at lysosomal pH [411]. Protein-based nanoparticles (albumin and silkworm 30Kc19 protein) have rendered enhanced uptake and stability of α -galactosidase (deficient in Fabry disease) in patient fibroblast cultures [412].

Overall, these approaches provide an avenue to enhance the circulation half-life of lysosomal enzymes, and/or their accumulation in peripheral organs and the CNS, while also enhancing lysosomal transport and activity in cellular models.

5.4. ICAM-1-Targeting of Lysosomal ERTs

Perhaps the first published strategy that attempted delivery of lysosomal enzymes using targeted polymer nanocarriers focused on intercellular adhesion molecule 1 (ICAM-1) [413] (Table 4). ICAM-1 is an immunoglobulin-like transmembrane glycoprotein that supports binding of leukocytes during inflammation [414, 415]. It possesses interesting characteristics in line with a good target for lysosomal ERT. For instance, ICAM-1 is expressed on the surface of most tissues and cell types (e.g., endothelial, epithelial, muscle, fibroblasts, neurons, etc.), which enables targeting of cells in peripheral tissues and the CNS [414]. As described above, this is important since lysosomes are ubiquitous organelles and, hence, LDs associate with broadly distributed dysfunctions [5, 45]. Many pathological factors, including oxidative stress, metabolic imbalance, and inflammation result in ICAM-1 overexpression [416, 417]. Because these local or systemic states are common to most pathologies, including LDs, this property provides targeting preference to sites affected by disease [414]. In fact, this represents a self-regulated targeting system, whereby amelioration of the disease condition at a particular body site would result in lower ICAM-1 expression at said site, which in turn would deviate treatment to areas in a higher need. With regard to accessibility from the administration site, because of its broad expression, ICAM-1 can be reached from the intravascular, intratracheal, intrathecal, intracerebral and other spaces [414], providing versatility regarding the selection of the route of administration.

Numerous works have focused on the development of therapeutic or diagnostic strategies targeting this molecule, including metabolic disease, cancer, cardiovascular and pulmonary pathologies, infections, inflammatory conditions, etc. [418–424]. ICAM-1 targeting by affinity antibodies or peptides has been shown in cell cultures, animal models and clinical trials [422, 424, 425–434], which have focused on delivery of contrast agents, isotopes, biosensors, biological therapeutics (including enzymes), protein conjugates, and several forms of drug carriers such as liposomes, dendrimers, nanoparticles, etc. [418–425].

In the past, ICAM-1 was not recognized as an endocytic receptor since no endogenous extracellular ligand had been shown to be endocytosed via this surface marker. However, interestingly, some pathogens were known to bind to ICAM-1 in order to invade cells, such as the case for major class human rhinoviruses, which enter epithelial cells in the upper airways by binding to this molecule [435]. Despite this knowledge, the mechanism by which ICAM-1 facilitates the uptake of said pathogens was unknown. Early work by Muro and Muzykantov focused on examining the interaction between ICAM-1 targeted nanoparticles and endothelial cells serendipitously rendered the identification of this endocytic route, which was called Cell Adhesion Molecule (CAM)-mediated endocytosis [436]. The CAM pathway was shown to be different from classical vesicular uptake via clathrin- or caveolar-mediated endocytosis and, although related, also differed from macropinocytosis and phagocytosis in terms of signaling cascade, actin cytoskeleton reorganization, markers involved, and inhibitor sensitivity [436, 437]. Subsequent work by the Muro lab has provided further understanding on the regulation of this pathway, which has been validated by others in the context of ICAM-1-mediated pathogen invasion, membrane recycling at the immune synapse, and leukocyte extravasation via the transcellular route [438]. Upon engagement, ICAM-1 associates with the sodium-proton exchanger NHE1 at membrane

regions enriched in cholesterol, sphingomyelin and gangliosides [436, 439]. Activation of protein kinase C, Src kinases, and Rho dependent kinase are central to the subsequent signaling cascade [436, 437, 440], which results in recruitment of intracellular sphingomyelinases at plasmalemma binding sites, with generation of ceramide [439]. This local increase in ceramide at binding sites, favors membrane vesiculation and actin reorganization into stress fibers, whereby said fibers are thought to engage with the cytosolic domain of ICAM-1 and NHE1 via ezrin-radixin-moesin family proteins, leading to uptake [436, 439]. Interestingly, this built-in mechanism to remodel not only the cytoskeletal organization but also the plasmalemma composition (hence, its biophysical properties) at ICAM-1 binding sites, provides flexibility in terms of the size and geometry of objects able to internalize via ICAM-1. As an illustration, polymer nanoparticles of different sizes and shapes, protein conjugates, and other drug delivery carriers have been shown to enter cells upon binding to ICAM-1 in cell culture and *in vivo*, while their efficiency of cellular uptake when targeted to classical clathrin- or caveolae-associated routes is much more restricted when exceeding 300 nm in size [441, 442]. This seems reminiscent of the ability of ICAM-1 in mediating the engulfment of leukocytes by endothelial cells during transcellular extravasation, which has recently been related to the built-in mechanism of ceramide generation at the binding site [443].

Also related to this role in transcellular (as opposed to paracellular) extravasation of leukocytes is the ability of ICAM-1 to mediate transcytosis across cellular linings, which has been shown for both endothelial and epithelial cells [444, 445]. In cellular models of these barriers, ICAM-1-targeted nanoparticles bearing enzymes (shown for alpha-galactosidase) and also direct antibody-enzyme conjugates (shown for model horseradish peroxidase) have been observed to transcytose from the apical to the basolateral surface of “barrier” cells [442, 444]. This occurred via the CAM pathway without disruption of the permeability barrier, as demonstrated by tracing lack of changes in the transcellular electrical resistance, lack of concomitant albumin leakage during the process, and lack of visible changes at the cell junction sites under electron microscopy visualization [406, 444, 445]. This phenomenon has been documented *in vivo*, including visualization of enzyme-bearing ICAM-1-targeted nanoparticles crossing the BBB and the pulmonary endothelial layer, which were then internalized by subjacent cells in the animal model for Types A-B NPD [406, 446]. This resulted, after a single injection, in a ~3–7-fold increase in the amount of acid sphingomyelinase delivered to the brain (Figure 3D), 6-fold increase in the case of α -glucosidase, and ~4-fold enhancement for α -galactosidase, illustrating the potential of this strategy [424, 426, 445, 447]. Similarly, acid sphingomyelinase delivery to the lungs in the animal model for Types A-B NPD resulted in 30-fold delivery enhancement (Figure 3D), with measurable enzyme activity in the tissue and marked degradation of the accumulated substrate after only 5 injections [446]. As shown for the case of acid sphingomyelinase delivery as an example, ICAM-1 targeting provided enhanced delivery to all other organs tested, including the liver, spleen, kidneys, heart, skeletal muscle, etc. (Figure 3D), which is of key importance for efficient treatment of multi-organ dysfunction in LDs, and was expected based on ICAM-1 broad expression [424, 426, 445, 447]. Furthermore, it was observed that said rapid targeting mediated by this ICAM-1 strategy resulted in fast disappearance (within minutes) of the enzyme cargo from the circulation [424, 426, 445,

447]. This, as said, did not affect ICAM-1 targeting throughout the body but was rather a consequence of it: in the case of α -galactosidase delivery, ICAM-1-targeted polymer nanoparticles reduced enzyme presence in the circulation from 50% for the “naked” enzyme to 5% for the carrier formulation, by 30 min after intravenous injection. Yet, ICAM-1-targeted nanoparticles resulted in 70% of the injected dose being delivered through the body by this time, while only 20% of the “naked” enzyme reached the tissues [445]. Hence, without compromising broad targeting, this strategy may help reduce systemic effects associated to circulating enzymes. Also, by providing such an enhancement in the delivery to multiple tissues and across endothelial barriers, this strategy may help reduce the dose and, perhaps, frequency of administration, altogether lowering side effects. To this extent, a thorough investigation is warranted in order to advance this strategy, yet preliminary results show that mice tolerate these injections well and lack gross effects, including histological appearance, lack of edema or decrease in the endothelial permeability, and actually reduction of endothelial release of soluble ICAM-1, a factor involved in inflammation [448].

Importantly, along with transcytosis across cellular monolayers that separate body compartments, ICAM-1 targeting also provides lysosomal trafficking in these cells and cells of subjacent tissues [446, 447, 449]. This is similar to dual trafficking (transcytosis and lysosomal transport) via other routes, such as the case for LDLR and transferrin receptor, associated to the clathrin route. However, because the clathrin route has been found to be altered in some cells in LDs (described in Section 4.2) [313–315, 319], ICAM-1 targeting may provide an alternative pathway, since it is overactive under inflammation pertaining to LDs and it also bypasses the M6PR and, hence, need for enzyme glycosylation [413]. Delivery of acid sphingomyelinase, α -glucosidase, and α -galactosidase by this route has resulted in enhanced degradation of lysosomal sphingomyelin, glycogen, and globotriaosylceramide, respectively, as compared to enzymes using the M6PR [413, 424, 447]. Therefore, this strategy holds considerable potential to improve and/or complement current therapeutic interventions for the treatment of LDs. Nevertheless, as for any other alternative strategy, concerns relative to potential acute or chronic side effects arising from targeting this cell surface marker or the particular formulations used for this matter (whether fusion enzymes, enzyme conjugates, or drug carrier driven) will need a careful and thorough investigation to discern the true applicability and overall benefit of this approach.

6. CONCLUSION

Due to their key function and broad distribution, lysosomes are central components of cells and play a relevant role in the maintenance of cellular and body-level homeostasis. LDs, caused by genetic deficiencies in lysosomal components cause aberrant accumulation of undigested substrates, leading to multi-system pathologies which are often fatal. ERT has offered an opportunity to improve the quality of life and survival rate of patients, yet its therapeutic value is hampered by side effects, resistance and, mainly the inability of recombinant enzymes to reach the CNS. These phenomena result in only a few diseases benefiting from ERT strategies. Numerous alternatives are under examination which provide proof of improvement, yet their road to translation will require arduous development and testing before truly revealing their overall benefit-to-risk balance and associated cost. It is becoming tangible, nonetheless, that a single treatment type will not serve the one-for-all

purpose given the different requirements, characteristics, targets, and disease states that associate with LDs. An interdisciplinary effort remains the best avenue to focus at different angles, including obtaining more detailed knowledge on the basic and translational fundamental factors influencing the progression and outcome of these diseases, the connections and relationships between the genotypes and phenotypes observed, the identification of the markers truly ruling the pathological state as well as its exacerbation, the anatomical barriers and transport routes available and accessible for therapeutic delivery, and multifaceted technology development, as well as feasible commercial production and regulatory hurdles.

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Abbreviations

ER	endoplasmic reticulum
ERT	enzyme replacement therapy
FDA	U.S. Food and Drug Administration
LD	lysosomal disease
M6P	mannose-6-phosphate
M6PR	mannose-6-phosphate receptor
MPS	mucopolysaccharidosis
NPC	Type C Niemann-Pick disease
NPD	Types A and B Niemann-Pick disease
Wt	wild-type

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Figure 1. Lysosome function and dysfunction

(A) Lysosomal components, including structural membrane proteins, H^+ -ATPase pump, membrane enzymes, channels and transporters, as well as luminal lysosomal enzymes. (B) Biosynthesis route for lysosomal enzymes, encompassing nuclear transcription, endoplasmic reticulum glycosylation (B_1), Golgi apparatus maturation (B_2 and B_3), and transport to endosomes (B_4) and lysosomes (B_5) via intracellular mannose-6-phosphate receptors. (C) Secretory route for lysosomal enzymes (C_1), also including endocytic uptake by cell surface mannose or mannose-6-phosphate receptors (C_2 and C_3), for delivery to lysosomes (C_4). (D) Some cellular functions in which lysosomes are involved.

Figure 2. Cross-correction effect of lysosomal enzymes

In a wild-type (Wt) cell, the nascent enzyme undergoes sequential post-translational modifications in the endoplasmic reticulum (A) and the Golgi apparatus (B), where the enzyme acquires mannose residues or mannose-6-phosphate (M6P) residues. A fraction of this enzyme is secreted extracellularly (C) and can bind to M6P receptor of a neighboring LD cell (D). The neighboring LD cell internalizes the functional lysosomal enzyme secreted by the healthy cell via M6P receptor endocytosis (E). Resulting endosomes containing the enzyme will eventually fuse with lysosomes (F), delivering the Wt enzyme in the LD cell and correcting the storage defect. This phenomenon is called cross-correction and forms the basis for lysosomal enzyme replacement therapy.

Figure 3. Examples of novel lysosomal enzyme replacement therapy approaches

(A) Enhanced enzyme activity in the heart and kidneys of the mouse model of Fabry disease, after intravenous injection with the same dose of α -galactosidase A recombinantly produced in tobacco cells (PRX-102; currently in Phase III clinical trial) versus CHO cell (clinically approved). Adapted from [210], with permission. (B) A fusion protein formed by recombinantly tagging the enzyme arylsulfatase A with a monoclonal antibody against the insulin receptor (HIRMAb-ASA; green), shows good fluorescence microscopy colocalization with lysosomes (red) when examined in metachromatic leukodystrophy (MLD) patients fibroblasts (top panel; 24 h). Radiotracing of this fusion protein also showed brain uptake 2 h after intravenous injection in Rhesus monkey (bottom panel). Adapted from [355], with permission. (C) PLGA nanoparticles targeted by g7 glycopeptide accumulate in the brain of Iduronidase knockout mice 2 h after intravenous injection, carrying albumin as a model cargo protein. Adapted from [407], with permission. (D) Enhanced brain and peripheral biodistribution of radiolabeled, recombinant acid sphingomyelinase (the enzyme deficient in Types A–B Niemann-Pick disease), 30 min after intravenous injection of ICAM-1-targeted polymer nanoparticles versus the “naked” enzyme currently in Phase III clinical trial (Localization Ratio (tissue-to-blood ratio) = percent of injected dose per gram of organ divided by the percent of the injected dose per gram of blood). Adapted from [426, 447], with permission.

Figure 4. Immunogenicity of enzymes administered for lysosomal enzyme replacement therapy
A lysosomal enzyme containing a catalytic site and a targeting moiety (mannose or M6P) can evoke an immune response after infusion in the patient, wherein either neutralizing antibodies (A) or non-neutralizing antibodies (B) are raised against the enzyme. Neutralizing antibodies can block the enzyme targeting site, preventing the enzyme from binding to mannose or M6P receptors on target cells and, hence, preventing their lysosomal delivery (A₁). Instead, neutralizing antibodies that bind to the enzyme catalytic site will permit its uptake by endocytosis and lysosomal trafficking into the target cell, but will block its catalytic function, preventing substrate reduction and disease attenuation (A₂). Instead, non-neutralizing antibodies do not affect these functions, but instead bind to the enzyme and tag it for removal by macrophages via Fc receptors (B).

Table 1

Examples of alterations leading to lysosomal diseases.

Defect	Consequence	LSD examples	Ref.
Mutations in the catalytic site of a lysosomal enzyme	Inactive enzyme	MPS, NPD-A/B, Pompe disease, Fabry disease	[31]
Mutations in the folding site of a lysosomal enzyme	Enzyme targeted to degradation pathway	Gaucher disease, Krabbe disease, Tay-Sach's disease	[32]
Mutations that affect enzyme processing in the ER	Inadequate post-translational modification of other enzymes	Multiple sulfatase deficiency	[33, 34]
Mutations in sorting enzymes	Enzymes secreted out of the cell	Mucopolidosis Type II (I-cell disease)	[24]
Mutations in lysosomal membrane transporters	Substrates cannot shuttle out of the lysosome	NPC, Salla disease, cystinosis	[35, 36]
Mutations in lysosomal membrane integral proteins	Lysosomal integrity disturbed	Danon disease	[38]
Reduced Rab7/Rab9 expression	Reduced endosomal mobility, M6PR recycling and lysosome biogenesis	NPC	[39, 40]
Mutations in calcium channel (Mucolipin)	Reduced lysosomal movement/secretion	Mucopolidosis Type IV	[37]

ER = endoplasmic reticulum; MPS = mucopolysaccharidosis; NPC = Type C Niemann-Pick Disease; NPD-A/B= Types A and B Niemann-Pick Disease.

Table 2

Therapies for lysosomal diseases.

Treatment	Mechanism of action	LSD examples	Ref.
Gene therapy	Integrate Wt sequences into host DNA- <i>in vivo</i> or <i>ex vivo</i>	Fabry disease, Pompe disease, Krabbe disease, several MPS, metachromatic leukodystrophy	[65, 68–78]
Genome editing	Remove faulty DNA sequences and replace Wt sequences by homologous recombination	Fabry disease, MPS I and II, and Gaucher disease	[87, 88]
Enzyme enhancement therapy	Assist enzymes to fold correctly in the ER and be trafficked to lysosomes	Gaucher and Fabry diseases, GMI gangliosidosis, NPC	[103–107]
Substrate reduction therapy	Inhibit biosynthesis of the accumulated substrate	Gaucher, Fabry, and Tay-Sachs' diseases, MPS, NPC, cystinosis	[94–96]
Organ transplantation	Alleviate phenotypic symptoms of LSDs	Gaucher, Pompe, and Krabbe diseases, MPS I	[65]
Hematopoietic stem cell transplantation	Cross-correct LSD cells by heterologous transplantation of Wt cells	MPS I and II, Krabbe disease, cystinosis	[90–93]
Enzyme replacement therapy	Cross-correct LSD cells and tissues by intravenous administration of exogenous Wt enzymes	Gaucher, Fabry, and Pompe diseases, MPS I, II, and IV	[262, 264–268]
Modulation of cytosolic molecules	Overexpress Rab7/Rab9 to restore endosome mobility	NPC	[108]
Lysosome acidification	Restore acidic lysosomal pH	Krabbe disease	[116]
Lysosome exocytosis	Secretion of lysosomes to dispose accumulated substrates outside the cell	Batten and Pompe diseases, MSD, MPS IIIA, NPD-A/B, NPC	[110–112, 115]

ER = endoplasmic reticulum; MPS = mucopolysaccharidosis; MSD = Multiple Sulfatase Deficiency; NPC = Type C Niemann-Pick Disease; NPD-A/B = Types A and B Niemann-Pick Disease; Wt = wild-type

Table 3

Clinical status of current enzyme replacement therapy for lysosomal diseases

LD	Deficient enzyme	Expression system	ERT	Clinical status
Gaucher's disease	β -glucocerebrosidase	Human placenta	Aglucerase (Ceredase TM)	1991-FDA
		CHO cells	Imiglutecase (Cerezyme TM)	1994-FDA; 1997-EMA; 2008-CPDA
		Fibrosarcoma cells	Velaglutecase (VPRIV TM)	2010-FDA, EMA; 2014-CPDA
		Carrot cells	Taliglucerase (Elelyso TM)	2012-FDA
Fabry disease	α -galactosidase	CHO cells	Agalsidase α (Fabrazyme TM)	2001-EMA
		Moss	Agalsidase β (Replagal TM)	2003-FDA, EMA
			moss- α Gal	Phase I
Pompe disease	α -glucosidase	Tobacco cells	Pegunigalsidase (PRX-102)	Phase III
		CHO cells	Aglicosidase (Myozyme TM)	2006-FDA, EMA, CPDA
		Rabbit milk	Aglicosidase (Lumizyme TM)	2010-FDA
			rhAGLU	Phase II
			Laromidase (Aldurazyme TM)	2003-FDA, EMA; 2006-CPDA
MPS I (Hurler syn.)	α -L-iduronidase	CHO cells	Idursulfase (Elaprase TM)	2006-FDA; 2007-EMA, CPDA
MPS II (Hunter syn.)	Iduronate-2-sulfatase	CHO cells	SBC-103	Phase I/II
MPS III B (Sanfilippo syn.)	α -N-acetylglucosaminidase	Egg white/hens	Elosulfase Alfa (Vimizim TM)	2014-FDA
MPS IV A (Morquio A syn.)	N-acetylglucosamine 6-sulfatase	CHO cells	Galsulfase (Naglazyme TM)	2005-FDA; 2006-EMA; 2008-CPDA
MPS VI (Maroteaux-Lamy syn.)	arylsulfatase B	Mouse L cells	UX-003	Phase III
MPS VII (Sly syn.)	β -glucuronidase	Egg white/hens	Sebelipase α (Kanuma TM)	2015-FDA, EMA; 2016-CPDA
Lysosomal acid lipase deficiency	Lysosomal acid lipase	CHO cells	Olipudase alfa @	Phase II/III
Niemann-Pick Type B	Acid sphingomyelinase			

CPDA: China Food and Drug Administration; EMA: European Medical Agency; FDA: U.S. Food and Drug Administration; PMDA: Pharmaceutical and Medicals Devices Agency of Japan.

Table 4

Examples of novel approaches for lysosomal enzyme replacement therapies

Drug delivery system	Application/Advantage in LD treatment	Examples	Ref.
Peptide-enzyme fusion proteins	Glycosylation independent binding, uptake, and/or transcytosis, yet still dependent on the M6PR	IGF-II- β -glucuronidase for MPS VII	[334]
	Glycosylation independent binding, uptake, and/or transcytosis by targeting receptors other than M6PR	RAP- α -glucosidase for Pompe disease	[335]
		RAP- α -L-iduronidase for MPS I	[335]
		Tat-glucocerebrosidase for Gaucher disease	[336]
		Tat- β -glucuronidase for MPS VII	[337, 338]
		Tat-galactocerebrosidase for Krabbe disease	[339]
		ApoE-I-aryl/sulfatase A for MLD	[361]
Antibody-enzyme conjugates and fusion proteins	Glycosylation independent binding, uptake, and/or transcytosis by targeting receptors other than M6PR	ApoB-aryl/sulfatase A for MLD	[361]
		Transferrin receptor MAb conjugated to β -galactosidase model	[292]
		Insulin receptor MAb-iduronate-2-sulfatase for MPS II	[354]
		Insulin receptor MAb-aryl/sulfatase A for MLD	[355]
Nanocarriers	Insulin receptor MAb- α -L-iduronidase for MPS I	Insulin receptor MAb- α -L-iduronidase for MPS I	[351]
	<i>Liposomes:</i>	Non-targeted, β -glucuronidase for MPS VII	[395]
		Non-targeted, β -galactosidase for Krabbe disease	[394]
		RGD targeted, α -galactosidase for Fabry disease	[399]
		IgG targeted, ATP synthase subunit C for Batten disease	[397]
	ApoE targeted, ATP synthase subunit C for Batten disease	ApoE targeted, ATP synthase subunit C for Batten disease	[397]
		Octadecyl-rhodamine B targeted, glucocerebrosidase for Gaucher	[402]
	<i>Polymeric nanoparticles</i>	PLGA, non-targeted, glucocerebrosidase for Gaucher disease	[405]
		PLGA, g7 targeted, albumin model, generic disease	[407]
		PLGA and polystyrene models, ICAM-1 targeted, acid sphingomyelinase for types A/B Niemann Pick Disease	[406, 413, 426, 447]
		Polystyrene models, ICAM-1 targeted, α -galactosidase for Fabry disease	[424, 445]

Drug delivery system	Application/Advantage in LD treatment	Examples	Ref.
		Polystyrene models, ICAM-1 targeted, α -glucosidase for Pompe disease	[447]
		Albumin-silk protein, α -galactosidase for Fabry disease	[412]
		Trimethyl chitosan, α -galactosidase for Fabry disease	[411]
		<i>Quantum dots</i>	
		Guanidinylated-neomycin modified, β -glucuronidase for MPS VII	[410]
		Guanidinylated-neomycin modified, α -L-iduronidase for MPS I	[410]
Microparticles	Sustained release <i>in situ</i>	Alginate microspheres, glucocerebrosidase for Gaucher disease	[408]
Engineered exosomes	Transcytosis through the BBB	Coding mRNA, α -glucosidase for Pompe disease	[365]

ICAM-1 = intercellular cell adhesion molecule 1; IGF II= Insulin like growth factor; MPS = mucopolysaccharidosis; MLD = mucopolysaccharidosis; MAb = Monoclonal antibody; PLGA = poly-(lactide-co-glycolide); RAP = receptor-associated protein.